Development of a robust and fully validated method for the simultaneous determination of sweeteners (including neotame and steviol glycosides) in food

Food Standards Agency

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Executive Summary

Development of a validated, readily accessible method for the identification and quantification of permitted sweeteners in food is required to enable enforcement of food additive legislation and to protect the consumer from misuse of sweeteners in food. Intense sweeteners are often used in combination in foodstuffs, therefore there is a need for a method to simultaneously extract and determine the permitted intense sweeteners saccharin, aspartame, acesulfame K, neohesperidine dihydrochalcone (NHDC), sucralose, cyclamic acid, neotame and steviol glycosides (e.g. stevioside and rebaudioside A (Reb A)). There are several methods in existence for the determination of combinations of intense sweeteners but there are currently no validated methods for the determination of the intense sweeteners together with steviol glycosides.

An HPLC-UV method has been developed for the simultaneous determination of acesulfame K, aspartame, saccharin, NHDC, Reb A, stevioside and neotame. Biscuits, jam, fruit squash, carbonated soft drink and yoghurt were chosen for the validation as being representative of high carbohydrate, high aqueous and high fat foods. The in-house validation indicated that the method was suitable for the determination of all of the sweeteners, however the recoveries obtained for NHDC were generally lower in the high fat samples (yoghurt and biscuits).

The ruggedness of the developed method was evaluated. Four parameters (extractant pH, extraction procedure, HPLC column and HPLC column oven temperature) were investigated for fruit squash, jam and yoghurt. Statistical evaluation of the results indicated that the extractant pH and extraction procedure had no effects on sweetener determination in any of these matrices. The temperature of the HPLC column only had an effect on acesulfame K in the fruit squash. The major contributor to any variation in results was the HPLC column which had an effect on several sweeteners in all three matrices.

Aliquots of three of the matrices used to validate the method were sent to a second laboratory as a pre-study method check. The repeatability and reproducibility obtained by the second laboratory was compared to that obtained by LGC. At this stage of the project it became apparent that the determination of stevioside was subject to over recovery. Despite various investigations no definitive explanation was been found.

A total of 14 laboratories participated in the collaborative trial and analysed five matrices; jam, blackcurrant flavour juice drink concentrate, blackcurrant flavour juice drink diluted ‘ready-to-drink’, low fat yoghurt and high fat yoghurt. The results from the trial indicated that the method was suitable for the analysis of a range of artificial and natural sweeteners in jam and squash drinks. It was not recommended for the detection of neotame, NHDC or aspartame in yoghurt matrices. Several laboratories showed variation in results indicating further training or practise may be required to improve performance overall.

Development of a method for the simultaneous determination of sweeteners (including neotame and steviol glycosides) in food

Introduction

The concentration of permitted sweeteners in foods in the UK is regulated by Regulation (EC) No. 1333/2008 implemented in England by the Food Additives (England) Regulations 2009 (No. 3238) and equivalent in the other devolved administrations. Development of a validated, readily accessible method for the identification and quantification of permitted sweeteners in food is required to enable enforcement of legislation and to protect the consumer from misuse of sweeteners in food. Food surveillance is integral to improving the understanding of exposure through collation of information on the concentration and usage of sweeteners. This information is needed to monitor the concentration of permitted sweeteners in foods and patterns of use, and to fulfill European Community legislation requirements for Member States to monitor food additive intakes in order to ensure that the use of sweeteners is safe, i.e. intakes are below acceptable daily intakes (ADIs).

Intense sweeteners are often used in combination in foodstuffs, there is a need for a method to simultaneously extract and determine the permitted intense sweeteners saccharin, aspartame, acesulfame K, NHDC, sucralose, cyclamic acid, neotame and steviol glycosides e.g. stevioside and Reb A which are approved in the US and are now permitted in the Member States of the European Union, under Annex II to Regulation (EC) No 1333/2008 as amended.

There are several methods in existence for the determination of combinations of intense sweeteners but there are no validated methods for the determination of the intense sweeteners including steviol glycosides. Due to the diverse nature of the structures of the sweeteners in question a 'universal' detection system is required to simultaneously determine the nine sweeteners of interest.

Method Development

Isocratic separation

As no validated methods for the determination of the intense sweeteners including steviol glycosides were found during a literature search, the chromatography conditions described in a paper written by the Institute of Reference Materials and Measurements (IRMM) for the determination of acesulfame k, alitame, aspartame, cyclamic acid, dulcin, neotame, neohesperidine dihydrochalcone, saccharin and sucralose were used as a starting point for this project[1].

The method described in the IRMM paper, involved gradient elution of the sweeteners using a combination of methanol, formic acid at pH 4.5 and acetone. Whilst, according to this study, the conditions were satisfactory for use with evaporative light scattering detection (ELSD) the chromatograms obtained after UV and Refractive Index (RI) detection in this study were not.

At the low wavelength (<250nm) needed to detect the sweeteners of interest, UV detectors are very sensitive to changes in mobile phase composition resulting in the baselines obtained being unsatisfactory. Whilst some problems were expected, possibly exacerbated by the high UV cut-off of acetone, the effect was greater than anticipated. Figure 1 shows

[1] http://irmm.jrc.ec.europa.eu/activities/food_additives/Documents/eur22726en.pdf)

an example chromatogram where whilst peaks for several of the sweeteners can be seen, it is obvious that the chromatography is not suitable for accurate quantification.

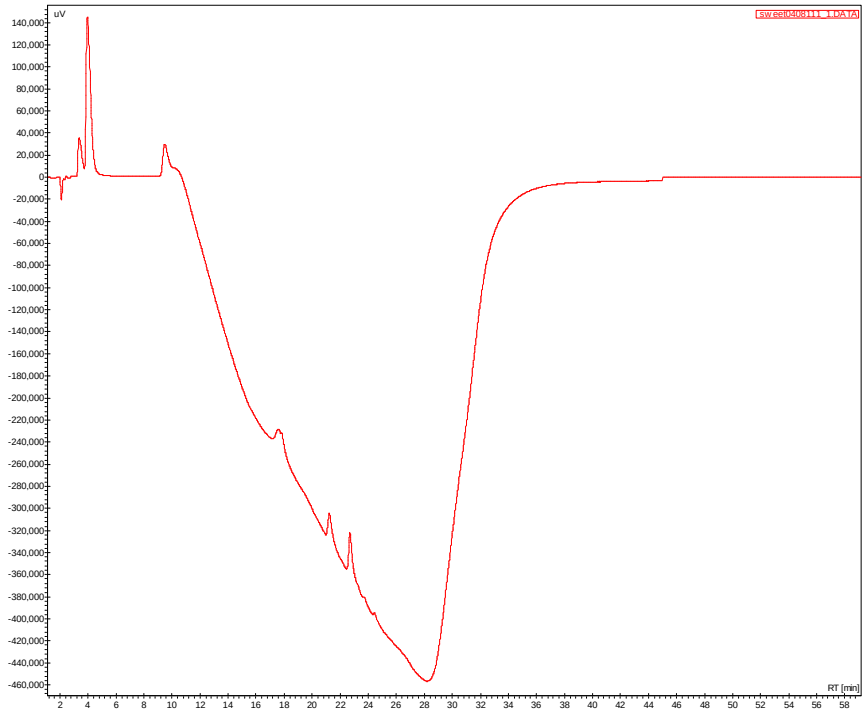


Figure 1: Standard solution containing 9 sweeteners analysed using methanol: formic acid: acetone gradient with UV detection (<250nm)

An additional problem was that refractive index (RI) detection is also known to be sensitive to changes in mobile phase composition and is not ideally suited to use with gradients. It was hoped, however, that the mobile phases detailed in the IRMM paper could be modified to obtain isocratic conditions suitable for use with an RI detector. Various combinations of methanol, formic acid and acetone were tried as mobile phases in an isocratic system, but satisfactory chromatography and separation of the sweeteners could not be obtained.

Due to the above problems, the results from the literature search were revisited and various chromatography conditions described were trialled to see if acceptable separation of the sweeteners could be obtained. Initially isocratic conditions were trialled to allow RI to be used for the detection of sucralose (sucralose cannot be detected using UV at the required concentrations) however, it was not possible to optimise the conditions sufficiently to allow complete separation / elution of all the sweeteners.

Isocratic conditions were also trialled for the UV HPLC system in an effort to eliminate the acetone cut-off effect and to find a method suitable for UV and RI detection at the same time. Figure 2 illustrates the chromatographic profile of the nine sweeteners achieved using UV detection with potassium dihydrogen phosphate, pH 5.0 and acetonitrile as an isocratic mobile phase. The percentage of acetonitrile and the pH of the buffer were altered but no significant improvement in the chromatography was achieved.

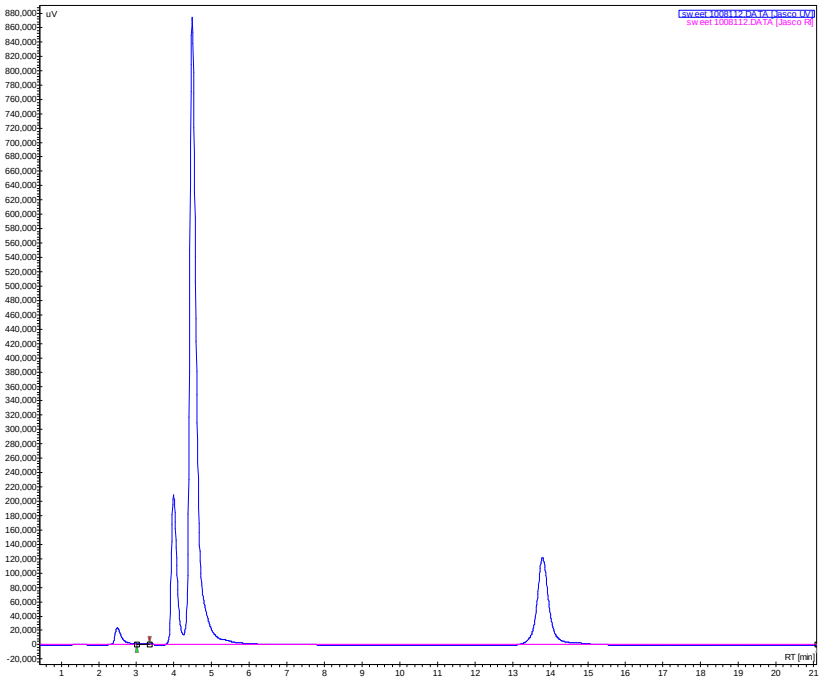


Figure 2: Isocratic elution of a mixed standard containing all 9 sweeteners (UV detection)

Despite assessing several different isocratic conditions, no suitable conditions were found that adequately separated all nine sweeteners of interest without the use of gradient elution.

Use of gradients for separation

Gradients can improve separation of analytes co-eluting in an isocratic system however there can be some disadvantages such as baseline drift. To offset any problems that may be observed with the baseline due to the use of gradient elution a compromise had to be made between maximum peak absorbance and degradation of the baseline. In addition to this, RI detection was excluded due to its incompatibility with the gradient systems required to achieve satisfactory separation of the sweeteners of interest. Since sucralose is not visible in the UV range the practicality of derivatising sucralose to obtain a compound suitable for detection by UV was explored. A derivatising agent which was considered to be suitable was p-nitrobenzoyl chloride which converts sucralose to a derivative with strong absorption at 260nm. Unfortunately, no satisfactory results were obtained.

Initially a gradient separation based on the method published by Lawrence et al. (1988) was assessed[2]. The article describes the separation of acesulfame-K, saccharin, sucralose and aspartame, however it was stated that stevioside could not be detected with this method. The method was adapted by increasing the percentage of acetonitrile in one of the mobile phases and amending the gradient. This resulted in the satisfactory separation of acesulfame-K, saccharin, aspartame, NHDC and neotame, however stevioside and reb A had co-eluted (figure 3). Cyclamate was seen as a small peak at a similar retention time to saccharin but the sensitivity was unlikely to be sufficient to accurately quantify this sweetener at concentrations currently permitted in foods. Sucralose gave a small peak at a retention time of less than 1 minute but only when injected at high concentrations and so the sensitivity was not considered sufficient for the permitted levels in foods. It was agreed with

[2] Determination of seven artificical sweeteners in diet food preparations by reverse-phase liquid chromatography with absorbance detection, J. Assoc. Off. Anal. Chem., Vol 71. No5, 1988

the FSA that method development should continue without the inclusion of sucralose or cyclamate.

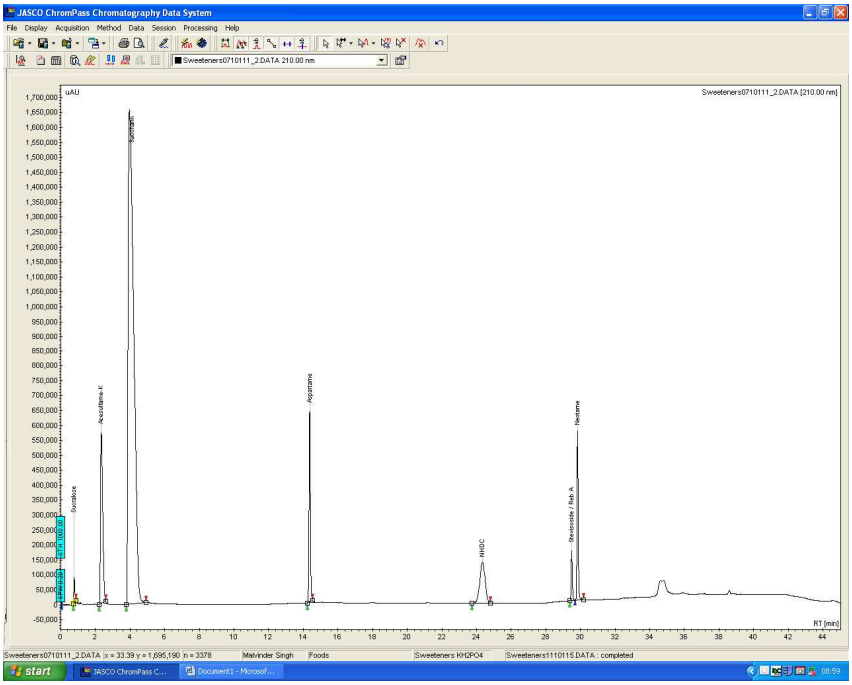


Figure 3: Mixed sweetener standard using potassium dihydrogen phosphate, acetonitrile gradient with UV detection

Since the recently approved sweeteners, stevioside and Reb A, were considered important, the chromatograpy conditions described above were abandoned in favour of developing a different system capable of separating the following seven sweeteners; acesulfame K, saccharin, aspartame, NHDC, neotame, Reb A and stevioside.

A range of mobile phases, gradients and HPLC columns were tested as listed in Table 1. These included normal phase hydrophilic interaction liquid chromatography (HILIC). Initial conditions involved a Luna C18 HILIC column with water and acetonitrile mobile phases. The gradient used was unsuccessful in separating the sweeteners. Various combinations of mobile phases were tried but acceptable separation could not be achieved for all of the sweeteners of interest. With HILIC chromatography, buffers, modifiers, sample solution and temperature can all greatly affect chromatography in addition to the percentage of water in the mobile phase. A range of conditions were tried, for example the addition of formic acid or ammonium formate to the mobile phases, modifying the sample solutions to include a higher percentage of acetonitrile and increasing the temperature of the column oven, but satisfactory separation of all the sweeteners still could not be achieved.

ChromaDex was also involved in trying to optimise a method suitable for separating all of the sweeteners. Their initial HPLC conditions consisted of a water: acetonitrile gradient and a Phenomenex Synergi Hydro-RP column. The chromatogram in Figure 4 is an example of the separation that can be achieved for a range of steviol glycosides.

Table 1: HPLC conditions evaluated

Mobile phase composition	Column type	Observations
A. 0.02M KH_2PO_4:ACN pH 5.0 (97:3) **B.** 0.02M KH_2PO_4:ACN pH 3.5 (80:20)	Polar RP Dimensions: 250 x 4.60mm	Only six peaks were obtained
A. 0.02M KH2PO4:ACN pH 5.0 (97:3) **B.** 0.02M KH2PO4:ACN pH 3.5 (80:20)	Luna C18 Dimensions:150 x 30mm	Only six peaks were obtained
A. MeOH: Buffer with formic acid: Acetone (69:24:7) **B.** MeOH: Buffer with formic acid: Acetone (11:82:7)	Luna C18 Phenyl- Hexyl Dimensions: 250 x 4.60mm	Stevioside and RebA not separated
H_2O:ACN (25:75)	Luna C18 Dimensions: 250 x 4.60 mm	Stevioside and RebA not separated
H_2O:ACN (25:75)	NH_2 Dimensions:150 x 30mm	Stevioside and RebA not separated
ACN: H_2O (85:15)	HILIC Dimensions: 250 x 4.60 mm	Stevioside and RebA not separated
ACN: H_2O with gradient system	HILIC Dimensions: 250 x 4.60 mm	Stevioside and RebA not separated
ACN: H_2O (95:5)	HILIC Dimensions: 250 x 4.60 mm	Stevioside and RebA not separated
ACN: H_2O (50:50)	HILIC Dimensions: 250 x 4.60 mm	Stevioside and RebA not separated
ACN: H_2O (95:5) + 0.1 % formic acid	HILIC Dimensions: 250 x 4.60 mm	Stevioside and RebA not separated
ACN: 10mM Ammonium formate, pH 3.0 (95:5)	HILIC Dimensions: 250 x 4.60 mm	Stevioside and RebA not separated

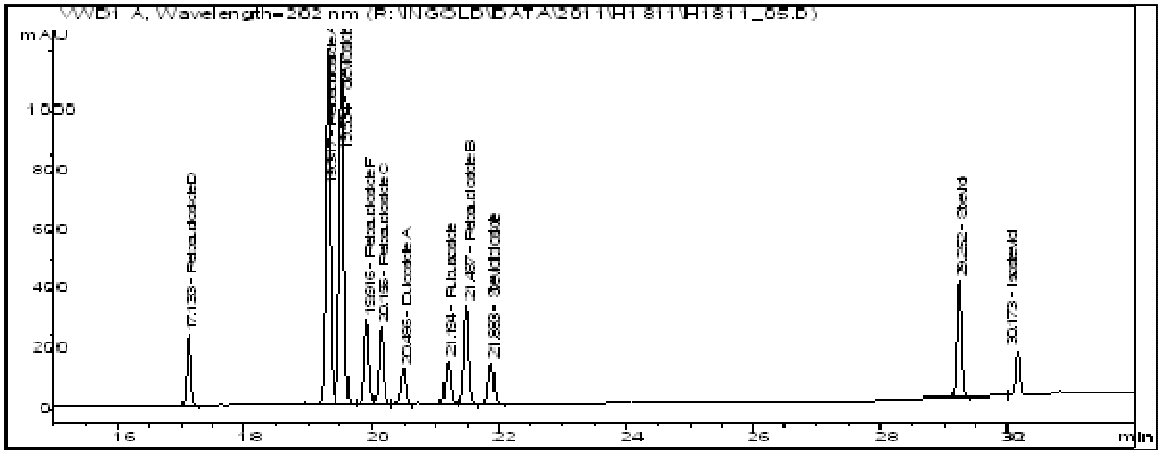


Figure 4: Chromatography of steviol glycosides

The ChromaDex conditions were modified to include 0.1 % phosphoric acid in the mobile phase and this enabled acesulfame-K, saccharin, aspartame, NHDC and neotame to be separated (Figure 5). Under these conditions reb A and stevioside elute around 19.2 and 19.4 minutes respectively (Figure 6). Although dulcoside A elutes at the same retention time as neotame, providing that dulcoside A is not present in any potential samples this should not cause any interference. Dulcoside A can be present in preparations of steviol glycosides but generally at lower concentrations that stevioside or Reb A. The two peaks detected for both acesulfame-K and saccharin are thought to be due to the composition of the solution the standard was prepared in, single peaks can be achieved for each sweetener by altering the pH or the percentage of organic solvent in the solution.

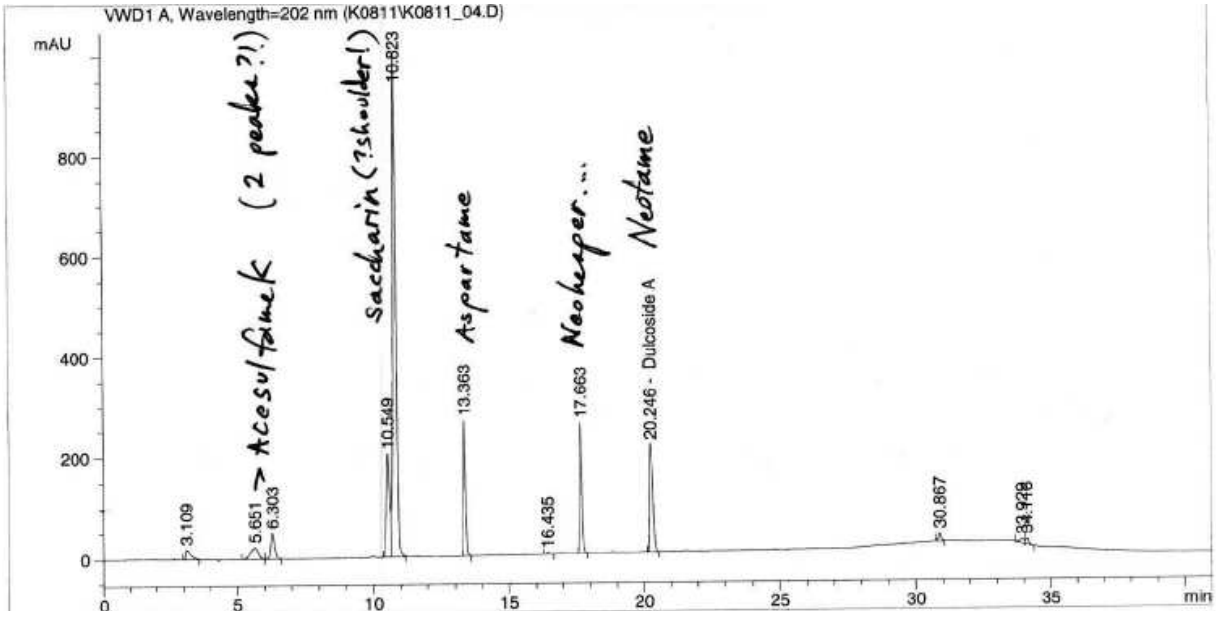


Figure 5: Separation of sweeteners using conditions suitable for separating all the major steviol glycosides

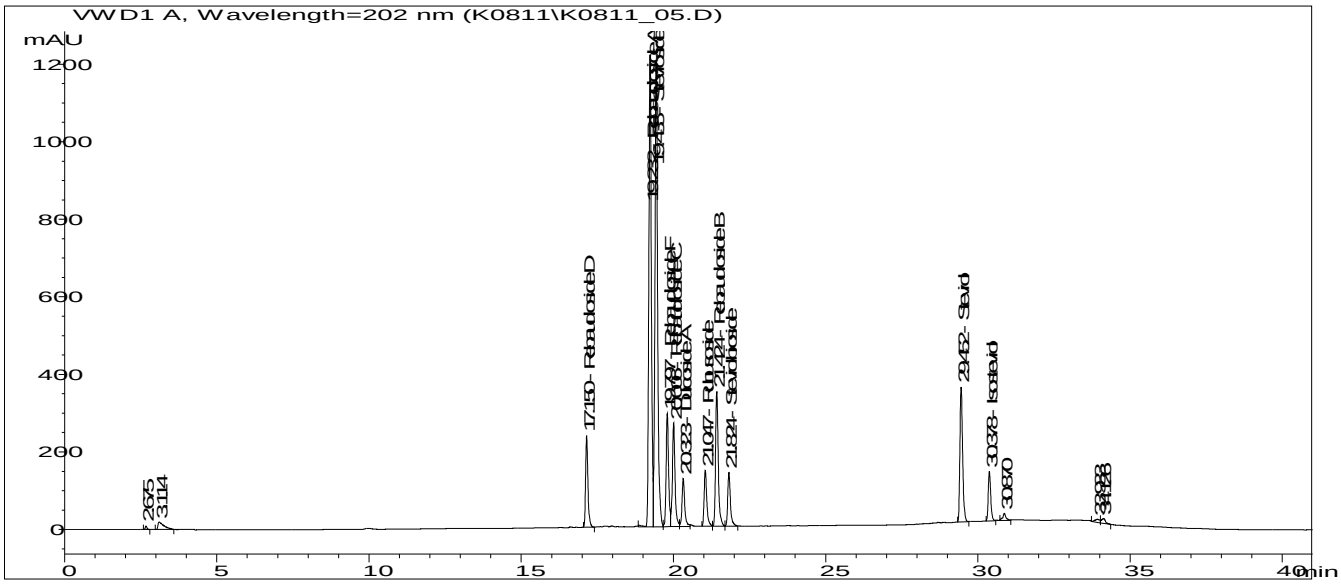


Figure 6: Steviol glycosides

In addition to the above separation, HPLC conditions based on a paper by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) were trialled. The conditions involved a Phenomenex Luna C18 column and isocratic elution with 32:68 acetonitrile: 10mM sodium phosphate buffer, pH 2.6. These conditions proved satisfactory for the majority of the sweeteners however acesulfame-K and saccharin co-eluted. After trying various combinations of these mobile phases the co-elution was resolved by the use of a gradient. Figure 7 shows the separation of the seven sweeteners.

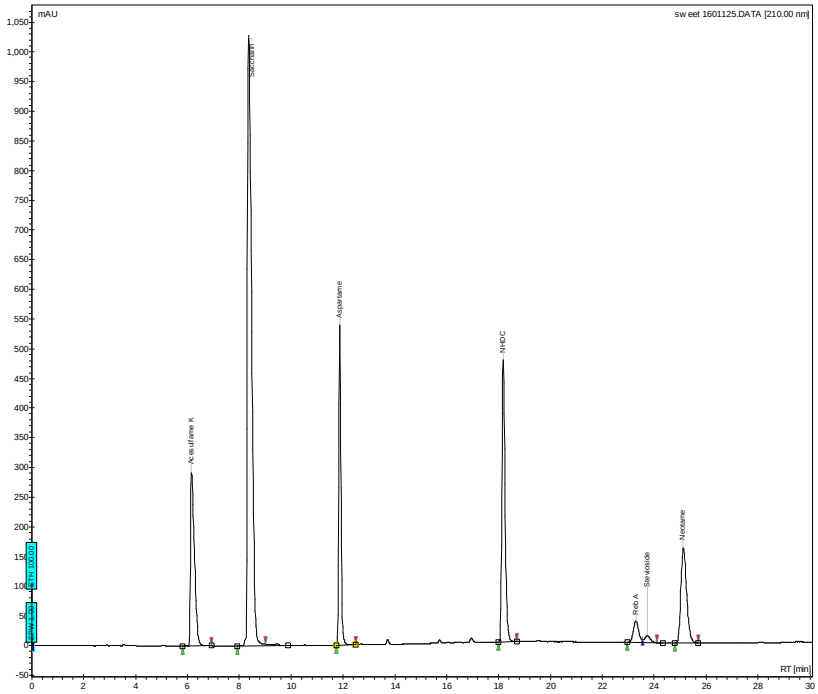


Figure 7: Chromatograph illustrating separation of acesulfame-K, saccharin, aspartame, NHDC, neotame, Reb A and stevioside

The final HPLC conditions which can achieve full separation of the seven sweeteners are shown in box 1.

Column: Luna C18, 5u, 250mm x 4.60mm 5 micron
Flow rate: 1.0 ml/min
Run time: 30 min
Column oven temperature: 40^{o}C
Injection volume: 10µl
Detection at UV 210nm
Mobile phase:
A: 10mM Sodium phosphate monobasic buffer, pH 2.6
B: Acetonitrile

Gradient program:

Time (min)	2	12	25	26	30
Mobile phase %A	90	70	70	90	90
Mobile phase %B	10	30	30	10	10

Box 1: Final chromatography conditions

A draft SOP was prepared describing the extraction (brief details) and the chromatography (HPLC-UV, conditions see Box 1) required to analyse food matrices for acesulfame K, saccharin, aspartame, NHDC, neotame, Reb A and stevioside. This method was taken forward to an in-house single laboratory validation study.

Single Laboratory Validation

Single laboratory validation of the optimised procedure was conducted according to harmonised IUPAC guidelines: The key parameters studied in the validation were:-

- Concentration Range and Applicability
- Detection Limit
- Accuracy
- Precision
- Matrix Effects

The lack of certified reference materials for all of the sweeteners of interest meant that the performance parameters for detection limit precision and accuracy could only calculated from recovery data on spiked blank materials.

Test Materials and Spiking Concentrations

The matrices used for the method validation was based on those foods cited in legislation (Regulation 1333/2008 as amended[3]). The matrices proposed represent a compromise between those in which sweeteners are permitted by legislation or could be present and the costs of a validation exercise that would be comprehensive. Following discussions with the FSA the matrices listed below were chosen to cover aqueous, carbohydrate (sugar and cereal), dairy and miscellaneous products:

- Fruit squash – Blackcurrant juice drink
- Jam – Seedless raspberry
- Biscuits – Rich tea
- Yoghurt (not low fat) – Natural style Greek yogurt
- Carbonated soft drink – Lemon and lime flavoured drink

None of the sweeteners of interest were listed as ingredients in the purchased samples.

Three spiking concentrations were chosen for the validation and are as follows:

1. at or close to the legislative limit to provide an indication of the method applicability for use in enforcement.
2. 50 % of the legislative limit.
3. 150 % of the legislative limit.

The spiking concentrations used for the biscuit matrix in the validation were based on those for breakfast cereal as they are both high carbohydrate products. The maximum permitted concentrations for breakfast cereal are generally lower than for 'fine bakery wares' and therefore the most challenging conditions were tested.

The concentrations listed for fruit squash are for ready-to-drink products. For the method development and validation stages of the project a concentrated fruit drink was purchased and diluted to the ready-to-drink concentration before analysis.

Table 2 illustrates the relevant legislative limits for each sweetener in each of the matrices chosen to be used in the validation:

	mg/kg or mg/l				
	Breakfast cereal	**Yoghurt**	**Jam**	**Fruit squash**	**Carbonate beverage**
Acesulfame K	350	350	1000	350	350
Saccharin	100	100	200	80	80
Aspartame	1000	1000	1000	600	600
NHDC	50	50	50	30	30
Neotame	32	32	32	20	20
Reb A	61	303	606	242	242
Stevioside	50	250	500	200	200

Table 2: Maximum permitted concentration of sweeteners in various foods

[3] Consolidated version available at

http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2008R1333:20130601:EN:HTML

The maximum permitted concentrations of steviol glycosides are specified in the Regulations as steviol equivalents. Table 3 shows the conversion factors needed for each steviol glycoside to calculate their relative concentrations as steviol equivalents.

Trivial name	Formula	Conversion factor
Steviol	$C_{20}H_{30}O_3$	1,00
Stevioside	$C_{38}H_{60}O_{18}$	0,40
Rebaudioside A	$C_{44}H_{70}O_{23}$	0,33
Rebaudioside C	$C_{44}H_{70}O_{22}$	0,34
Dulcoside A	$C_{38}H_{60}O_{17}$	0,40
Rubusoside	$C_{32}H_{50}O_{13}$	0,50
Steviolbioside	$C_{32}H_{50}O_{13}$	0,50
Rebaudioside B	$C_{38}H_{60}O_{18}$	0,40
Rebaudioside D	$C_{50}H_{80}O_{28}$	0,29
Rebaudioside E	$C_{44}H_{70}O_{23}$	0,33
Rebaudioside F	$C_{43}H_{68}O_{22}$	0,34

Table 3: Steviol glycosides conversion factors

Concentration Range and Applicability

Mixed standard solutions were prepared at concentrations between 1 and 60 µg/ml in the injected solutions for acesulfame K, saccharin, aspartame, reb A and stevioside. Due to the lower maximum permitted concentrations for NHDC and neotame, calibration standards for these two sweeteners were prepared at between 0.5 and 40 µg/ml in the injected solutions. The concentration of the sweeteners in the injected solutions is equivalent to the same concentration in the sample when the proposed extraction procedure is followed, for example a determined concentration of 20 µg/ml in the injected solution would be equivalent to 100 µg/g in the sample.

The calibration graphs for each of the sweeteners are shown in figure 8. An r2 value of greater than 0.99 indicates that the calibration was linear over the range tested for each of the analytes.

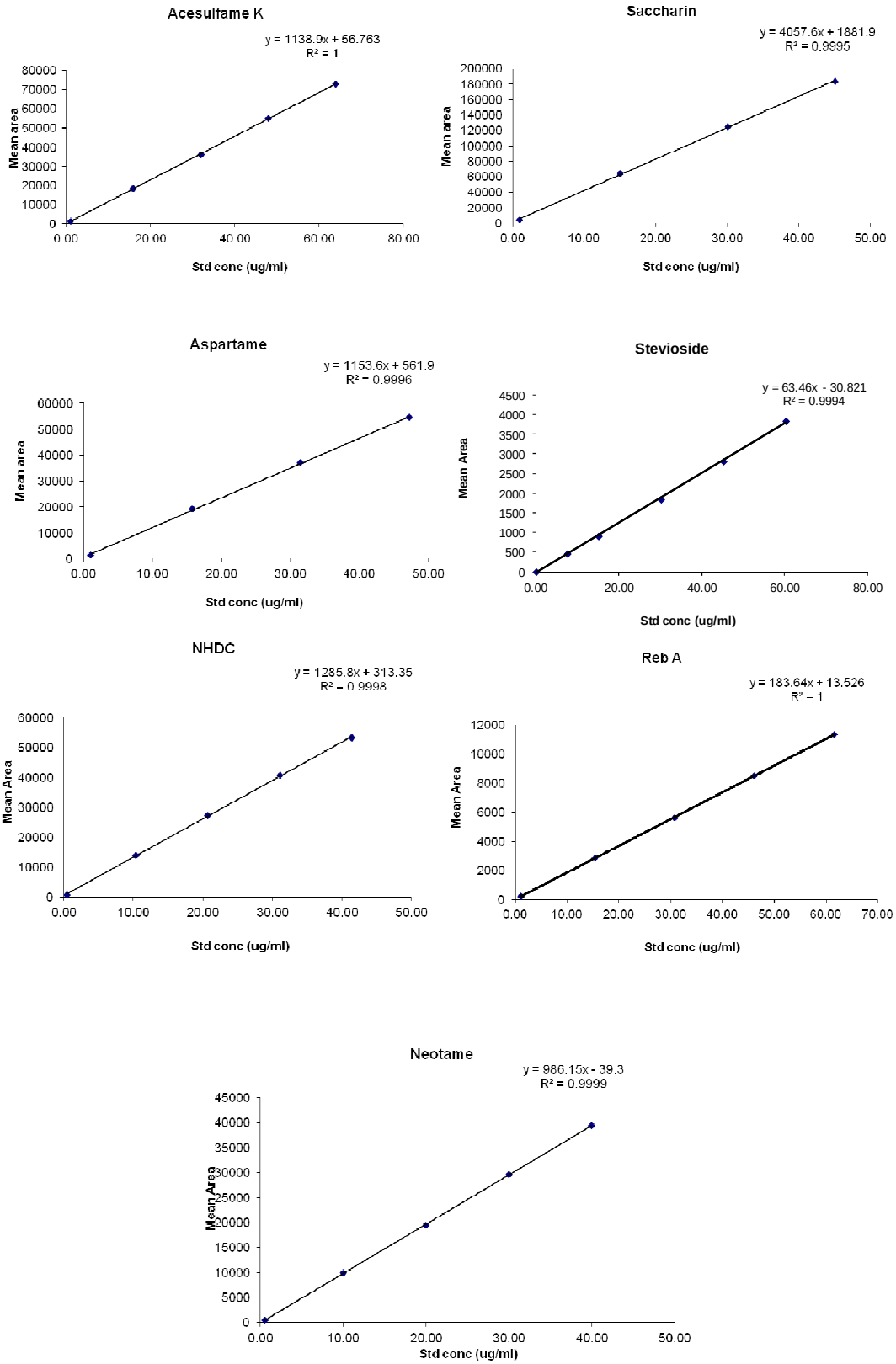


Figure 8. Calibration graphs obtained for each sweetener

Limit of Detection and Limit of Quantification

The standard deviation of the peak areas obtained from repeat injections of the lowest calibration standard was calculated. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3 times the standard deviation and 10 times the standard deviation respectively. Table 4 presents the LOD and LOQ for each of the sweeteners.

Sweetener	LOD (µg/g)	LOQ (µg/g)
Acesulfame K	1.9	6.2
Saccharin	2.0	6.7
Aspartame	1.8	6.1
NHDC	1.8	6.1
Reb A	2.0	6.7
Stevioside	2.7	9.1
Neotame	1.9	6.2

Table 4: Limit of detection (LOD) and limit of quantification for each of the sweeteners

Accuracy and Precision

Each of the five matrices were spiked with acesulfame K, saccharin, aspartame, NHDC, Reb A, stevioside and neotame at the maximum permitted concentration, half of this concentration and 1.5 times the maximum permitted concentration (see Table 2). Duplicate samples were prepared for each matrix. The percentage recoveries are presented in the tables 5 and 6.

Acceptable recoveries (60 – 120 %) were obtained for all sweeteners in all matrices except NHDC in yoghurt and biscuits. The recovery for NHDC in carbonated drinks was also slightly low (mean of 6 recoveries 53.9 %). It was also noted that whilst the recoveries for all of the sweeteners from the jam matrix were around 80 % when spiked at 50 % and 100 % of the legislative limit, the recoveries for the jam samples spiked at the highest level were generally lower indicating that there may be saturation problems at higher concentrations in the matrix.

			% Recovery						
Matrix	**Replicate**	**Level**	**Acesulfame K**	**Saccharin**	**Aspartame**	**NHDC**	**Reb A**	**Stevioside**	**Neotame**
Carbonated drink	A	0.5	76.9	72.7	80.0	53.7	76.6	72.5	75.7
Carbonated drink	B	0.5	76.5	71.1	79.4	56.6	75.7	75.1	64.3
Carbonated drink	A	1.0	75.2	78.5	74.1	64.5	77.9	72.6	66.0
Carbonated drink	B	1.0	77.2	78.1	72.6	53.8	77.7	71.4	66.8
Carbonated drink	A	1.5	74.9	84.9	69.2	49.9	82.5	75.8	71.0
Carbonated drink	B	1.5	73.7	84.5	67.2	45.1	82.6	75.3	70.9
Biscuit	A	0.5	106.6	102.2	80.9	14.1	74.3	67.9	101.2
Biscuit	B	0.5	102.5	99.7	78.2	12.1	75.8	78.6	101.4
Biscuit	A	1.0	93.9	106.1	80.0	13.1	87.8	104.4	93.1
Biscuit	B	1.0	95.8	111.4	81.5	14.0	101.9	108.8	94.2
Biscuit	A	1.5	97.3	113.1	83.0	11.1	102.4	97.7	95.6
Biscuit	B	1.5	99.7	89.7	85.4	26.9	120.1	106.7	108.1
Jam	A	0.5	85.5	93.0	84.3	85.0	84.9	80.9	71.1
Jam	B	0.5	89.9	92.7	89.2	85.7	84.5	82.4	74.3
Jam	A	1.0	82.6	95.4	81.2	81.9	81.6	78.0	75.0
Jam	B	1.0	84.7	100.8	85.1	89.2	85.3	82.7	85.4
Jam	A	1.5	68.1	87.6	68.5	74.6	67.3	66.5	73.8
Jam	B	1.5	67.5	84.1	68.4	72.6	69.3	67.5	73.1
Fruit squash	A	0.5	77.2	77.9	65.2	61.6	80.3	79.4	70.6
Fruit squash	B	0.5	76.6	74.8	64.3	53.7	79.2	75.8	65.3
Fruit squash	A	1.0	77.0	87.9	66.5	70.7	80.9	74.8	82.7
Fruit squash	B	1.0	76.7	87.2	67.3	67.2	78.6	74.1	87.3
Fruit squash	A	1.5	73.8	86.4	62.5	66.5	76.6	74.3	69.4
Fruit squash	B	1.5	71.7	85.4	62.6	67.0	77.4	72.5	70.8
Yoghurt	A	0.5	76.0	73.8	62.4	37.4	86.0	76.6	72.5
Yoghurt	B	0.5	75.0	70.9	68.1	33.0	93.2	69.9	64.2
Yoghurt	A	1.0	74.0	74.8	68.5	36.0	81.3	71.1	67.5
Yoghurt	B	1.0	75.4	76.9	65.1	39.7	83.7	72.3	67.6
Yoghurt	A	1.5	73.0	77.8	65.2	42.5	75.0	70.3	69.3
Yoghurt	B	1.5	73.2	77.1	67.2	41.9	75.5	68.5	71.1

Table 5: Recoveries obtained

	Mean % Recovery						
	Acesulfame K	**Saccharin**	**Aspartame**	**NHDC**	**Reb A**	**Stevioside**	**Neotame**
Carbonated drink	75.7	78.3	73.8	53.9	78.8	73.8	69.1
Biscuit	99.3	103.7	81.5	15.2	93.7	94.0	98.9
Jam	79.7	92.3	79.4	81.5	78.8	76.3	75.5
Fruit squash	75.5	83.3	64.7	64.5	78.8	75.2	74.4
Yoghurt	74.4	75.2	66.1	38.4	82.4	71.4	68.7

Table 6: Mean recoveries for each matrix

Matrix effects

Further investigations were carried out into the low recoveries for NHDC in yoghurt and biscuits. A common factor between these two matrices was the fat content (yoghurt 11 % fat, biscuits 15 % fat), therefore an additional step was added to the procedure to remove the fat prior to extraction of the sweeteners. Spiked replicates of both matrices were shaken with hexane to remove the fat, after centrifugation the hexane was removed and the sample residue dried with a gentle stream of nitrogen. The extraction was then carried out as previous. The NHDC recovery for both yoghurt and biscuits was not improved by this additional step (yoghurt approximately 44 % recovery, biscuit approximately 14 %).

It was thought that the one step where sweeteners may be lost during the extraction procedure was during the SPE clean-up stage. Earlier analyses had shown that if the conditions were not optimum, sweeteners could be washed from the SPE cartridge. Spiked aliquots of yoghurt and biscuit were extracted as previous but the elution from the SPE cartridge was carried out with 10ml of methanol instead of 6ml. Similar recoveries were obtained for NHDC when 10ml was used compared to 6 ml indicating that either there was no NHDC remaining on the cartridge or it could not be easily removed when present in these particular sample extracts.

Design of ruggedness test

The potentially critical steps of the method were identified. For example, as the sweeteners are a wide range of compounds with diverse chemical and physical properties, it was thought that the pH of the extractant may be the key to efficient extraction. HPLC column was chosen as a variable to be investigated as it was envisaged that, if a laboratory did not own the exact column specified in the SOP, a near alternative may be used. It was decided not to include SPE cartridges in the ruggedness testing as it had been determined during the method development stage that the cartridge size and packing was crucial to the successful extraction of the sweeteners.

Therefore the parameters investigated in the ruggedness test of the developed method were as follows:

- pH of extractant
- extraction procedure
- HPLC column
- HPLC column oven temperature

Following discussions with LGC's statistics team the experimental plan presented in Table 7 was drawn up to enable these four parameters to be investigated. The conditions described in the SOP are denoted as A in the table and are replicated in order to evaluate repeatability.

No	Run Order	pH of extractant	Extraction	HPLC column	HPLC column temperature
1	6	A	A	A	A
2	1	A	B	B	B
3	2	A	C	C	C
4	5	B	A	B	C
5	10	B	B	C	A
6	12	B	C	A	B
7	7	C	A	C	B
8	9	C	B	A	C
9	3	C	C	B	A
10	4	A	A	A	A
11	8	A	A	A	A
12	11	A	A	A	A

Table 7: Ruggedness experimental plan

Descriptions of the variables, A, B and C, for each of the parameters investigated are presented in Table 8.

	A	B	C
pH of extractant	pH 4.5	pH 3.5	pH 5.5
Extraction procedure	Sonicate for 15 minutes	Shake by hand	Sonicate for 30 minutes
HPLC column	Phenomenex Luna C18, 5µm, 250 x 4.60 mm Part number 00G-4252-E0	Waters Spherisorb, ODS2, 5µm, 250 x 4.60mm Part number PSS831915	Waters Symmetry, C18, 5µm, 250 x 4.60mm Part number WAT054275
HPLC column temperature	40℃	30℃	50℃

Table 8: Variables investigated during the ruggedness experiments

Individual standard solutions of each of the sweeteners were injected onto each of the three HPLC columns to ensure that the elution order was not altered by the column packing.

The experimental design required all of the extracts to be injected randomly, in order to eliminate any possible drift throughout the run, however, as one of the parameters under investigation was HPLC column, the design could not be followed exactly as it was not feasible to change columns between every injection. Following discussions with LGC's statistics team it was agreed that the extracts should be injected in a random order but grouped by column. Table 9 presents the exact ruggedness experimental plan that was followed.

Extraction Number	Run Order	Run order sorted by column	pH of extractant	Extraction	HPLC column	HPLC column temperature
10	4	1	A	A	A	A
1	6	2	A	A	A	A
11	8	3	A	A	A	A
8	9	4	C	B	A	C
12	11	5	A	A	A	A
6	12	6	B	C	A	B
2	1	1	A	B	B	B
9	3	2	C	C	B	A
4	5	3	B	A	B	C
3	2	1	A	C	C	C
7	7	2	C	A	C	B
5	10	3	B	B	C	A

Table 9: Ruggedness experimental plan grouped by HPLC column

The matrices used to evaluate the ruggedness of the method were jam, fruit squash and yoghurt. These matrices were chosen to represent a high aqueous sample (fruit squash), high carbohydrate sample (jam) and high fat sample (yoghurt). Before extraction, aliquots of the sweetener standard solutions were added to each matrix to obtain spiking concentrations at the maximum permitted concentration for each sweetener.

Results and discussions of ruggedness evaluation

Tables 10, 11 and 12 illustrate the results obtained for each of the seven sweeteners for the three matrices.

pH of Extractant	Extraction	HPLC column	HPLC column temperature	Acesulfame K (ug/g)	Saccharin (ug/g)	Aspartame (ug/g)	NHDC (ug/g)	Reb A (ug/g)	Stevioside (ug/g)	Neotame (ug/g)
4.5	15 mins	Luna	40℃	321	102	445	37.5	212	217	14.4
4.5	15 mins	Luna	40℃	312	114	430	44.9	255	254	18.2
4.5	15 mins	Luna	40℃	305	101	423	38.9	220	224	14.6
5.5	Shake	Luna	50℃	321	208	485	37.8	271	212	21.1
4.5	15 mins	Luna	40℃	335	104	471	41.8	246	245	18.5
3.5	30 mins	Luna	30℃	307	86.4	463	28.6	210	209	14. 7
4.5	Shake	Spherisorb	30℃	156	85.3	379	267	227	206	0.0
5.5	30 mins	Spherisorb	40℃	300	91.5	450	316	215	22 7	0.0
3.5	15 mins	Spherisorb	50℃	288	102	491	337	240	260	0.0
4.5	30 mins	Symmetry	50℃	306	90.4	370	43.5	269	228	0.0
5.5	15 mins	Symmetry	30℃	219	9.3	423	34.3	229	194	14.2
3.5	Shake	Symmetry	40℃	257	71.9	387	33.2	127	379	0 .0

Table 10: Ruggedness results for fruit squash

pH of Extractant	Extraction	HPLC column	HPLC column temperature	Acesulfame K (ug/g)	Saccharin (ug/g)	Aspartame (ug/g)	NHDC (ug/g)	Reb A (ug/g)	Stevioside (ug/g)	Neotame (ug/g)
4.5	15 mins	Luna	40℃	739	192	645	15.3	353	340	18.1
4.5	15 mins	Luna	40℃	525	181	508	12.6	277	319	16.9
4.5	15 mins	Luna	40℃	739	264	647	22.9	362	375	28.8
5.5	Shake	Luna	50℃	607	208	542	18.9	332	306	23.4
4.5	15 mins	Luna	40℃	741	261	654	52.4	0.0	548	16.3
3.5	30 mins	Luna	30℃	588	250	627	17.0	397	135	0.0
4.5	Shake	Spherisorb	30℃	641	98.9	746	21.2	470	445	0.0
5.5	30 mins	Spherisorb	40℃	682	93.7	568	16.2	352	1 016	0.0
3.5	15 mins	Spherisorb	50℃	676	69.4	622	21.8	399	3 87	0.0
4.5	30 mins	Symmetry	50℃	548	130	526	23.5	205	571	0.0
5.5	15 mins	Symmetry	30℃	630	125	657	24.1	353	337	27.7
3.5	Shake	Symmetry	40℃	490	120	512	26.4	289	295	25 .8

Table 11: Ruggedness results for jam

pH of Extractant	Extraction	HPLC column	HPLC column temperature	Acesulfame K (ug/g)	Saccharin (ug/g)	Aspartame (ug/g)	NHDC (ug/g)	Reb A (ug/g)	Stevioside (ug/g)	Neotame (ug/g)
4.5	15 mins	Luna	40℃	203	115	568	0.0	233	0.0	0.0
4.5	15 mins	Luna	40℃	238	89.5	637	8.0	252	245	0.0
4.5	15 mins	Luna	40℃	224	64.5	638	4.8	174	161	0.0
5.5	Shake	Luna	50℃	198	80.1	539	6.2	213	221	21.4
4.5	15 mins	Luna	40℃	201	112	568	9.3	259	271	28.7
3.5	30 mins	Luna	30℃	198	80.1	542	6.4	200	216	19.1
4.5	Shake	Spherisorb	30℃	213	66.7	653	12.8	212	190	0.0
5.5	30 mins	Spherisorb	40℃	255	96.0	676	11.0	304	2 17	0.0
3.5	15 mins	Spherisorb	50℃	264	51.2	673	9.5	227	20 8	0.0
4.5	30 mins	Symmetry	50℃	186	84.6	488	12.1	97.2	43 9	0.0
5.5	15 mins	Symmetry	30℃	174	67.5	504	17.2	149	166	21.7
3.5	Shake	Symmetry	40℃	186	87.6	532	15.2	153	151	2 9.2

Table 12: Ruggedness results for yoghurt

Examples of the chromatograms achieved for the calibration standards and the matrices for each of the HPLC columns are shown in Appendix 1. A visual inspection of the results indicated that column B (Waters Spherisorb) was unsuitable for the determination of neotame as an acceptable calibration curve could not be achieved and no peaks could be detected in the sample extracts at the same retention time as neotame. This is due to the fact that the peak obtained for neotame on column B was very wide which meant that the low concentrations in the sample extracts could not be detected.

For extraction 5 (pH 3.5, shaken by hand, Symmetry column at 40℃) for the fruit squash and extraction 3 (pH4.5, 30 minutes extraction, Symmetry column at 50℃) for the yoghurt it was noted that the Reb A result was slightly low and the Stevioside was slightly high. Both these extracts were run using a Waters Symmetry column where the Reb A and Stevioside peaks elute close together. It is thought that these anomalous results may be due to incomplete separation.

Statistical evaluation of the data indicated that the extraction process had no effect on the results obtained for any of the sweeteners in any of the matrices. The pH of the extractant was also shown to have no effect on any of the sweeteners in any of the matrices. Acesulfame K in fruit squash was the only matrix / sweetener combination which was affected by HPLC column temperature. The major contributor to variation in the results for several sweeteners in all three matrices was the HPLC column.

The data was investigated to predict a set of values which would, theoretically, show the combination of the four parameters which would allow the maximum yield for each sweetener to be achieved. For fruit squash the software predicted that the optimum conditions were pH 5.5 extractant shaken by hand and HPLC column A (Phenomenex Luna C18) at 50℃. These conditions did not provide the highest result for NHDC but on further examination of the chromatograms it was thought that the peak originally identified as NHDC was at a slightly earlier retention time than seen for the standards and may not be NHDC.

The optimum conditions predicted for jam also involved pH 5.5 extractant shaken by hand and HPLC column A, however a column temperature of 30℃ was suggested.

For yoghurt, extraction at pH 5.5 was suggested but with a more vigorous extraction procedure (sonication for 30 minutes). The maximum yield for the majority of the

sweeteners was predicted to occur with column B (Waters Spherisorb) at 50℃, however it was predicted that the yield for NHDC and neotame would increase if column C (Waters Symmetry) was used.

Conclusions on ruggedness

The extraction procedure described in the draft SOP for the determination of acesulfame K, aspartame, saccharin, NHDC, Reb A, stevioside and neotame has proved to be sufficiently rugged. The temperature of the HPLC column was found to have an effect on the determination of acesulfame K in fruit squash with the results for temperature B (30℃) being generally lower. It will be stated in the SOP that a column temperature of less than 40℃ should not be used for this matrix. The major contributor to any variation in results was the HPLC column. Column A (Phenomenex Luna) was found to be optimum for the majority of the sweeteners but it was noted that the recovery for NHDC in yoghurt increased when column C (Waters Symmetry) was used.

The method developed is suitable for the determination of the seven sweeteners. However, if NHDC is detected in a matrix with a high-fat content it may be advisable to re-run the extracts with alternative chromatography conditions to confirm the quantity of the sweetener present.

Transfer of the Method to a Second laboratory

A second laboratory validation / pre-study method check was performed by ChromaDex to ensure that the method validated at LGC could be transferred to another laboratory.

Design of pre-study method check

Following discussions with LGC's statistics team, aliquots of yoghurt, jam and fruit squash were included in the pre-study method check. As with the ruggedness evaluation, these matrices were chosen as they represented a high fat matrix (yoghurt), a high carbohydrate matrix (jam) and a high aqueous matrix (fruit squash). The sample of fruit squash was diluted to the ready-to-drink concentration before being dispatched. The second laboratory, ChromaDex, was asked to analyse each matrix spiked in duplicate at 0.5 and 1.5 the maximum permitted limit. This analysis was carried out on each of three days. A copy of the SOP was sent together with the samples and ChromaDex was asked to follow this without deviations. The conditions specified in the SOP were those described as A in the ruggedness evaluation, i.e. pH 4.5 extraction buffer, extraction for 15 minutes with sonication and a Phenomenex Luna C18 HPLC column at a temperature of 40℃. A copy of the SOP is presented in Appendix 2.

Pre-study method check

Presented in Table 13 are the recoveries obtained by ChromaDex for the pre-study method check. The analysis undertaken by ChromaDex was also carried out at LGC and the results are presented in Table 14.

				% Recovery						
Matrix	**Spike Level**	**Replicate**	**Day**	**Acesulfame K**	**Saccharin**	**Aspartame**	**NHDC**	**Reb A**	**Stevioside**	**Neotame**
Yoghurt	0.5 limit	A	1	99.3	105.8	16.1	67.7	105.9	100.2	39.9
Yoghurt	0.5 limit	B	1	95.7	102.4	15.1	58.1	103.9	97.1	36.3
Yoghurt	1.5 limit	A	1	100.4	99.7	17.8	64.0	112.0	107.5	45.5
Yoghurt	1.5 limit	B	1	102.9	102.2	22.5	66.9	115.7	105.5	48.0
Yoghurt	0.5 limit	A	2	102.6	100.8	39.3	74.5	111.3	102.2	81.2
Yoghurt	0.5 limit	B	2	102.5	101.1	31.9	74.5	115.7	104.8	73.5
Yoghurt	1.5 limit	A	2	110.8	99. 6	42.8	76.1	125.2	119.4	92.4
Yoghurt	1.5 limit	B	2	105.8	100.9	43.2	76.1	118.6	121.1	93.5
Yoghurt	0.5 limit	A	3	106.1	108.4	74.1	81.7	118.0	98.7	105.1
Yoghurt	0.5 limit	B	3	107.5	111.2	73.5	84.5	122.9	104.1	98.8
Yoghurt	1.5 limit	A	3	105.8	111.2	90.2	77.8	115.7	97.4	103.4
Yoghurt	1.5 limit	B	3	106.2	105.5	92.1	82.1	113.2	105.0	109.7
Jam	0.5 limit	A	1	85.0	83.6	70. 8	57.7	86.1	83.1	86.8
Jam	0.5 limit	B	1	98.3	96.9	81.6	61.9	101.2	95.4	98.6
Jam	1.5 limit	A	1	106.7	110.3	94.9	90.6	110.0	107.6	97.3
Jam	1.5 limit	B	1	106.9	101.4	94.0	91.8	110.8	109.2	99.4
Jam	0.5 limit	A	2	97.9	97.0	89.7	82.7	103.1	97.8	96.6
Jam	0.5 limit	B	2	86.7	87.8	83. 7	85.4	96.7	93.1	101.8
Jam	1.5 limit	A	2	91.6	94.7	91.0	92.5	101.4	98.4	92.8
Jam	1.5 limit	B	2	96.2	100.9	94.1	97.7	108.3	103.1	100.2
Jam	0.5 limit	A	3	92.4	89.5	90.8	84.6	103.8	96.2	98.8
Jam	0.5 limit	B	3	94.3	91.7	91.7	86.5	107.4	101.3	102.1
Jam	1.5 limit	A	3	105.0	105.1	104.0	104.1	120.7	106.2	101.7
Jam	1.5 limit	B	3	104.6	107.0	105.5	101.8	120.7	113.7	103.6
Fruit squash	0.5 limit	A	1	103.4	101.8	63.3	85.0	101.7	100.4	101.4
Fruit squash	0.5 limit	B	1	106.1	105.4	63.4	108.1	103.8	101.1	102.9
Fruit squash	1.5 limit	A	1	100.2	103.5	59.2	93.3	80.8	100.9	100.4
Fruit squash	1.5 limit	B	1	100.0	104.7	59.4	89.7	80.6	100.6	99.0
Fruit squash	0.5 limit	A	2	105.6	97.0	75.4	87.7	106.7	101.2	111.6
Fruit squash	0.5 limit	B	2	113.9	108.1	48.1	108.6	113.5	105.7	108.6
Fruit squash	1.5 limit	A	2	105.9	100.4	64.7	108.2	107.8	110.4	104.8
Fruit squash	1.5 limit	B	2	99.6	94.4	87.8	98.7	103.0	101.2	97.4
Fruit squash	0.5 limit	A	3	111.8	110.2	79.5	99.1	120.3	106.0	114.1
Fruit squash	0.5 limit	B	3	107.7	108.8	97.5	104.1	118.6	111.0	114.9
Fruit squash	1.5 limit	A	3	107.3	103.2	87.8	96.1	114.3	105.6	112.4
Fruit squash	1.5 limit	B	3	103.5	99.3	90.3	112.5	110.5	106.0	111.8

Table 13: Pre-study method check results from ChromaDex

				% Recovery						
Matrix	**Spike Level**	**Replicate**	**Day**	**Acesulfame K**	**Saccharin**	**Aspartame**	**NHDC**	**Reb A**	**Stevioside**	**Neotame**
Yoghurt	0.5 limit	A	1	106.5	91.0	104.6	53.1	100.7	189.7	104.0
Yoghurt	0.5 limit	B	1	98.6	77.6	93.8	48.2	93.8	183.4	80.4
Yoghurt	1.5 limit	A	1	106.3	105.4	101.3	59.8	110.1	232.6	112.7
Yoghurt	1.5 limit	B	1	99.2	97.6	93.8	51.6	103.7	239.6	98.9
Yoghurt	0.5 limit	A	2	102.6	96. 8	92.0	64.5	88.5	93.2	80.6
Yoghurt	0.5 limit	B	2	96.6	90.4	85.0	61.0	80.3	86.8	74.1
Yoghurt	1.5 limit	A	2	99.0	96.6	87.8	70.8	103.1	98.5	96.7
Yoghurt	1.5 limit	B	2	95.6	93.2	82.0	77.4	108.2	88.9	87.3
Yoghurt	0.5 limit	A	3	96.0	86.9	92.1	58.6	97.4	171.7	78.2
Yoghurt	0.5 limit	B	3	100.1	92.6	94.3	62.9	104.0	163.2	81.6
Yoghurt	1.5 limit	A	3	99.2	96.4	86.6	71.0	96.6	111.5	98.3
Yoghurt	1.5 limit	B	3	99.2	96.2	93.6	68.9	96.2	102.3	92.9
Jam	0.5 limit	A	1	101.2	93.4	105.9	81.6	98.6	146.5	104.5
Jam	0.5 limit	B	1	88.7	81.9	93.1	73.3	90.5	132.1	79.4
Jam	1.5 limit	A	1	82.5	91.5	96.4	91.6	101.4	149.0	105.8
Jam	1.5 limit	B	1	78.6	95.6	97.0	93.3	101.6	133.5	102.9
Jam	0.5 limit	A	2	96.7	110.9	104.3	77. 8	90.7	95.5	87.4
Jam	0.5 limit	B	2	102.8	102.8	101.7	76. 0	90.6	88.1	91.9
Jam	1.5 limit	A	2	87. 8	87. 8	97.5	84.3	90.1	95.4	84.8
Jam	1.5 limit	B	2	84.1	84.1	96.1	87.6	90.2	94.6	93.5
Jam	0.5 limit	A	3	112.1	118.5	110.3	90.5	80.2	114.1	93.3
Jam	0.5 limit	B	3	99.8	99.8	100.1	87.5	86.6	102. 8	87.5
Jam	1.5 limit	A	3	45.4	45.4	60.8	60.9	54.2	77.4	62.9
Jam	1.5 limit	B	3	79. 9	79. 9	103.0	98.1	89.9	95.6	106.3
Fruit squash	0.5 limit	A	1	105.1	82.4	99.8	86.5	107.4	149.4	129.5
Fruit squash	0.5 limit	B	1	106.3	106.3	90.0	81.1	100.4	139.6	120.4
Fruit squash	1.5 limit	A	1	100.1	100.1	95.6	90.0	105.5	145. 8	105.9
Fruit squash	1.5 limit	B	1	110.7	110.7	97. 5	101.0	100.5	112.6	119.0
Fruit squash	0.5 limit	A	2	95.1	114.0	104.3	102.1	71.0	62.3	70.3
Fruit squash	0.5 limit	B	2	95.7	114.5	104.0	106.6	83.5	106.9	85.0
Fruit squash	1.5 limit	A	2	118.4	108.2	99.5	110.5	86.4	92.4	90.7
Fruit squash	1.5 limit	B	2	117.4	103.8	97. 9	108.8	85.7	92.7	94.7
Fruit squash	0.5 limit	A	3	101.3	100.8	99.1	100.5	94.0	139.2	82.7
Fruit squash	0.5 limit	B	3	105.8	105.1	105.5	105.4	98.6	128.1	87.7
Fruit squash	1.5 limit	A	3	109.0	111.1	104.7	110.4	99. 7	132. 7	110.9
Fruit squash	1.5 limit	B	3	130. 8	131.4	122.6	117.2	117.4	157.6	134.5

Table 14: Pre-study method check results from LGC

The mean recoveries obtained for each sweetener and matrix combination for both ChromaDex and LGC are presented in Table 15. The mean recovery reported by

ChromaDex for aspartame in yoghurt is low because, for unknown reasons, the recoveries for this sweetener in this particular matrix were very low for the first two batches (mean recoveries of 17.9 and 39.4 % for batches 1 and 2 respectively), however the recoveries obtained in the third batch were acceptable (mean 82.5 %). Low recoveries were also reported for neotame in yoghurt for the first batch (mean 42.4 %). These anomalous recoveries, obviously, had an effect on the calculated value for the reproducibility as can be seen in Table 16. High stevioside recoveries were also observed for the analysis carried out by LGC.

		ChromaDex	LGC
Matrix	**Sweetener**	**Mean Recovery**	**Mean Recovery**
Yoghurt	Acesulfame K	104	100
Yoghurt	Saccharin	104	93
Yoghurt	Aspartame	47	92
Yoghurt	NHDC	74	62
Yoghurt	Reb A	115	99
Yoghurt	Stevioside	105	147
Yoghurt	Neotame	77	90
Jam	Acesulfame K	97	88
Jam	Saccharin	97	91
Jam	Aspartame	91	97
Jam	NHDC	86	84
Jam	Reb A	106	89
Jam	Stevioside	100	110
Jam	Neotame	98	92
Fruit squash	Acesulfame K	105	108
Fruit squash	Saccharin	103	107
Fruit squash	Aspartame	73	102
Fruit squash	NHDC	99	102
Fruit squash	Reb A	105	96
Fruit squash	Stevioside	104	122
Fruit squash	Neotame	107	103

Table 15: Pre-study method check –Mean recoveries

The repeatability and reproducibility for the method were determined for each matrix. As no variation was seen between the performance of the different sweeteners in jam, a single repeatability and reproducibility figure was determined which is applicable to all of the six sweeteners in this matrix. Individual repeatability and reproducibility values were determined for each individual sweetener for the yoghurt and fruit squash matrix (Table 16). As the high recoveries seen for stevioside were not consistent, repeatability and reproducibility was not calculated for this sweetener.

		ChromaDex		LGC	
Matrix	**Sweetener**	**Repeatability**	**Intermediate precision**	**Repeatability**	**Intermediate precision**
Yoghurt	Acesulfame K	2.5	5.1	3.3	3.9
Yoghurt	Saccharin	3.9	7.7	5.9	7.1
Yoghurt	Aspartame	26.1	52.3	5.4	6.5
Yoghurt	NHDC	5.0	10.0	9.5	11.3
Yoghurt	Reb A	4.8	9.7	6.9	8.2
Yoghurt	Neotame	24.8	49.7	8.5	10.2
Jam	Acesulfame K	6.0	7.2	12.5 (7.3)*	12.9 (7.3)*
Jam	Saccharin				
Jam	Aspartame				
Jam	NHDC				
Jam	Reb A				
Jam	Neotame				
Fruit squash	Acesulfame K	3.5	5.8	8.0	9.6
Fruit squash	Saccharin	5.4	8.9	10.1	12.1
Fruit squash	Aspartame	13.3	21.8	6.4	7.6
Fruit squash	NHDC	8.8	14.5	7.1	8.5
Fruit squash	Reb A	8.7	14.2	13.5	16.1
Fruit squash	Neotame	3.1	5.1	22.0	26.2

Table 16: Pre-study method check - Repeatability and reproducibility

*It was noted that the recoveries obtained by LGC for replicate A of the jam sample spiked at 1.5 times the maximum permitted limit on day three were significantly lower than for the other replicates. The figures above take into account the low recoveries but if this replicate were to be considered as an outlier the repeatability and reproducibility would be 7.3 and 0.8 respectively.

Stevioside

The pre-study check highlighted a problem with the recovery measured for stevioside on some occasions. This had not been apparent previous to the pre-study check. The mean recovery for stevioside obtained by ChromaDex for the three matrices was close to 100 % (103.3 %) whereas the mean obtained by LGC was significantly higher (126.3 %). The method was amended as described in Appendix 3 to try and improve its robustness but although the amendments improve the method in that they may extend the life of the HPLC system and improve injection repeatability, consistent recoveries for stevioside within the acceptable quality criteria of 60 to 120 % were not obtained. The amendments to the method included pre-mixing the mobile phases at the initial gradient conditions, i.e. 90:10 sodium phosphate buffer: acetonitrile and using this solution to prepare the standard solutions and sample extracts, and an increase in the injection volume from 10 to 50 µl.

The analysis carried out for the pre-study method check was repeated at LGC using the amended SOP, i.e. the three matrices were spiked in duplicate at two concentrations and were analysed on each of three days. The mean results are shown in Table 17 and show that the recovery for stevioside did not improve significantly when the amended method was employed.

	Mean % recovery of duplicate extractions of samples spiked at 2 different concentrations on each of 3 days						
	Acesulfame K	**Saccharin**	**Aspartame**	**NHDC**	**Reb A**	**Stevioside**	**Neotame**
Yoghurt - Original method	100	93	92	62	99	147	90
Jam - Original method	88	91	97	84	89	110	92
Fruit squash- Original method	108	107	102	102	96	122	103
Yoghurt - Amended method	96	92	95	30	96	144*	87
Jam - Amended method	98	99	90	100	91	121	91
Fruit squash- Amended method	100	88	83	87	92	128	94

* Two outliers removed due to possible problems during the spiking procedure.

Table 17: Repeat of pre-study method check at LGC

Various investigations were carried out to try and establish the reasons behind the over recovery of stevioside and these are detailed in Appendix 4. As, to date, an acceptable explanation for the over recovery of stevioside has not been found, it is recommended that the results for stevioside be corrected for recovery. A comment has been added to the SOP stating that for each batch, at least one sample of each matrix type should be spiked at the maximum permitted concentration of each of the sweeteners and the results obtained for the samples be corrected for recovery.

Conclusions

A method for the simultaneous determination of acesulfame K, aspartame, saccharin, neohesperidine dihydrochalcone, rebaudioside A and neotame has been developed involving extraction with aqueous pH 4.5 buffer followed by SPE clean-up and HPLC with UV detection. The method is also suitable for the determination of stevioside, however recoveries above the acceptable range of 60 to 120 % have been observed for this sweetener.

Appendix 1: Example chromatograms of each matrix on each column used for the ruggedness evaluation

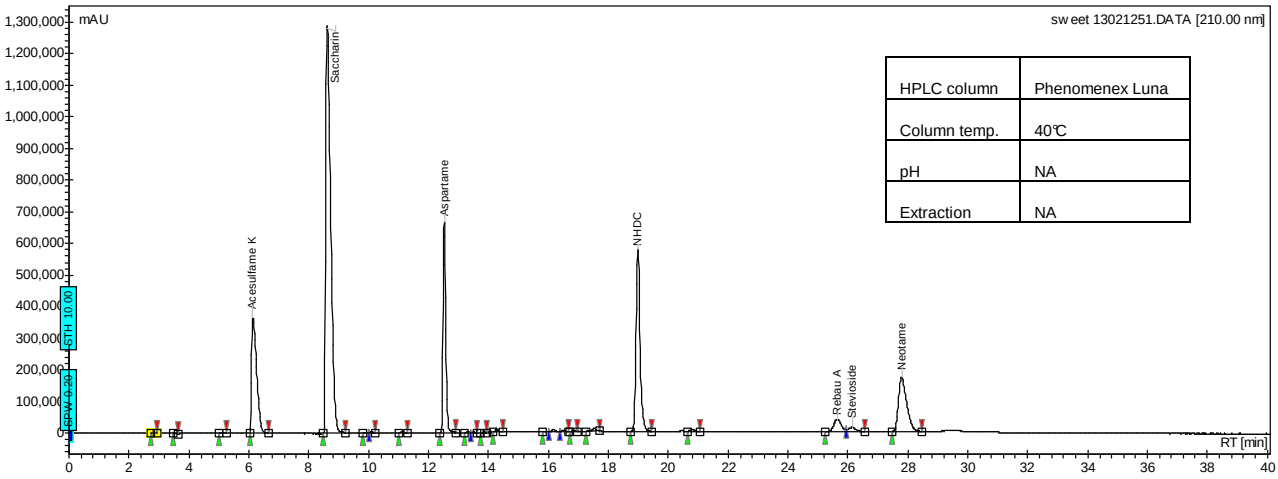


Figure 1: Standard – Phenomenex Luna

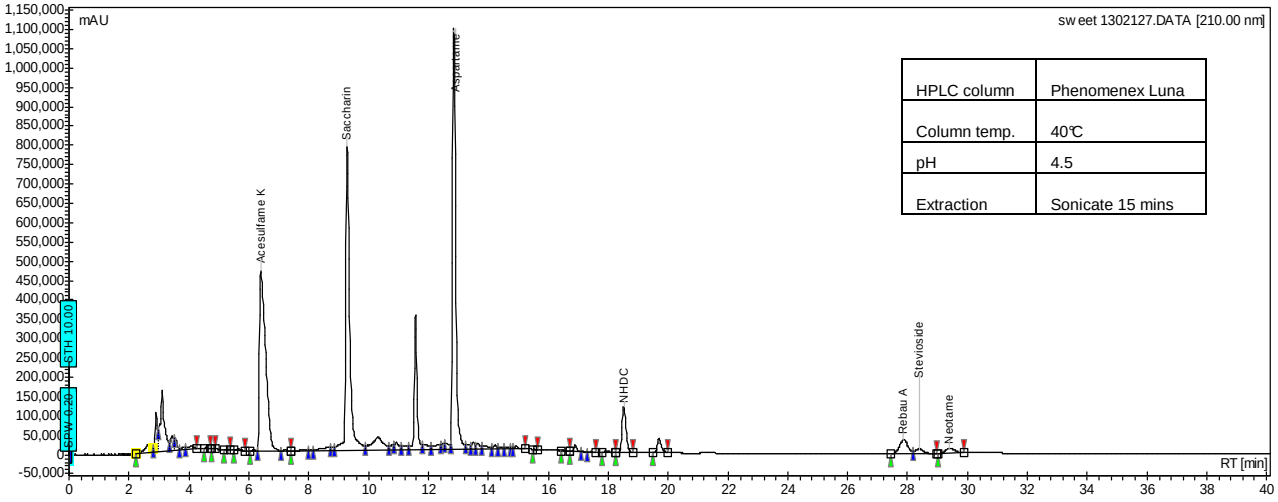


Figure 2: Fruit squash – Phenomenex Luna

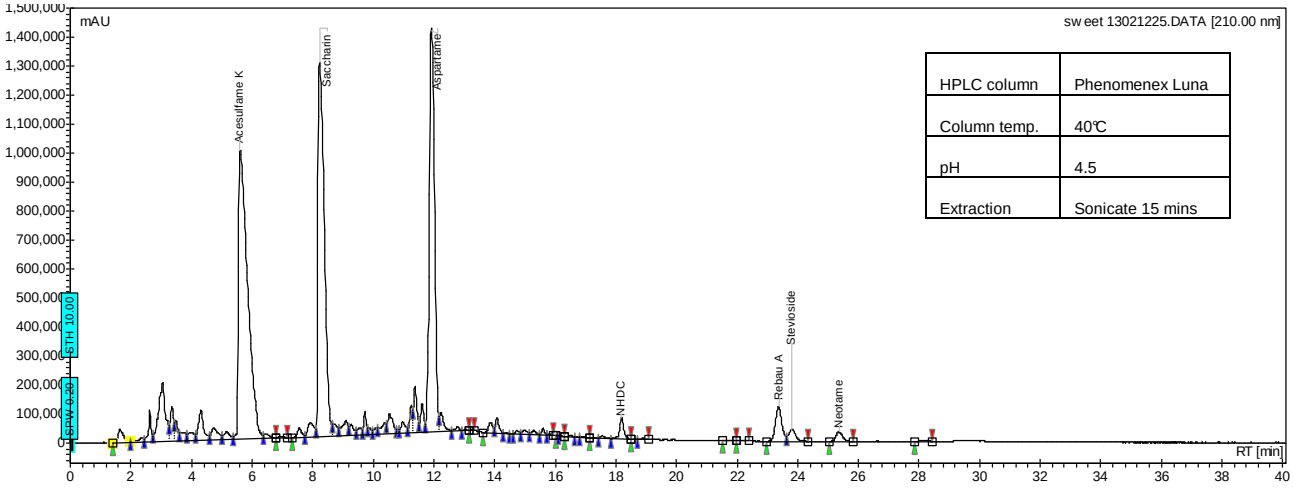


Figure 3: Jam – Phenomenex Luna

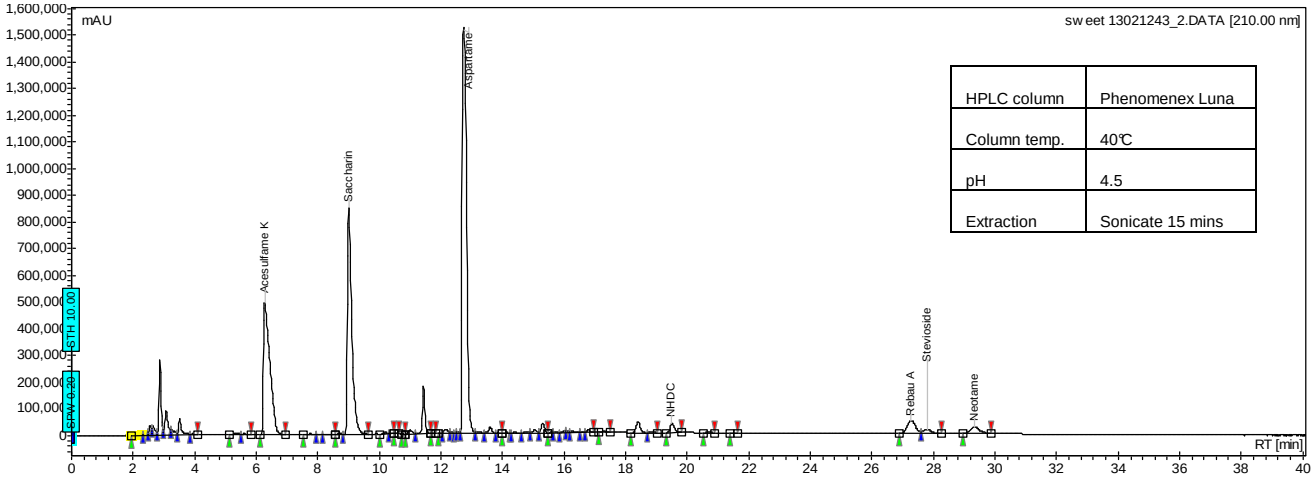


Figure 4: Yogurt – Phenomenex Luna

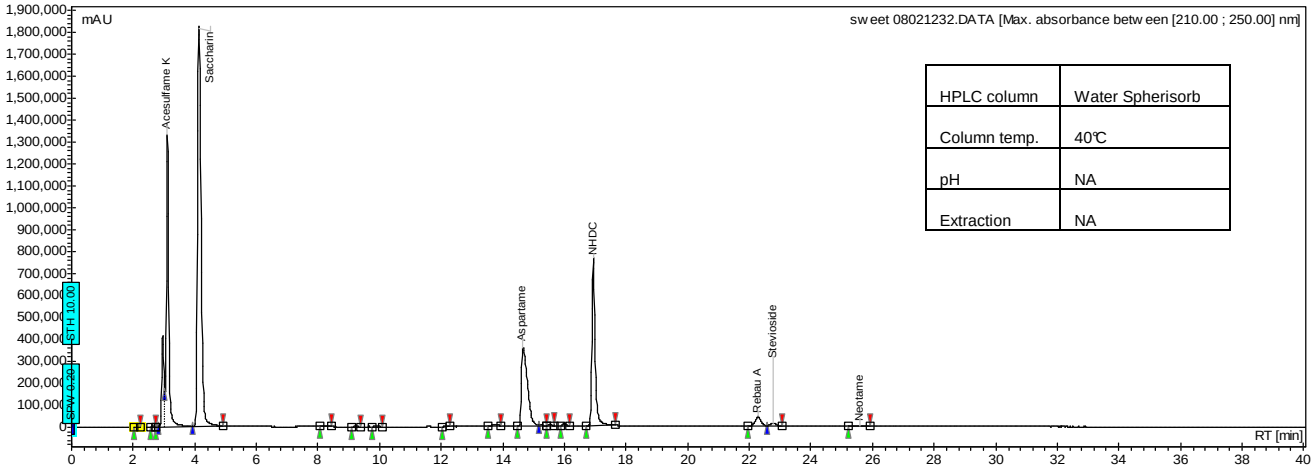


Figure 5: Standard – Waters Spherisorb

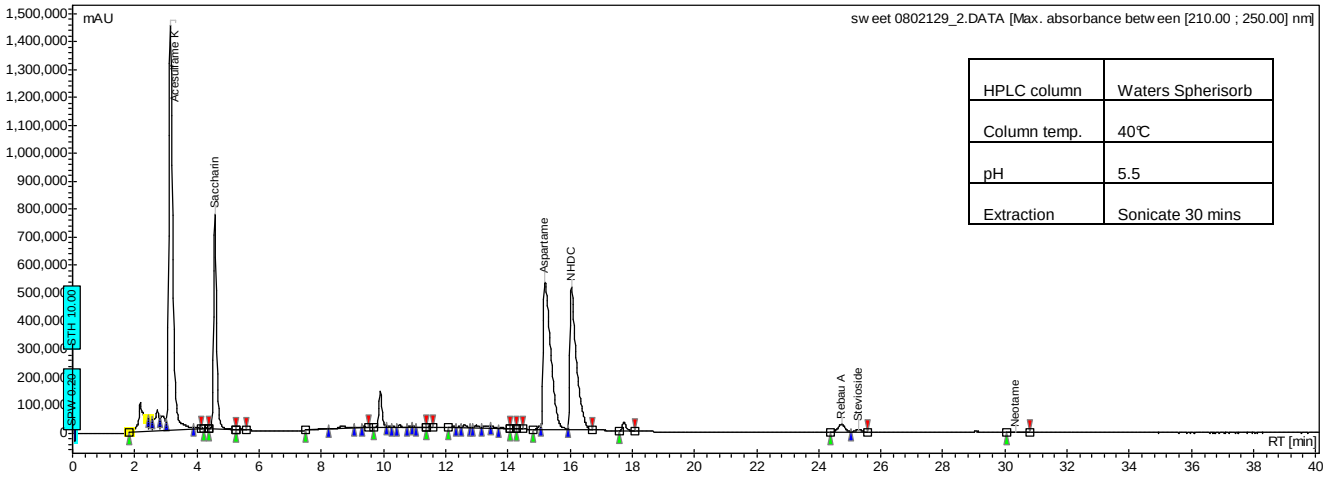


Figure 6: Fruit squash – Waters Spherisorb

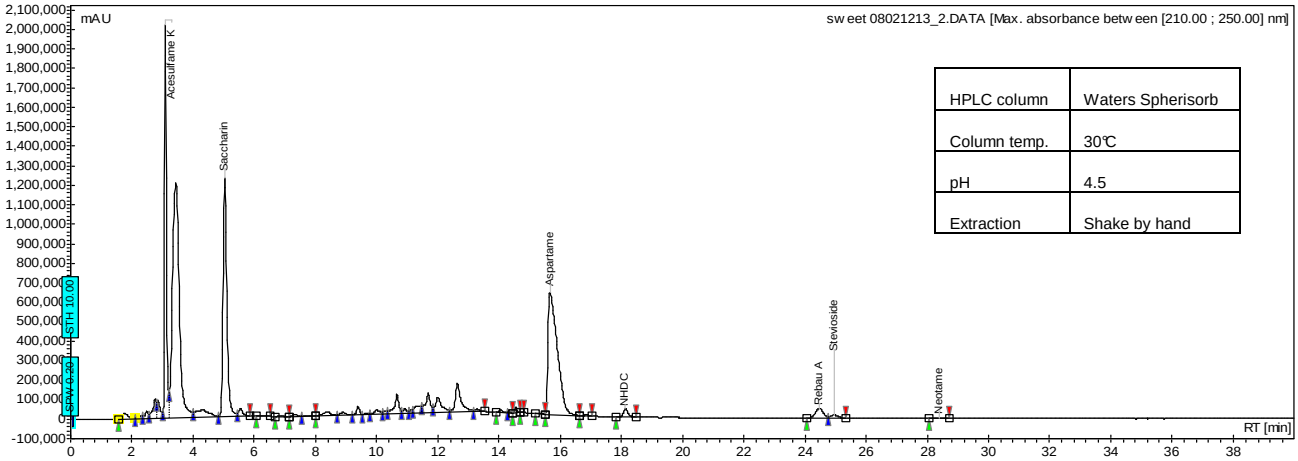


Figure 7: Jam – Waters Spherisorb

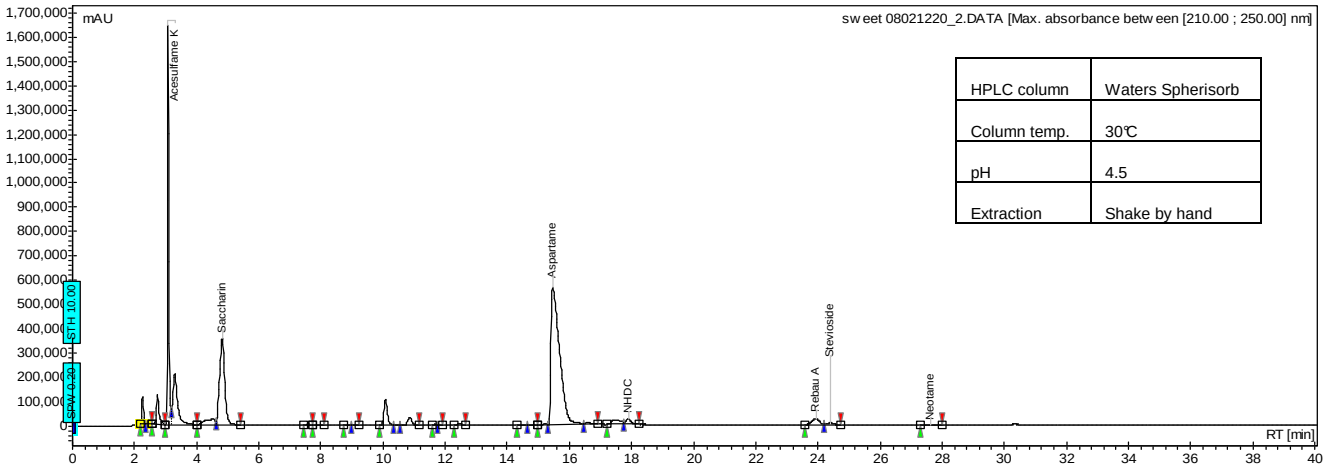


Figure 8: Yoghurt – Waters Spherisorb

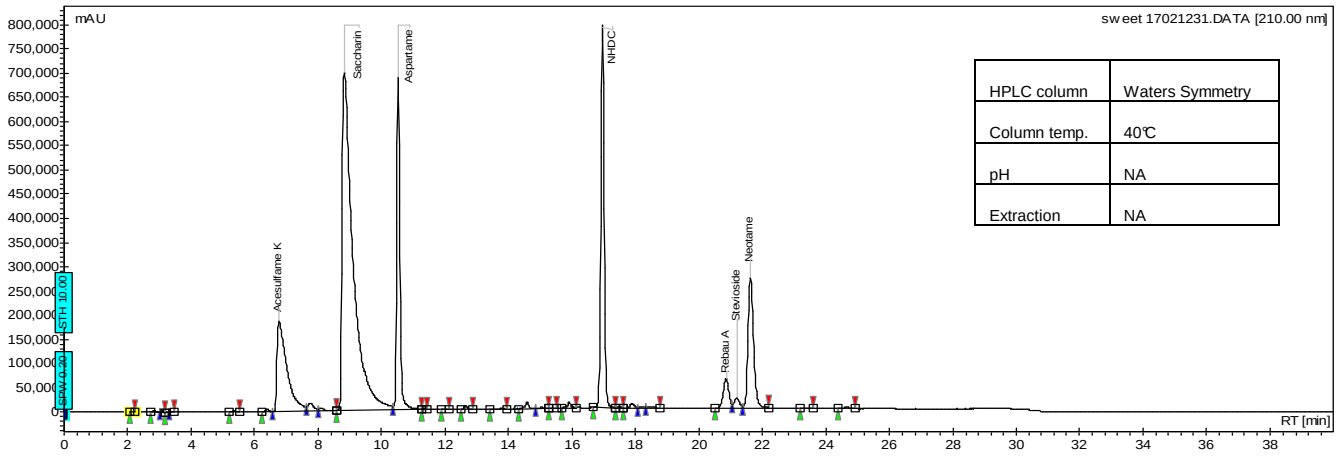


Figure 9: Standard – Waters Symmetry

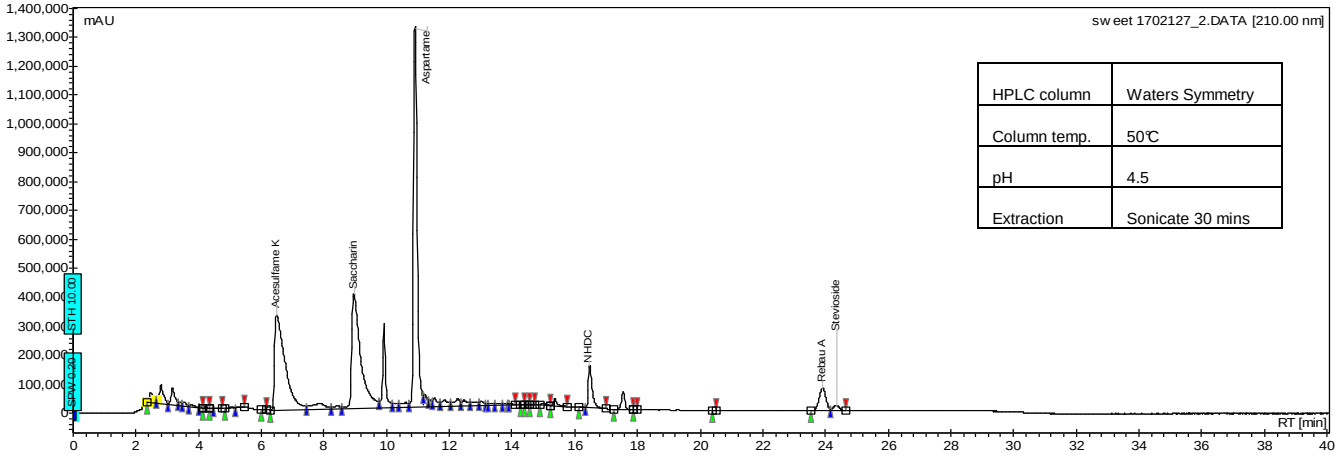


Figure 10: Fruit squash – Waters Symmetry

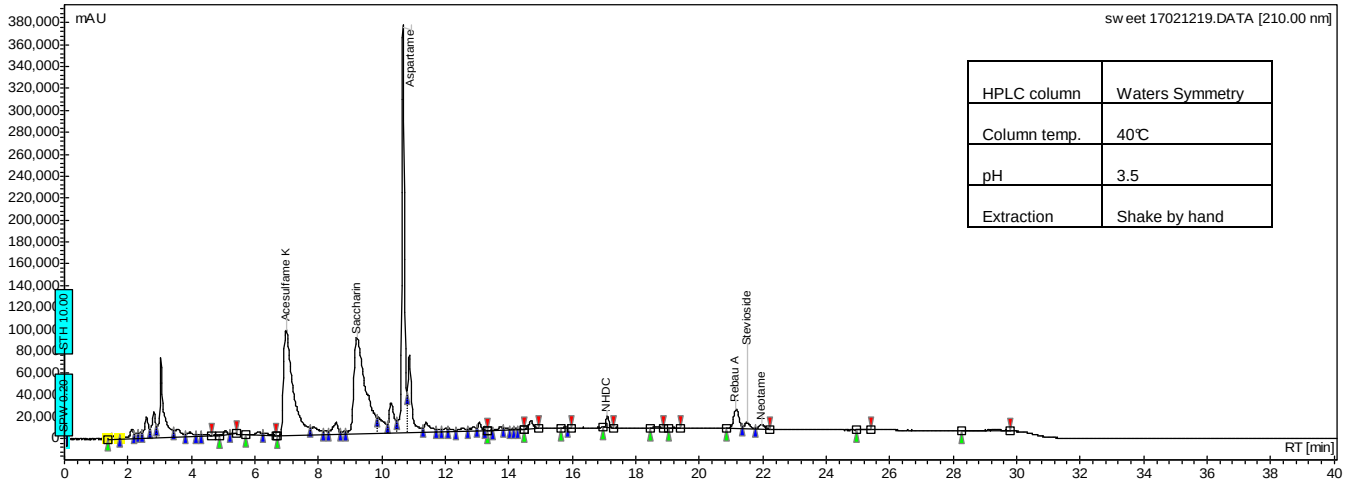


Figure 11: Jam – Waters Symmetry

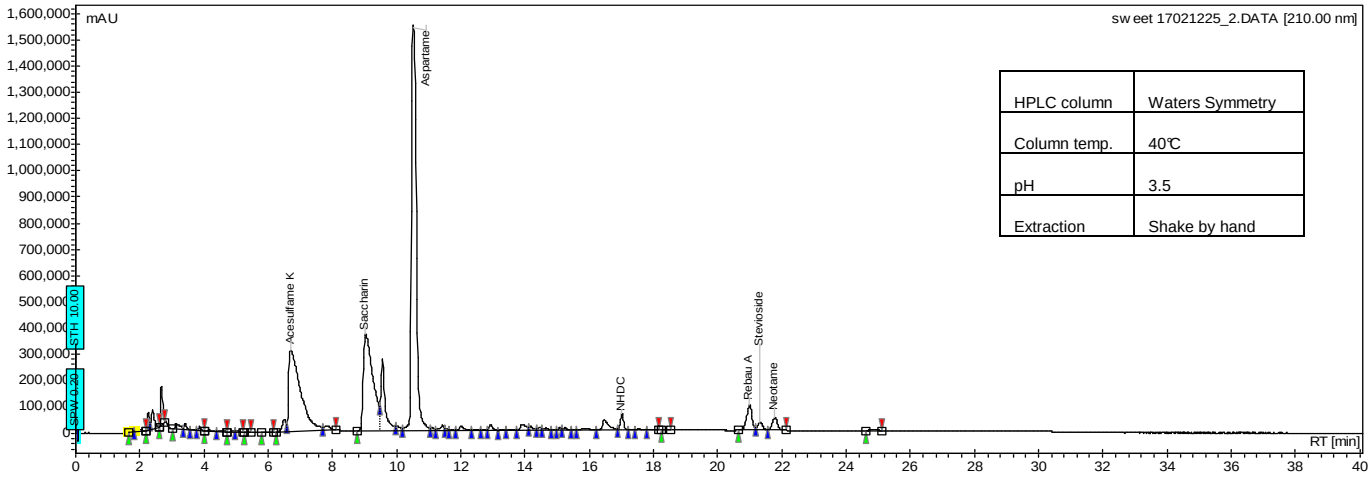


Figure 12: Yoghurt – Waters Symmetry

Appendix 2: SOP for the Simultaneous determination of seven sweeteners by high performance liquid chromatography

LGC Limited

STANDARD OPERATING PROCEDURE

Number: **FFF/**

Title: Simultaneous determination of seven sweeteners by high performance liquid chromatography

Issue number: **1 Draft**

Author: Julia Vitzilaiou

Issue date:

Next Review Date	Reviewed by	Date

Authorised by: **Peter Colwell**

Authorising signature:

UNCONTROLLED IF NOT SIGNED BY STATED AUTHORISED PERSON OR SOP CO-ORDINATOR

Copy Control: Copy Number _____

UNCONTROLLED IF NOT COMPLETE

METHOD: Simultaneous determination of seven sweeteners by high performance liquid chromatography

1. SCOPE

This method describes a high performance liquid chromatographic method for the simultaneous determination of seven sweeteners, i.e. Acesulfame K (ACS-K), Aspartame (ASP), Saccharin (SAC), Neohesperidine dihydrochalcone (NHDC), Rebaudioside A (REB-A), Stevioside (STE) and Neotame (NEO) in fruit squash, carbonated soft drinks, yogurt, biscuits and jam.

2. REFERENCES

- Wasik, A., and Buchgraber, M., *Foodstuffs-Simultaneous determination of nine sweeteners by high performance liquid chromatography and evaporative light scattering detection,* **IRMM :** p. 35, 2007
- Steviol Glycosides, Prepared at the 73rd JECFA (2010), published in FAO JECFA Monographs, 10 (2010).

3. METHOD PRINCIPLE

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

4. REAGENTS

NB Unless otherwise stated all reagents are of analytical grade quality, and should be prepared using UHP water.

4.1. **Acesulfame K**, e.g. Product No. 04054-25G, Sigma Aldrich

4.2. **Saccharin**, e.g. Product No. 240931-50G, Sigma Aldrich

4.3. **Aspartame**, e.g. Product No. 4-7135-500mg, Sigma Aldrich

4.4. **Neohesperidin dihydrochalcone (NHDC)**, e.g. Product No. N8757-1G, Sigma Aldrich

4.5. **Rebaudioside A**, e.g. Product No. 01432-10G, Sigma Aldrich

4.6. **Stevioside**, e.g. Product No. OS093961001, Carbosynth

4.7. **Neotame**,

4.8. **Formic acid**, > 98 %

4.9. **Water**

4.10. **Triethylamine,** > 99 %

4.11. **Methanol** (HPLC grade)

4.12. **Buffer solution for extraction** (pH 4.5)

Dissolve 4ml of formic acid (4.8) in 5 L of water (4.9). Adjust to pH 4.5 with approximately 12.5 ml triethylamine (4.10)

4.13. **Acetonitrile (HPLC grade),** ≥ 99.8 %

4.14. **10 mM Sodium phosphate monobasic buffer,** pH 2.6 (Product No. S5011-500G, Sigma Aldrich)

Dissolve 2.4 g of sodium phosphate monobasic in 2 L of water. Adjust the pH at 2.6 ± 0.1 with orthophosphoric acid (4.15)

4.15. **Orthophosphoric acid**, ≥ 84.0 %

4.16. **HPLC mobile phase A,** 10 mM sodium phosphate buffer (pH 2.6) (4.14). Degas by sonication for 10 minutes.

4.17. **HPLC mobile phase B,** Acetonitrile (4.13) Degas by sonication 1 litre of acetonitrile for 10 minutes.

5. STANDARD PREPARATION

5.1. **Stock Solution** (2500ug/ml)

5.1.1. Weigh 125 mg of each analyte into a separate 50 ml volumetric flask. Dissolve in water then make up to the mark with water. The exact weight taken should be recorded in the appropriate workbook.

Note: Some stevioside standards also contain reb A. When first using a new supply of stevioside standard material an individual standard should be prepared and analysed to confirm the presence of any reb A. If Reb A is found to be present the stevioside can still be used but an allowance should be made for its purity. If both reb A and stevioside are to be quantified, the reb A concentration should be adjusted to correct for the quantity of reb A in the stevioside standard.

5.2. **Calibration Standards Solutions**

The following volumes of each individual stock standard solution should be pipetted into a 10 ml volumetric flask and diluted to volume with 32:68 Acetonitrile (4.17): 10mM sodium phosphate buffer (4.16).

	Vol of stock – Aspartame, Saccharin, Acesulfame K, Stevioside, Reb A	**Vol of stock – NHDC, Neotame**	**Final Volume**
	mL	**mL**	**mL**
Standard 1	0.02	0.01	10
Standard 2	0.04	0.02	10
Standard 3	0.4	0.04	10
Standard 4	0.8	0.4	10
Standard 5	1.2	0.8	10

This will give standards containing the following concentration of each analyte*.

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
Aspartame, Saccharin, Acesulfame K, Stevioside, Reb A	5	10	100	200	300
NHDC, Neotame	2.5	5	10	100	200

** The calibration levels are indicative and can be adjusted to fit the concentrations expected in the samples.*

5.3. **Quality Control Standard**

There are currently no reference materials available containing all of the sweeteners described in this SOP. In each analysis batch at least one sample should be spiked at the maximum permitted concentration for each of the sweeteners of interest as shown in the table below.

	mg/kg or mg/l				
	Biscuits	**Yoghurt**	**Jam**	**Fruit squash**	**Carbonated beverage**
Acesulfame K	350	350	1000	350	350
Saccharin	100	100	200	80	80
Aspartame	1000	1000	1000	600	600
NHDC	50	50	50	30	30
Neotame	32	32	32	20	20
Reb A	61	303	606	242	242
Stevioside	50	250	500	200	200

These spiking concentrations can be achieved by adding the following volumes of 2500 µg/ml stock solutions to 5 g of sample.

	ml 2500ug/ml stock solution				
	Biscuits	**Yoghurt**	**Jam**	**Fruit squash**	**Carbonated beverage**
Acesulfame K	0.7	0.7	2.0	0.7	0.7
Saccharin	0.2	0.2	0.4	0.16	0.16
Aspartame	2.0	2.0	2.0	1.2	1.2
NHDC	0.1	0.1	0.1	0.06	0.06
Neotame	0.064	0.064	0.064	0.04	0.04
Reb A	0.122	0.606	1.212	0.484	0.484
Stevioside	0.1	0.5	1.0	0.4	0.4

6. APPARATUS

Standard laboratory glassware, together with the following:-

6.1. **Grade A laboratory glassware.**

6.2. **Analytical balance,** capable of weighing to 0.0001 g.

6.3. **Falcon tubes**, 50 ml

6.4. **Food blender,** suitable for homogenisation of food samples

6.5. **Ultrasonic bath**

6.6. **Centrifuge,** capable of maintaining 4000rpm

6.7. **SPE Vacuum system,** or equivalent

6.8. **Turbovap,** capable of maintaining 40^{o}C ± 0.5

6.9. **pH meter**

6.10. **Bond elut C18-OH cartridges,** 1g/ 6ml (Crawford Scientific, Product No. 12256040)

6.11. **Disposable HPLC vials** for use with the autosampler

6.12. **50 ml volumetric flasks**

6.13. **Pipettes,** different volumes

6.14. **Disposable plastic syringes,** 10ml

6.15. **HPLC System**

- Column: Luna C18, 5u, 250mm x 4.60mm 5 micron
- Flow rate: 1.0 ml/min
- Run time: 30 min
- Oven temperature: 40^{o}C
- Injection volume: 10ul
- Mobile phase: Line A: 10mM Sodium phosphate monobasic buffer, pH 2.6 (4.16)
  Line B: Acetonitrile (4.17)

- Gradient program:

Time (min)	2	12	25	26	30
Mobile phase % A	90	70	70	90	90
Mobile phase % B	10	30	30	10	10

- Detection at UV 210nm

7. PREPARATION

7.1. **Preparation of test sample**

Comminute the entire test sample to give a homogenous suspension (6.4). Liquid samples may be subjected directly to the extraction procedure. Fruit squash samples should be diluted to their ready-to-drink concentration prior to extraction.

7.2. **Extraction and clean-up**

7.2.1. Weigh 5 g of the homogenised test sample (7.1) into a 50 ml volumetric flask. Make up to the mark with buffer solution (4.12), mix thoroughly to obtain a homogenous suspension and sonicate (6.5) for 15 min. The exact weight of sample taken should be recorded in the appropriate workbook.

7.2.2. Transfer the obtained suspension to a 50 ml Falcon tube (6.3). Centrifuge at 4000 rpm for 10 min.

Note: *If the test solution is clear, this step can be ignored.*

7.2.3. Condition the SPE cartridge (6.10) by applying 3 ml methanol (4.11) and let it pass through the cartridge 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 (1mm).

7.2.4. Equilibrate the SPE cartridge by applying 6 ml of buffer solution (4.12) and let it pass through the cartridge 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).

7.2.5. Load the SPE cartridge with 10 ml of sample extract (7.2.2) and let it pass through the cartridge 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).

7.2.6. Wash the SPE cartridge with 3 ml of buffer solution (4.12) and let it pass through the cartridge 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).

7.2.7. Elute the sweeteners from the SPE cartridge by applying 6 ml of methanol (4.11) and collect the eluate in a 10 ml test tube. Use a slight vacuum to obtain a flow rate of 1 ml/min. Make sure to let the SPE cartridges run dry this time.

7.2.8. Evaporate the eluate to dryness in a turbovap (6.8).

7.2.9. Dissolve the residue in 1 ml of 32:68 acetonitrile (4.17): 10mM sodium phosphate buffer (4.16) and transfer to an HPLC vial ready for injection on the UV-HPLC system. If required, dilute the extract with 32:68 acetonitrile (4.17): 10mM sodium phosphate buffer (4.16) to ensure that the expected concentration of each sweetener is within the calibration range. It may be necessary to prepare several dilutions of each extract.

7.2.10. Calibration standards should be injected at the beginning and end of the run and at suitable intervals throughout the run.

8. CALCULATION OF RESULTS

8.1.1. Using a suitable Excel spreadsheet construct a linear regression curve using the areas or heights obtained for the calibration standards and determine both the slope (m) and intercept (c) of the curve. From the responses for the sample (y), determine the concentration of each sweetener in the injected solution using the following equation:

$$x\ (ug/ml) = \frac{y - c}{m}$$

Calculate the concentration of each sweetener in the sample using the following equation:

$$\text{Concentration of sweetener in the sample } (\mu g/g) = \frac{Y \times V \times D \times W}{M \times Z}$$

where:

Y = concentration of the sweetener in the injected solution (ug/ml)

V = volume of extractant (ml)

D = dilution factor

W = final volume (ml)

Z = volume of aliquot taken through SPE clean-up (ml)

M = weight of test portion of the sample (g)

9. ANALYTICAL QUALITY CONTROL

9.1. Quality Control sample

The percent recovery for each sweetener should be between 60 and 130 %. If any result falls outside this range consult the project manager and record any actions taken.

Document history

Document changes and acknowledgment of staff awareness to these changes-

Issue	Change	Date

All staff carrying out this procedure should sign below (on the master copy) to record that they have read and understood this SOP.

Staff name	Signature	Date

Appendix 3: Amended SOP for the Simultaneous determination of seven sweeteners by high performance liquid chromatography

LGC Limited

STANDARD OPERATING PROCEDURE

Screening method for the simultaneous determination of Acesulfame K, Aspartame, Saccharin, Neohesperidine dihydrochalcone, Rebaudioside A, Stevioside and Neotame by high performance liquid chromatography

Issue number: **1 Draft**

METHOD: Screening method for the simultaneous determination of Acesulfame K, Aspartame, Saccharin, Neohesperidine dihydrochalcone, Rebaudioside A, Stevioside and Neotame by high performance liquid chromatography

1. SCOPE

This method describes a high performance liquid chromatographic method for the simultaneous determination of seven sweeteners, i.e. Acesulfame K (ACS-K), Aspartame (ASP), Saccharin (SAC), Neohesperidine dihydrochalcone (NHDC), Rebaudioside A (REB-A), Stevioside (STE) and Neotame (NEO) in fruit squash, carbonated soft drinks, yoghurt, biscuits and jam.

2. REFERENCES

- Wasik, A., and Buchgraber, M., *Foodstuffs-Simultaneous determination of nine sweeteners by high performance liquid chromatography and evaporative light scattering detection,* **IRMM :** p. 35, 2007
- Steviol Glycosides, Prepared at the 73rd JECFA (2010), published in FAO JECFA Monographs, 10 (2010).

3. METHOD PRINCIPLE

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

4. REAGENTS

NB Unless otherwise stated all reagents are of analytical grade quality, and should be prepared using UHP water.

4.1. **Acesulfame K**, e.g. Product No. 04054-25G, Sigma Aldrich

4.2. **Saccharin**, e.g. Product No. 240931-50G, Sigma Aldrich

4.3. **Aspartame**, e.g. Product No. 4-7135-500mg, Sigma Aldrich

4.4. **Neohesperidin dihydrochalcone (NHDC)**, e.g. Product No. N8757-1G, Sigma Aldrich

4.5. **Rebaudioside A**, e.g. Product No. 01432-10G, Sigma Aldrich

4.6. **Stevioside**, e.g. Product No. ASB-00019351, ChromaDex

4.7. **Neotame**, e.g. Product No. USP 1460204, LGC Standards

4.8. **Formic acid**, > 98 %

4.9. **Water**

4.10. **Triethylamine,** > 99 %

4.11. **Methanol** (HPLC grade)

4.12. **Buffer solution for extraction** (pH 4.5)

Dissolve 4ml of formic acid (4.8) in 5 L of water (4.9). Adjust to pH 4.5 with approximately 12.5 ml triethylamine (4.10)

4.13. **Acetonitrile (HPLC grade),** ≥ 99.8 %

4.14. **10 mM Sodium phosphate monobasic buffer,** pH 2.6

Dissolve 2.4 g of sodium phosphate monobasic (Product No. S5011-500G, Sigma Aldrich) in 2 L of water. Adjust the pH at 2.6 ± 0.1 with orthophosphoric acid (4.15)

4.15. **Orthophosphoric acid**, ≥ 84.0 %

4.16. **HPLC mobile phase A,** 90:10 10 mM sodium phosphate buffer (pH 2.6) (4.14): acetonitrile (4.13).

Using a measuring cylinder, add 900ml of 10 mM sodium phosphate buffer (pH 2.6) (4.14) and 100ml acetonitrile into a suitable container and mix well. Degas by sonication for 10 minutes.

4.17. **HPLC mobile phase B,** Acetonitrile (4.13) Degas by sonication 1 litre of acetonitrile for 10 minutes.

5. STANDARD PREPARATION

5.1. **Stock Standard Solutions** (2500 µg/ml, 1000 µg/ml NHDC)

5.1.1. Weigh 125 mg of Aspartame, Saccharin, Acesulfame K, Stevioside, Reb A and Neotame into separate 50 ml volumetric flasks. Dissolve in HPLC mobile phase A (90:10 10 mM sodium phosphate buffer (pH 2.6): acetonitrile) (4.16) then make up to the mark with mobile phase A. The exact weight taken should be recorded in the appropriate workbook.

Note: Some stevioside standards also contain reb A. When first using a new supply of stevioside standard material an individual standard should be prepared and analysed to confirm the presence of any Reb A. If Reb A is found to be present the stevioside can still be used but an allowance should be made for its purity. If both reb A and stevioside are to be quantified, the reb A concentration should be adjusted to correct for the quantity of reb A in the stevioside standard.

5.1.2. Weigh 50 mg of NHDC into a 50 ml volumetric flask. Dissolve in HPLC mobile phase A (90:10 10 mM sodium phosphate buffer (pH 2.6): acetonitrile) (4.16) then make up to the mark with mobile phase A. The exact weight taken should be recorded in the appropriate workbook.

5.2. **Intermediate Mixed Standard Solutions**

5.2.1 Intermediate Mixed Standard 1 (250 µg/ml Aspartame, Saccharin, Acesulfame K, Stevioside, Reb A)

Pipette 1 ml each of the stock standard solutions (2500 µg/ml) for Aspartame, Saccharin, Acesulfame K, Stevioside and Reb A into a 10 ml volumetric flask and dilute to volume with mobile phase A (4.16).

5.2.2 Intermediate Mixed Standard 2 (250 µg/ml Neotame and NHDC)

Pipette 1 ml of the 2500 µg/ml Neotame stock solution and 2.5 ml 1000 µg/ml NHDC stock solution into a 10 ml volumetric flask and dilute to volume with mobile phase A (4.16).

5.3. **Calibration Standards Solutions**

The following volumes of each individual stock standard solution should be pipetted into a 10 ml volumetric flask and diluted to volume with mobile phase A (4.16).

	Vol of Intermediate Mixed Standard 1	**Vol of Intermediate Mixed Standard 2**	**Final Volume**
	ml	**ml**	**ml**
Standard 1	0.04	0.02	10
Standard 2	0.6	0.4	10
Standard 3	1.2	0.8	10
Standard 4	1.8	1.2	10
Standard 5	2.4	1.6	10

This will give standards containing the following concentration of each analyte*.

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
Aspartame, Saccharin, Acesulfame K, Stevioside, Reb A	1	15	30	45	60
NHDC, Neotame	0.5	10	20	30	40

** The calibration levels are indicative and can be adjusted to fit the concentrations expected in the samples.*

5.4. **Quality Control Standard**

There are currently no reference materials available containing all of the sweeteners described in this SOP. In each analysis batch at least one sample of each matrix type should be spiked at the maximum permitted concentration for each of the sweeteners of interest as shown in the table below. All sample results should be corrected for the recovery obtained for the relevant sweetener in the same matrix type.

	mg/kg or mg/l				
	Biscuits	**Yoghurt**	**Jam**	**Fruit squash**	**Carbonate beverage**
Acesulfame K	350	350	1000	350	350
Saccharin	100	100	200	80	80
Aspartame	1000	1000	1000	600	600
NHDC	50	50	50	30	30
Neotame	32	32	32	20	20
Reb A	61	303	606	242	242
Stevioside	50	250	500	200	200

These spiking concentrations can be achieved by adding the following volumes of 2500 µg/ml (1000 µg/ml NHDC) stock solutions to 5 g of sample.

	ml 2500 ug/ml stock solution, 1000 µg/ml NHDC				
	Biscuits	**Yoghurt**	**Jam**	**Fruit squash**	**Carbonate beverage**
Acesulfame K	0.7	0.7	2.0	0.7	0.7
Saccharin	0.2	0.2	0.4	0.16	0.16
Aspartame	2.0	2.0	2.0	1.2	1.2
NHDC	0.25	0.25	0.25	0.15	0.15
Neotame	0.064	0.064	0.064	0.04	0.04
Reb A	0.122	0.606	1.212	0.484	0.484
Stevioside	0.1	0.5	1.0	0.4	0.4

6. APPARATUS

Standard laboratory glassware, together with the following:-

6.1. **Grade A laboratory glassware.**

6.2. **Analytical balance,** capable of weighing to 0.0001 g.

6.3. **Falcon tubes**, 50 ml

6.4. **Food blender,** suitable for homogenisation of food samples

6.5. **Ultrasonic bath**

6.6. **Centrifuge,** capable of maintaining 4000rpm

6.7. **SPE Vacuum system,** or equivalent

6.8. **Turbovap,** capable of maintaining $40^{o}C \pm 0.5$

6.9. **pH meter**

6.10. **Bond elut C18-OH cartridges,** 1g/ 6ml (Crawford Scientific, Product No. 12256040)

6.11. **Disposable HPLC vials** for use with the autosampler

6.12. **50 ml volumetric flasks**

6.13. **Pipettes,** different volumes

6.14. **Disposable plastic syringes,** 10ml

6.15. **HPLC System**

- Column: Luna C18, 5u, 250mm x 4.60mm 5 micron
- Flow rate: 1.0 ml/min
- Run time: 30 min
- Oven temperature: 40^{o}C
- Injection volume: 50ul
- Mobile phase: Line A: 90:10 10mM sodium phosphate buffer (pH 2.6): acetonitrile (4.16)

  Line B: Acetonitrile (4.17)
- Gradient program:

Time (min)	2	12	25	26	30
Mobile phase % A	100	75	75	100	100
Mobile phase % B	0	25	25	0	0

- Detection at UV 210nm

7. SAMPLE PREPARATION AND EXTRACTION

7.1. **Preparation of test sample**

Comminute the entire test sample to give a homogenous sample. Liquid samples may be subjected directly to the extraction procedure. Fruit squash samples should be diluted to their ready-to-drink concentration prior to extraction.

7.2. **Extraction and clean-up**

7.2.1. Weigh 5 g of the homogenised test sample (7.1) into a 50 ml volumetric flask. Make up to the mark with buffer solution (4.12), mix thoroughly to obtain a homogenous suspension and sonicate (6.5) for 15 min. The exact weight of sample taken should be recorded in the appropriate workbook.

7.2.2. Transfer the obtained suspension to a 50 ml Falcon tube (6.3). Centrifuge at 4000 rpm for 10 min.

Note: *If the test solution is clear, this step can be ignored.*

7.2.3. Condition the SPE cartridge (6.10) by applying 3 ml methanol (4.11) and let it pass through the cartridge 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 (1mm).

7.2.4. Equilibrate the SPE cartridge by applying 6 ml of buffer solution (4.12) and let it pass through the cartridge 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).

7.2.5. Load the SPE cartridge with 10 ml of sample extract (7.2.2) and let it pass through the cartridge 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).

7.2.6. Wash the SPE cartridge with 3 ml of buffer solution (4.12) and let it pass through the cartridge 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).

7.2.7. Elute the sweeteners from the SPE cartridge by applying 6 ml of methanol (4.11) and collect the eluate in a 10 ml test tube. Use a slight vacuum to obtain a flow rate of 1 ml/min. Make sure to let the SPE cartridges run dry this time.

7.2.8. Evaporate the eluate to dryness in a turbovap (6.8).

7.2.9. Dissolve the residue in 5 ml of mobile phase A (4.16) and transfer to an HPLC vial ready for injection on the UV-HPLC system. If required, dilute the extract with mobile phase A (4.16) to ensure that the expected concentration of each sweetener is within the calibration range. It may be necessary to prepare several dilutions for each extract.

7.2.10. Calibration standards should be injected at the beginning and end of the run and at suitable intervals throughout the run.

8. CALCULATION OF RESULTS

8.1.1. Using a suitable Excel spreadsheet construct a linear regression curve using the areas or heights obtained for the calibration standards and determine both the slope (m) and intercept (c) of the curve. From the responses for the sample (y), determine the concentration of each sweetener in the injected solution using the following equation:

$$x\ (ug/ml) = \frac{y - c}{m}$$

Calculate the concentration of each sweetener in the sample using the following equation:

$$\text{Concentration of sweetener in the sample } (\mu g/g) = \frac{Y \times V \times D \times W}{M \times Z}$$

where:

Y = concentration of the sweetener in the injected solution (ug/ml)

V = volume of extractant (ml)

D = dilution factor

W = final volume (ml)

Z = volume of aliquot taken through SPE clean-up (ml)

M = weight of test portion of the sample (g)

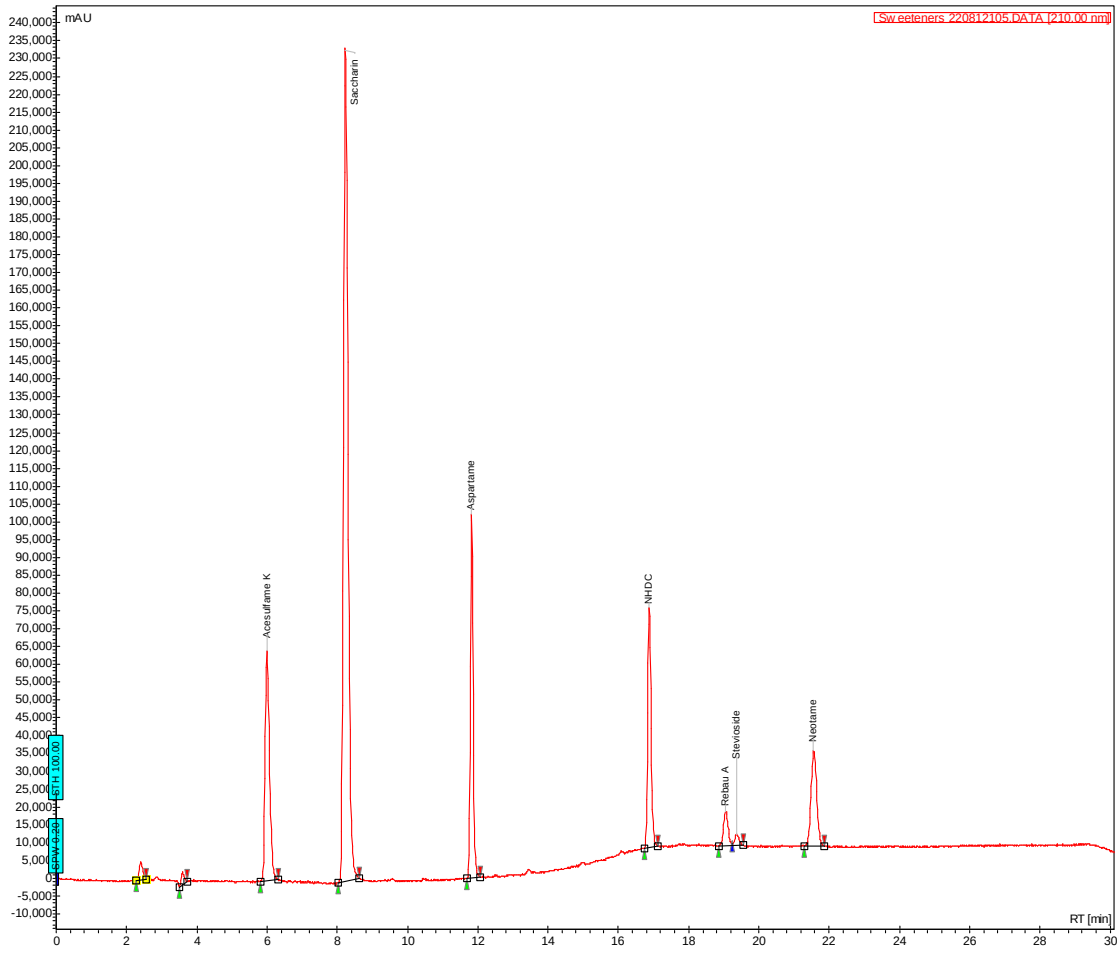
9. ANALYTICAL QUALITY CONTROL

9.1. Quality Control sample

The percent recovery for each sweetener should be between 70 and 130 %. If any result falls outside this range consult the project manager and record any actions taken.

Full quality control criteria will be added on the completion of the collaborative trial.

10. EXAMPLE CHROMATOGRAM



Sweetener	Approximate retention time (Minutes)
Acesulfame K	5.9
Saccharin	8.2
Aspartame	11.8
NHDC	16.9
Reb A	19.1
Stevioside	19.4
Neotame	21.6

Appendix 4: Stevioside investigations

The pre-study check highlighted a problem with the recovery measured for stevioside on some occasions. This had not been apparent previous to the pre-study check and various investigations were carried out to try and establish the reasons behind the over recovery of stevioside.

Initially, aliquots of fruit squash were spiked in duplicate at 200 µg/g and 600 µg/g stevioside and taken through the extraction procedure. Each solution was diluted to two different concentrations and anlaysed by HPLC. The concentrations were measured at different points of the calibration line (between 40 and 190 µg/ml) to see if the intercept of the calibraion line significantly affected the results. All of recoveries were between 92 and 108 % implying that the calibration line and its associated intercept was not the source of the high stevioside results.

Next, duplicate aliquots of yoghurt were spiked at 1800 µg/g stevioside and were taken through the extraction procedure. Three dilutions were made for each extract and the mean recovery was 89 %. These results indicated that stevioside could be accurately determined in yoghurt in the absence of other sweeteners, the implication being that high recoveries for stevioside were only observed when it was determined in the presence of the other sweeteners in this study.

To establish if the over recovery for stevioside was due to an interaction with one of the other sweeteners an aliquot of stevioside, without any matrix, was taken through the extraction procedure. The mean of the recoveries obtained was 104 %. Aliquots of fruit squash were then spiked with stevioside only and taken through the extraction, again the mean recovery was 104 %. Next, the six sweeteners other than stevioside (acesulfame k, saccharin, aspartame, NHDC, reb A and neotame) were spiked together into one flask and taken through extraction procedure as described in the amended SOP. No peaks were detected in these extracts at the same retention time as stevioside indicating that the over recovery of stevioside was not due to any impurity in the other sweeteners that eluted at the same retention time as stevioside and so increase the apparent stevioside concentration.

The final fruit squash extracts, which had been spiked with the six sweeteners apart from stevioside before extraction, were spiked with aliquots of stevioside directly prior to injection onto the HPLC. The mean recovery for stevioside was 97 % indicating that the HPLC determination was not the source of the over recovery.

The next stage of the investigation involved taking aliquots of the individual stock sweetener solutions through the extraction to confirm that no artefacts from the extraction procedure produced peaks that eluted at the same time as stevioside and thus increase the apparent concentration of stevioside. Table 1 shows the percentage recovery for each of the individual sweeteners, the figures presented are the mean values of duplicate extractions on each of two days. As can be seen, the recoveries for each of the individual sweeteners, including that for stevioside, are close to 100 %. However, when stevioside was determined in the presence of the other six sweeteners a recovery of 121 % was obtained.

Description	% Recovery Acesulfame K	% Recovery Saccharin	% Recovery Aspartame	% Recovery NHDC	% Recovery Reb A	% Recovery Stevioside	% Recovery Neotame
Acesulfame K	103						
Saccharin		106					
Aspartame			101				
NHDC				101			
Reb A					71		
Neotame							104
Mixed spike	104	104	99	93	99	121	102
Stevioside						105	
Standard deviation	3.2	2.3	0.9	5.8	2.0	4.0	10.9

Table 1: Recoveries for individual sweeteners taken through the extraction procedure

As high recoveries were observed for stevioside only when it was present in combination with other sweeteners, a series of extractions were carried out with stevioside in addition to one of each of the other sweeteners in turn. Table 2 presents the results of this experiment, with the highest recovery for stevioside being observed when it was determined in the presence of Reb A.

Description	% Recovery Acesulfame K	% Recovery Saccharin	% Recovery Aspartame	% Recovery NHDC	% Recovery Reb A	% Recovery Stevioside	% Recovery Neotame
Stevioside + Acesulfame K	104					108	
Stevioside + Saccharin		97				95	
Stevioside + Aspartame			97			89	
Stevioside + NHDC				94		110	
Stevioside + Reb A					95	116	
Stevioside + Neotame						104	96
Mixed standard	106	101	102	85	90	108	77
Stevioside						98	

Table 2: Mean recoveries for stevioside in combination with one other sweetener

Throughout the project discussions were held with ChromaDex concerning the method and the recoveries obtained. Their response was that there was potentially a possibility of degradation of the steviol glycosides at low pH. The extraction buffer for this developed method is aqueous formic acid at pH 4.5 and the buffer portion of the mobile phase is at pH 2.6. Whilst these solutions are fairly acidic there are several published methods for the

determination of steviol glycosides that use similar pHs, for example 69th JECFA (2008) for the determination of stevioside and reb A and recommends a mobile phase of 80:20 acetonitrile water adjusted to pH 3.0 with phosphoric acid, which implies that these low pHs are suitable for the determination of stevioside.

The stevioside standard material used throughout this project was supplied by Carbosynth and had a stated purity of 90 %, with a significant amount of the other 10 % being reb A. One consideration was whether the over recovery of stevioside was possibly due to other impurities (other steviol glycosides) in the stevioside standard that degraded during extraction to produce a compound that eluted at the same retention time as stevioside. To explore this possibility ChromaDex were asked to repeat the above trial extractions of stevioside in addition to one other sweetener in turn, both with the Carbosynth stevioside used at LGC and a high purity stevioside standard supplied by ChromaDex themselves. Tables 3 and 4 show the mean recoveries of duplicate extractions for each of the sweeteners in combination with stevioside. All of the recoveries are close to 100 % and those in Table 3 that were prepared using the Carbosynth stevioside are very similar to the recoveries in Table 4 for the ChromaDex stevioside.

Description	% Recovery Acesulfame K	% Recovery Saccharin	% Recovery Aspartame	% Recovery NHDC	% Recovery Reb A	% Recovery Stevioside	% Recovery Neotame
Stevioside + Acesulfame K	95					93	
Stevioside + Saccharin		99				93	
Stevioside + Aspartame			95			97	
Stevioside + NHDC				95		97	
Stevioside + Reb A					90	99	
Stevioside + Neotame						97	103
Mixed standard	94	100	96	97	92	100	105
Stevioside						96	

Table 3: Stevioside (Carbosynth) in combination with one other sweetener. Extractions performed by ChromaDex

Description	% Recovery Acesulfame K	% Recovery Saccharin	% Recovery Aspartame	% Recovery NHDC	% Recovery Reb A	% Recovery Stevioside	% Recovery Neotame
Stevioside + Acesulfame K	97					90	
Stevioside + Saccharin		100				99	
Stevioside+ Aspartame			99			99	
Stevioside + NHDC				95		93	
Stevioside + Reb A					106	101	
Stevioside + Neotame						97	106
Mixed standard	97	101	99	93	96	104	103
Stevioside						97	

Table 4: Stevioside (ChromaDex) in combination with one other sweetener. Extractions performed by ChromaDex

As, to date, an acceptable explanation for the over recovery of stevioside has not been found, it is recommended that the results for stevioside be corrected for recovery. A comment has been added to the SOP stating that for each batch, at least one sample of each matrix type should be spiked at the maximum permitted concentration of each of the sweeteners and the results obtained for the samples be corrected for recovery.

ANNEX 1. Collaborative Trial of Method – Draft Report

Participants

A total of 14 laboratories completed the collaborative trial, this included 13 Public Analyst laboratories (12 from the UK, 1 from Ireland) and LGC (see Appendix to Annex 1).

Pre-Trial

A pre-trial was carried out to allow the laboratories to familiarise themselves with the method. Each laboratory was sent two aliquots of jam and asked to analyse each sample in duplicate and report their findings. The SOP, results sheet, standards, and appropriate SPE cartridges were supplied. All 14 laboratories returned results (The SOP and pre-trial instructions are included in the Appendix).

The majority of the data was acceptable albeit with an overall trend to lower recovery than that measured by LGC in the same samples. The results are summarised in the 'Evaluation of the results for the sweetener pretrial' which was sent to each participant with the instructions for the main trial (see Appendix).

Main Trial

Samples

A total of five matrices were selected; jam, blackcurrant flavour juice drink concentrate, blackcurrant flavour juice drink diluted 'ready-to-drink', low fat yoghurt and high fat yoghurt. These products were bought from local supermarkets; none had any added sweeteners listed in the ingredients.

Sample	Jam	Blackcurrant Juice Drink conc.	Blackcurrant Juice Drink diluted	Low Fat Yoghurt	High Fat Yoghurt
Acesulfame K	930	158	385	322	340
Aspartame	930	270	660	920	970
Saccharin	186	36	88	92	97
Stevioside	465	90	220	230	243
Rebaudioside	564	136	333	279	294
Neotame	30	9	22	29	31
NHDC	47	14	33	46	49

Table 1. Spike concentration for each sweetener per matrix (mg/kg or mg/L)

Each matrix, with the exception of the concentrated juice drink, was spiked with the seven different sweeteners around the legislative limit for each sweetener in that matrix (Table 1). The concentrated juice drink was spiked at a much lower level of each sweetener (around half the legislative limit for the diluted drink). Samples were mixed well and then individual aliquots prepared.

Homogeneity

Ten individual aliquots were selected at random and analysed in duplicate to assess the homogeneity of the spiked matrices. The results are shown in Table 2 below. Overall the homogeneity was acceptable with the variation between aliquots being less than 10 % CV. A peak co-eluting with NHDC in the concentrated blackcurrant juice matrix prevented the measurement of this sweetener in this matrix.

Sample	Description	Units		Acesulfame K	Saccharin	Aspartame	NHDC	Reb A	Stevioside	Neotame
1	Jam	mg/Kg	Mean	979	188	929	50	588	476	25
			SD	8.96	2.02	10.99	0.85	6.35	9.61	0.45
			CV%	0.9	1.1	1.2	1.7	1.1	2.0	1.8
2	Blackcurrent Juice Drink concentrate	mg/L	Mean	156	38	224	N/A	134	81.4	6.6
			SD	3.36	0.89	11.04	N/A	4.21	4.42	0.38
			CV%	2.2	2.3	4.9	-	3.1	5.4	5.7
3	Blackcurrrent Juice Drink diluted	mg/L	Mean	336	76.4	549	55.5	298	199	20
			SD	3.99	0.82	14.81	2.31	6.16	5.87	0.4
			CV%	1.2	1.1	2.7	4.2	2.1	2.9	2.0
4	Low-fat yoghurt	mg/Kg	Mean	263	68	673	37	231	202	22
			SD	7.56	2.44	31.03	1.37	10.14	13.18	0.90
			CV%	2.9	3.6	4.6	3.7	4.4	6.5	4.1
5	High-fat yoghurt	mg/Kg	Mean	295	88.8	746	44.9	252	225	25
			SD	4.17	1.75	29.02	1.44	4.34	4.71	0.58
				1.4	2.0	3.9	3.2	1.7	2.1	2.3

Table 2. Homogeneity of spiked matrices

Materials and Instructions

Each participating laboratory was provided with feedback from the main trial, main trial instructions, the analytical SOP and a results sheet (see Appendix).

Each laboratory was sent two samples of each spiked matrix (supplied as blind duplicates) and asked to analyse each sample once (see diagram 1). In addition, an aliquot of blank matrix was supplied for each of the matrix types and each laboratory was required to prepare a spiked blank sample for each matrix. Standards, to be used to prepare spiked matrices and for calibration, were supplied by LGC along with the appropriate SPE cartridges for the clean-up step.

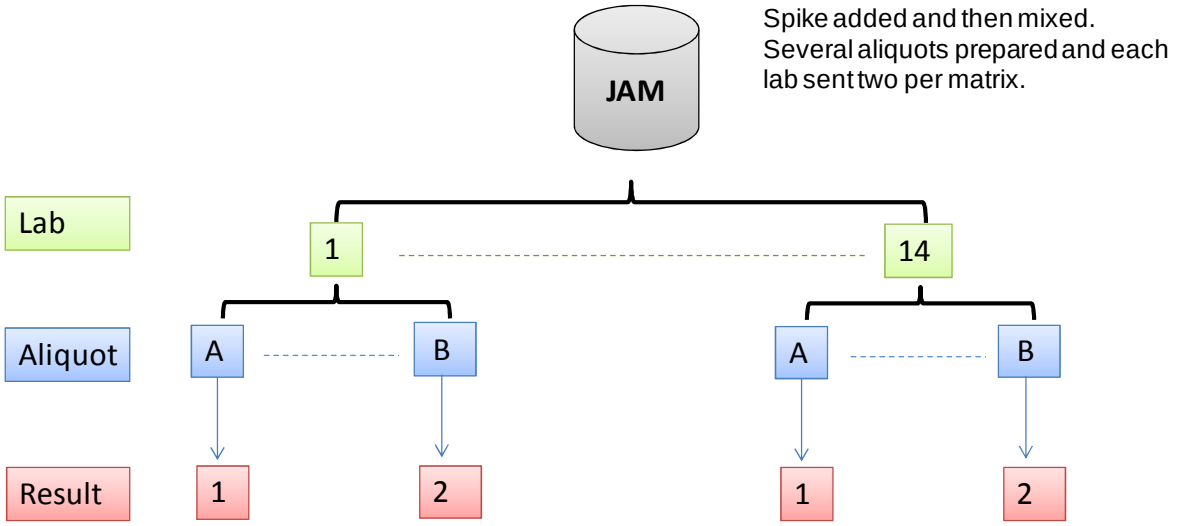


Diagram 1. Flow Diagram of sample preparation and analysis

Results

All 14 laboratories returned data.

Mandel statistics were used to test the consistency of the laboratories taking part in the trial; seven laboratories were identified as outliers, for one or more sweetener. Cochran's and Grubb's tests were also used to examine the within and between laboratory consistency of data, nine laboratories were identified as outliers for one or more sweeteners. Overall a total of 36 pairs of data were removed from the final analysis (out of 490 pairs of data). One laboratory showed frequent poor performance, valid data from this laboratory was included in the final data set but consideration could be given to removing this laboratory entirely on technical grounds.

Neotame and NHDC were the two analytes with the most outliers removed (8 pairs of data each out of a possible 70).

The recovery, repeatability and reproducibility were calculated from the remaining data set (Table 3). The majority of the analytes gave good recovery (60-100 %) in each of the matrices tested. The exceptions were low recovery for aspartame and neotame in both yoghurt samples and NHDC in the low fat yoghurt. The recovery for NHDC in concentrated blackcurrant juice was a low and the results very variable (CV 44 %) probably due to the co-eluting interference, the spike concentration of NHDC was low and this would have compounded the effect of the interference.

Table 3. Statistical Analysis of Results by Matrix

Matrix	Sweetener	Spike level (mg/kg or mg/L)	Measured value (mg/kg or mg/L) (Mean, Range)		% Recovery	Within Lab repeatability (mg/kg or mg/L)	Between lab reproducibility (mg/kg or mg/L)	RSDr	% CV
Jam	Acesulfame K	930	889	822 - 952	96	23.0	31.7	0.026	2.6
	Aspartame	930	815	579 - 1013	88	50.8	104.0	0.062	6.2
	Neotame	30	26	22 - 28	86	0.9	1.0	0.034	3.4
	NHDC	47	43	30 - 53	92	1.7	5.6	0.038	3.8
	Rebaudioside	564	594	432 - 841	105	25.1	85.6	0.042	4.2
	Saccharin	186	173	127 - 229	93	8.2	21.3	0.047	4.7
	Stevioside	465	456	314 - 598	98	21.8	52.3	0.048	4.8
Blackcurrant Juice conc.	Acesulfame K	158	135	102 - 177	86	5.6	19.7	0.042	4.2
	Aspartame	270	184	80 - 262	68	8.0	47.9	0.043	4.3
	Neotame	9	7	5 - 9	72	0.3	1.1	0.050	5.0
	NHDC	14	9	0 - 27	64	14.7	62.5	0.446	**44.6**
	Rebaudioside	136	124	43 - 184	91	9.2	28.0	0.074	7.4
	Saccharin	36	33	23 - 48	93	2.6	7.9	0.077	7.7
	Stevioside	90	70	35 - 97	77	3.1	15.1	0.044	4.4
Blackcurrant Juice dilute	Acesulfame K	385	330	284 - 363	86	11.6	17.4	0.035	3.5
	Aspartame	660	537	399 - 615	81	30.2	44.8	0.056	5.6
	Neotame	22	18	14 - 21	82	1.2	0.9	0.065	6.5
	NHDC	33	29	18 - 45	89	2.2	5.2	0.073	7.3
	Rebaudioside	333	319	141 - 517	96	26.2	65.5	0.082	8.2
	Saccharin	88	81	66 - 94	92	4.5	6.2	0.056	5.6
	Stevioside	220	203	166 - 241	92	9.3	17.2	0.046	4.6
Yoghurt Low Fat	Acesulfame K	322	281	259 - 317	87	5.6	12.7	0.020	2.0
	Aspartame	920	41	0 - 152	**4**	6.2	49.7	0.150	15.0
	Neotame	29	0	-	-	-	-	-	-
	NHDC	46	19	12 - 23	**42**	1.7	2.4	0.089	8.9
	Rebaudioside	279	278	250 - 344	100	4.8	30.7	0.017	1.7
	Saccharin	92	116	64 - 271	**126**	2.6	78.0	0.022	2.2
	Stevioside	230	232	177 - 342	101	5.3	42.1	0.023	2.3
Yoghurt High Fat	Acesulfame K	340	298	266 - 327	88	11.4	4.5	0.038	3.8
	Aspartame	970	159	0 - 704	**16**	22.5	255.0	0.142	14.2
	Neotame	31	6	0 - 27	**19**	0.6	9.8	0.107	10.7
	NHDC	49	34	21 - 62	69	4.5	10.5	0.133	13.3
	Rebaudioside	294	302	210 - 438	103	26.0	41.7	0.086	8.6
	Saccharin	97	86	36 - 121	89	3.0	20.1	0.035	3.5
	Stevioside	243	248	159 - 361	102	8.4	43.7	0.034	3.4

Table 4. Statistical Analysis of Results by Sweetener

Estimates and variance components by sweetener and matrix

Sweet.	*Matrix*	*N Labs rejected*	*Mean*	s_r	s_R	*r*	*R*	RSD_r	RSD_R	H_{or}	H_{oR}
Acesulfame K	1	2	889.17	23.00	39.15	63.74	108.52	0.03	0.04	0.67	0.76
	2	1	135.37	5.63	20.52	15.60	56.88	0.04	0.15	0.82	1.98
	3	0	330.21	11.56	20.93	32.03	58.01	0.03	0.06	0.79	0.95
	4	1	280.58	5.58	13.83	15.46	38.34	0.02	0.05	0.44	0.72
	5	1	298.16	11.39	12.23	31.57	33.90	0.04	0.04	0.84	0.60
Aspartame	1	0	815.25	50.85	115.79	140.95	320.95	0.06	0.14	1.60	2.44
	2	1	183.68	7.96	48.59	22.07	134.68	0.04	0.26	0.89	3.62
	3	0	536.96	30.16	53.98	83.60	149.61	0.06	0.10	1.36	1.62
	4	3	41.32	6.20	50.12	17.20	138.93	0.15	1.21	2.46	13.28
	5	0	158.75	22.52	255.97	62.43	709.52	0.14	1.61	2.85	21.61
Neotame	1	2	25.65	0.87	1.35	2.40	3.73	0.03	0.05	0.52	0.53
	2	1	6.52	0.33	1.19	0.91	3.30	0.05	0.18	0.63	1.51
	3	1	18.14	1.17	1.50	3.25	4.16	0.06	0.08	0.94	0.80
	4	3	0	0	0	0	0	NA	NA	NA	NA
	5	1	6.01	0.64	9.87	1.78	27.36	0.11	1.64	1.31	13.46
NHDC	1	1	43.20	1.66	5.83	4.60	16.16	0.04	0.13	0.64	1.49
	2	2	8.61	3.84	6.21	10.63	17.23	0.45	0.72	5.78	6.24
	3	1	29.36	2.16	5.66	5.98	15.68	0.07	0.19	1.15	2.00
	4	4	19.10	1.71	2.95	4.73	8.18	0.09	0.15	1.30	1.51
	5	0	33.90	4.50	11.41	12.47	31.62	0.13	0.34	2.11	3.58
Rebaudioside	1	1	594.44	25.10	89.17	69.57	247.16	0.04	0.15	1.04	2.45
	2	0	123.88	9.19	29.49	25.46	81.75	0.07	0.24	1.44	3.07
	3	0	319.44	26.19	70.59	72.61	195.66	0.08	0.22	1.83	3.29
	4	2	277.97	4.75	31.02	13.17	85.99	0.02	0.11	0.37	1.63
	5	0	301.77	26.01	49.13	72.09	136.18	0.09	0.16	1.91	2.40
Saccharin	1	0	173.07	8.21	22.78	22.76	63.15	0.05	0.13	0.97	1.79
	2	1	33.49	2.59	8.29	7.19	22.97	0.08	0.25	1.23	2.62
	3	0	81.01	4.51	7.68	12.50	21.28	0.06	0.09	1.01	1.15
	4	0	116.05	2.57	78.07	7.13	216.40	0.02	0.67	0.43	8.60
	5	2	86.17	2.99	20.28	8.30	56.21	0.03	0.24	0.64	2.88
Stevioside	1	1	455.98	21.84	56.70	60.54	157.17	0.05	0.12	1.13	1.95
	2	1	69.61	3.09	15.46	8.57	42.84	0.04	0.22	0.79	2.63
	3	1	203.28	9.31	19.60	25.81	54.33	0.05	0.10	0.96	1.34
	4	1	231.88	5.33	42.40	14.77	117.54	0.02	0.18	0.49	2.59
	5	1	247.70	8.36	44.52	23.16	123.39	0.03	0.18	0.73	2.58

Note: Matrix ID's: 1=Jam, 2=Blackcurrant juice drink concentrate, 3= Blackcurrant juice drink diluted, 4=Low fat yogurt, 5=High fat yogurt.

Conclusion

This method is suitable for screening a range of artificial and natural sweeteners in jam and squash drinks. It is not recommended for the detection of neotame, NHDC or aspartame in yoghurt matrices. As is typical for a method such as this, 70 – 120% is deemed to be an acceptable range for recovery, apart for the sweetener matrix combinations listed above. Several laboratories showed variation in results, further training or practise may be required to improve performance overall. It is therefore recommended that the method be validated in–house before use.

Appendix to ANNEX 1.

List of documents

1. List of Participating Laboratories
2. Standard Operating Procedure
3. Instructions for Pre-trial
4. Evaluation of the results for the sweetener pre-trial
5. Main trial instructions and results sheet
6. Results

1. Participating Laboratories

Aberdeen Scientific Services Laboratory
Cardiff – Minton, Treharne & Davies Ltd
Cardiff Scientific Services
Dundee City Council, Scientific Services
Edinburgh Scientific Services
Glasgow Scientific Services
Kent Scientific Services
Lancashire County Scientific Services
LGC Ltd
Public Analyst's Laboratory, Dublin
Staffordshire County Laboratory and Scientific Services
West Wales – Minton, Treharne & Davies Ltd
West Yorkshire Analytical Services
Wolverhampton Public Analyst Laboratory

Analytical method for Validation by Collaborative Study.

Simultaneous Determination of Seven Sweeteners by High Performance Liquid Chromatography with UV detection.

Note: This method protocol includes some aspects that are specific to the collaborative study being carried out. It should not be assumed that it can be used for general application until the study has been completed.

Simultaneous Determination of Seven Sweeteners by High Performance Liquid Chromatography with UV detection.

1. SCOPE

This method describes a high performance liquid chromatographic method for the simultaneous determination of seven sweeteners in fruit squash, carbonated soft drinks, yoghurt, biscuits and jam. The sweeteners are Acesulfame K, Aspartame, Neotame, Saccharin, Neohesperidine dihydrochalcone (NHDC), Stevioside, and Rebaudioside A.

For the purposes of this study, the samples should be analysed as received, however fruit squash should normally be diluted for consumption as directed by the manufacturer, before analysis.

2. REFERENCES

- Wasik, A., and Buchgraber, M., *Foodstuffs-Simultaneous determination of nine sweeteners by high performance liquid chromatography and evaporative light scattering detection,* **IRMM :** p. 35, 2007
- Steviol Glycosides, Prepared at the 73rd JECFA (2010), published in FAO JECFA Monographs, 10 (2010).

3. METHOD PRINCIPLE

The sweeteners are extracted into an aqueous buffer solution. The sample extracts are purified using solid-phase extraction cartridges before HPLC analysis with UV detection.

4. REAGENTS

NB Unless otherwise stated all reagents are of analytical grade quality, and should be prepared using purified water.

4.1. **Acesulfame K**, e.g. Product No. 04054-25G, Sigma Aldrich

4.2. **Saccharin**, e.g. Product No. 240931-50G, Sigma Aldrich

4.3. **Aspartame**, e.g. Product No. 4-7135-500mg, Sigma Aldrich

4.4. **Neohesperidin dihydrochalcone (NHDC)**, e.g. Product No. N8757-1G, Sigma Aldrich

4.5. **Rebaudioside A**, e.g. ASB-00018226-100mg,Rebaudioside A (rebiana)(P); ChromaDex Inc.

4.6. **Stevioside**, e.g. Product No. ASB-00019351-100mg, Stevioside (P), ChromaDex Inc.

4.7. **Neotame**, e.g. Product No. USP 1460204, LGC Standards

4.8. **Formic acid**, > 98 %

4.9. **Water**
4.10. **Triethylamine,** > 99 %

4.11. **Methanol** (HPLC grade)

4.12. **Extraction solution (pH 4.5)**
Dissolve 1.6 mL of formic acid (4.8) in 1800mL of water (4.9) in a 2 litre beaker. Using a pH meter, adjust to pH 4.5 ± 0.1 with approximately 5 ml triethylamine (4.10). Transfer to a 2L volumetric flask and make to volume with water.

4.13. **Acetonitrile (HPLC grade),** ≥ 99.8 %

4.14. **Sodium phosphate, monobasic (NaH2PO4)** (e.g. Product No. S50110G, Sigma Aldrich)

4.15. **Orthophosphoric acid**, ≥ 84.0 %

4.16. **Sodium phosphate buffer, 10 mM, pH 2.6**
Dissolve 2.4 g of sodium phosphate (4.14) in 1800 mL of water. Using a pH meter, adjust the pH to 2.6 ± 0.1 with orthophosphoric acid (4.15). Transfer to a 2L volumetric flask and make to volume with water.

4.17. **HPLC mobile phase A,** sodium phosphate buffer (4.16): acetonitrile (4.13); 90/10 ratio.
Using a measuring cylinder, add 900ml of sodium phosphate buffer (4.16) and 100ml acetonitrile into a suitable container and mix well. Degas before or during use.

4.18. **HPLC mobile phase B,** Acetonitrile (4.13) Degas before or during use.

5. STANDARD PREPARATION

5.1 Stock Standard Solutions *(Supplied*)*

5.1.1. Weigh 100 mg of Aspartame, Acesulfame K, Stevioside, Rebaudioside A and Neotame into separate 50 ml volumetric flasks and dissolve in water. The exact weight taken should be recorded in the appropriate workbook. (~ 2000 g/mL)

5.1.2. Weigh 50 mg of Saccharin into a 50 ml volumetric flask and dissolve in water. The exact weight taken should be recorded in the appropriate workbook. (~ 1000 µg/mL)

5.1.3. Weigh 40 mg of NHDC into a 100 ml volumetric flask and dissolve in water. The exact weight taken should be recorded in the appropriate workbook. (~400 µg/mL)

N*ote: Some stevioside standards also contain Rebaudioside A (Reb A). When first using a new supply of Stevioside standard material, an individual standard should be prepared and analysed to confirm the presence of any Reb A. If Reb A is found to be present the Stevioside can still be used but an allowance should be made for its purity. If both Reb A and Stevioside are to be quantified, the Reb A concentration should be adjusted to correct for the quantity of Reb A in the stevioside standard.*

***Although the stock standards are not very labile, they should be stored in a fridge.**

5.2. **Intermediate Mixed Standard Solutions**

5.2.1 Intermediate Mixed Standard 1 (200 µg/ml Aspartame, Saccharin, Acesulfame K, Stevioside, Reb A)

Pipette 2.0 ml each of the stock standard solutions for Aspartame, Acesulfame K, Stevioside and Reb A (5.1.1) and 4ml of the stock standard solution for saccharin (5.1.2) into a 20 ml volumetric flask and dilute to volume with water.

5.2.2 Intermediate Mixed Standard 2 (200 µg/ml Neotame & NHDC)

Pipette 2.0mL of the stock standard solution for Neotame and 10mL of the stock standard solution for NHDC into a 20mL volumetric flask and dilute to volume with water.

These mixed standards may be stored in a fridge for at least two weeks and probably longer.

5.3. **Calibration Standards Solutions**

The following volumes of the intermediate standard solution should be pipetted into 10 ml volumetric flasks and diluted to volume with mobile phase A (4.17).

Table 1: Preparation of Intermediate standard solutions

	Vol of Mixed intermediate 1	**Vol of Mixed intermediate 2**	**Final Volume**
	ml	**ml**	**ml**
Standard 1	0.05	0.025	10
Standard 2	1	0.5	10
Standard 3	1.5	1.0	10
Standard 4	2	1.5	10
Standard 5	3	2.0	10

This will give standards containing the following concentration of each analyte*.

Table 2: Sweetener concentrations in calibration standards.

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
	µg/mL	µg/mL	µg/mL	µg/mL	µg/mL
Aspartame, Saccharin, Acesulfame K, Stevioside, Reb A	1	20	30	40	60
NHDC, Neotame	0.5	10	20	30	40

* *The calibration levels are indicative and can be adjusted to fit the concentrations expected in the samples.*

Calibration standards should be prepared on the day of use until stability is established.

5.4. **Quality Control Standard**

There are currently no reference materials available containing all of the sweeteners described in this SOP. Recovery will be assessed by spiked additions.

For the purposes of this study, a blank matrix has been provided for each of the test samples. Each blank sample is to be spiked (in duplicate) with each of the standard stock solutions as shown in Table 3 below:

Table 3: Spiking volumes to be added to blank sample

Sample	Pre-trial Sample	Test Sample 1	Test Sample 2	Test Sample 3	Test Sample 4	Test Sample 5
Blank Aliquot	**5 g**	**5 g**	**5 ml**	**5 ml**	**5 g**	**5 g**
Volume of individual stock standards to add (µl)						
Acesulfame K	2500	2500	875	875	875	875
Saccharin	1000	1000	400	400	500	500
Aspartame	2500	2500	1500	1500	2500	2500
NHDC	625	625	375	375	625	625
Neotame	80	80	50	50	80	80
Reb A	1515	1515	605	605	758	758
Stevioside	1250	1250	500	500	625	625

Add the blank aliquot into the volumetric flask and then add the spikes directly into the flask using appropriate, calibrated pipettes. Proceed with the analysis as described.

6. APPARATUS

Standard laboratory glassware, together with the following:-

6.1. **Grade A laboratory glassware.**
6.2. **Analytical balance,** capable of weighing to 0.0001 g.
6.3. **Falcon tubes**, 50 ml
6.4. **Food blender,** suitable for homogenisation of food samples
6.5. **Ultrasonic bath**
6.6. **Centrifuge,** capable of maintaining 4000rpm
6.7. **SPE Vacuum system,** or equivalent
6.8. **Turbovap Evaporator,** capable of maintaining 40^{o}C ± 0.5 *(A rotary evaporator may also be used)*
6.9. **pH meter**
6.10. **Bond-Elut C18-OH cartridges,** 1g/ 6ml (Crawford Scientific, Product No. 12256040) ***- Supplied***
6.11. **Disposable HPLC vials**
6.12. **50 ml volumetric flasks**
6.13. **Pipettes,** different volumes
6.14. **Disposable plastic syringes,** 10ml
6.15. **HPLC System**

- Column: Luna C18, 5u, 250mm x 4.60mm 5 micron
- Flow rate: 1.0 ml/min

- Run time: 30 min
- Oven temperature: 40^{o}C
- Injection volume: 50ul
- Mobile phase: A: 10mM sodium phosphate buffer, pH 2.6 (4.16) : acetonitrile [90:10]

  B: Acetonitrile (4.17)
- Gradient program:

Time (min)	2	12	25	26	30
Mobile phase % A	100	75	75	100	100
Mobile phase % B	0	25	25	0	0

- Detection: UV at 210nm wavelength.

7. SAMPLE PREPARATION AND EXTRACTION

7.1. **Preparation of test sample**

Blend the test sample if required, to produce a homogenous sample. Liquid samples may be subjected directly to the extraction procedure. Fruit squash samples should be diluted to their ready-to-drink concentration prior to extraction.
For the purposes of this study, the test samples should be analysed directly as received. Samples should be mixed well before analysis but no dilution is required before extraction.

7.2. **Extraction and clean-up**

7.2.1. Weigh 5 g (5 mL for liquid samples) of the homogenised test sample (7.1) into a 50 ml volumetric flask. Make up to the mark with buffer solution (4.12), mix thoroughly to disperse the sample and sonicate for 15 min. The exact weight of sample taken should be recorded in the appropriate workbook.

7.2.2. Transfer the extract to a 50 ml Falcon tube . Centrifuge at 4000 rpm for 10 min.
Note: *If the test solution is clear, this step can be ignored.*

7.2.3. Condition the SPE cartridge (6.10) by applying 3 ml methanol (4.11). Elute the methanol to waste.

7.2.4. Equilibrate the SPE cartridge by applying 6 ml of buffer solution (4.12) and elute to waste, as above.

7.2.5. Load the SPE cartridge with 10 ml of sample extract (7.2.2) and elute to waste.

7.2.6. Wash the SPE cartridge with 3 ml of buffer solution (4.12) and elute to waste.

7.2.7. Elute the sweeteners from the SPE cartridge by applying 6 ml of methanol (4.11) and collect the eluate in a 10mL tube.
Note: For all steps 7.2.3 – 7.2.7, the flow-rate should be approx. 1 – 2 mL/min. Use a slight vacuum if necessary. Ensure that the sorbent bed is not allowed to dry out between each stage. Stop the elution when the eluent just reaches the sorbent bed.

7.2.8. Evaporate the eluate to dryness.(6.8).

7.2.9. Dissolve the residue in 5 ml of mobile phase A (4.16) and transfer to an HPLC vial ready for injection on the UV-HPLC system. If required, dilute the extract with

mobile phase A (4.16) to ensure that the expected concentration of each sweetener is within the calibration range. It may be necessary to prepare several dilutions for each extract.

7.2.10. Calibration standards should be injected at the beginning and end of the run and at suitable intervals throughout the run.

8. CALCULATION OF RESULTS

8.1.1. Using a suitable Excel spreadsheet construct a linear regression curve using the areas obtained for the calibration standards and determine both the slope (m) and intercept (c) of the curve. From the responses for the sample (y), determine the concentration of each sweetener in the injected solution using the following equation:

$$x\ (ug/ml) = \frac{y - c}{m}$$

Calculate the concentration of each sweetener in the sample using the following equation:

$$\text{Concentration of sweetener in the sample } (\mu g/g) = \frac{x \times v \times d \times w}{m \times z}$$

where:

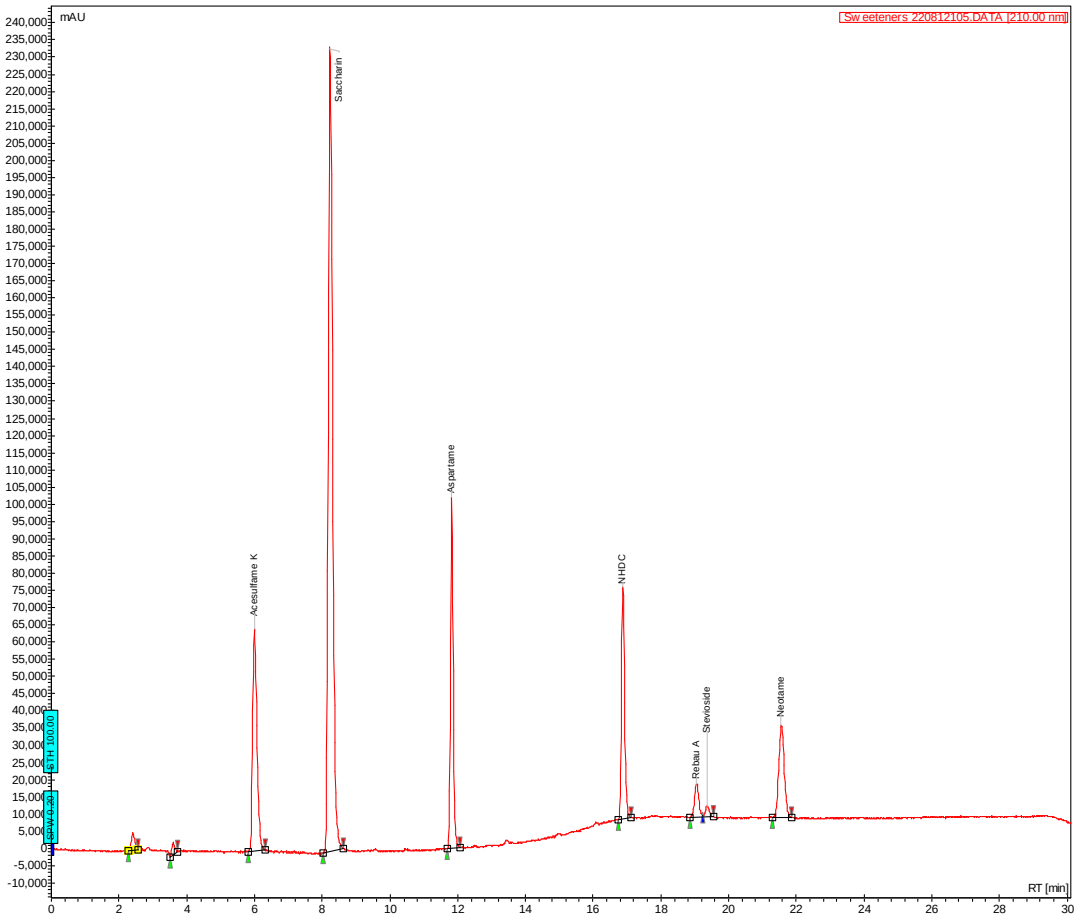
x = concentration of the sweetener in the injected solution (ug/ml)
v = volume of sample extract (ml)
d = dilution factor (if used)
w = final volume after SPE (ml)
z = volume of aliquot taken through SPE clean-up (ml)
m = weight of test portion of the sample (g)

9. ANALYTICAL QUALITY CONTROL

9.1. **Spike recoveries**

The percent recovery for each sweetener should be calculated and should lie between 80 and 120 %. The acceptable range for recovery is to be established. Please report the recovery obtained for each sweetener but do NOT correct the sample results

10. EXAMPLE CHROMATOGRAM



Sweetener	Approximate retention time (Minutes)
Acesulfame K	5.9

Saccharin	8.2
Aspartame	11.8
NHDC	16.9
Reb A	19.1
Stevioside	19.4
Neotame	21.6

3. Instructions for Pre-trial

Collaborative Validation of a Method for Simultaneous Determination of Seven Sweeteners by High Performance Liquid Chromatography.

Study Instructions:

Materials Supplied:

- 2 units of test sample 1.
- 1 unit of blank test sample
- 1 unit of each sweetener stock standard (in water)
- Eight SPE Columns

The samples and standards should be stored in a fridge until required for analysis.

Standards:

The stock standards supplied are prepared as shown in Section 5.1 of the SOP and have the following concentrations:

Standard	Concentration ug/ml
Aspartame	2002
Acesulfame K	2002
Rebaudioside	2000
Stevioside	2002
Neotame	2001
Saccharin	1001
NHDC	402

Test Samples:

Each of the test samples should be analysed, in duplicate according to the SOP.

Blank Sample :
The blank sample should be analysed in duplicate, according to the SOP.

In-house Spiked Sample:
The blank sample should be spiked in duplicate with the supplied stock standards using the volumes shown in Section 5.4 of the SOP (Table 3- Pretrial sample). The spiked samples should be analysed according to the SOP.

A 5g sample weight should be used in all cases. Samples, spiked samples and blanks should be analysed in a single batch. The SOP should be followed without deviation

Information required.

- The results obtained should be entered onto the supplied results sheet.
- The in-house recoveries for each sweetener should be entered onto the supplied results sheet.
- Please supply a copy of the chromatograms obtained for the top standard (Standard 5), a sample, blank, and a spiked blank and examples of the standard calibration lines.
- Please provide the HPLC conditions used and provide details of any deviations from the SOP (however small).

The SOP and results sheet will be sent separately by email.

Results can be sent by email with scanned copies of chromatograms etc if desired or by post to Paul Lawrance, Food & Consumer Safety, LGC, Queens Road, Teddington, Middx. TW11 0LY. (Email: paul.lawrance@lgcgroup.com) before the 26th July 2013.

Pre-trial Results Sheet

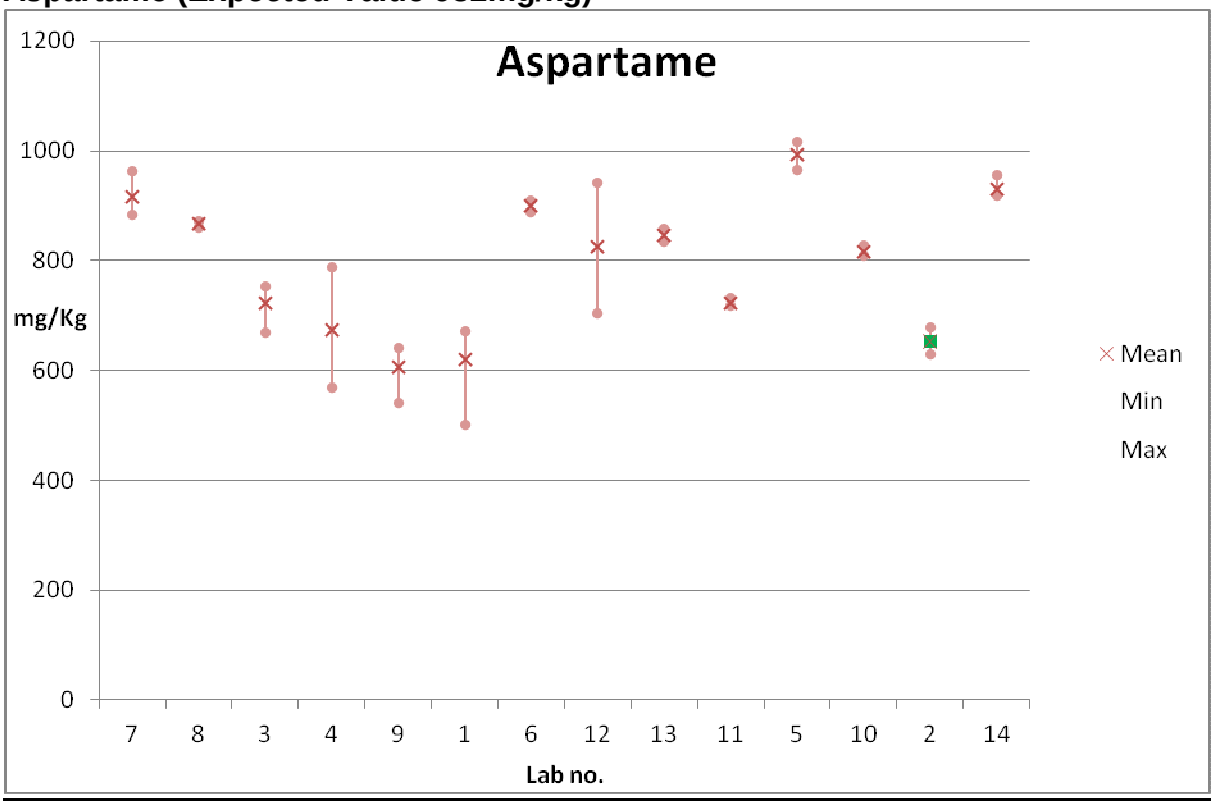
Sweetener	Sweetener Concentration (µg/g)								In-house recovery	
	Test Sample (a)		Test Sample (b)		Blank		Spiked Blank		%	%
	i	ii	i	ii	i	ii	i	ii	i	ii
Aspartame										
Acesulfame K										
Rebaudioside										
Stevioside										
Neotame										
Saccharin										
NHDC										

Analysis Date

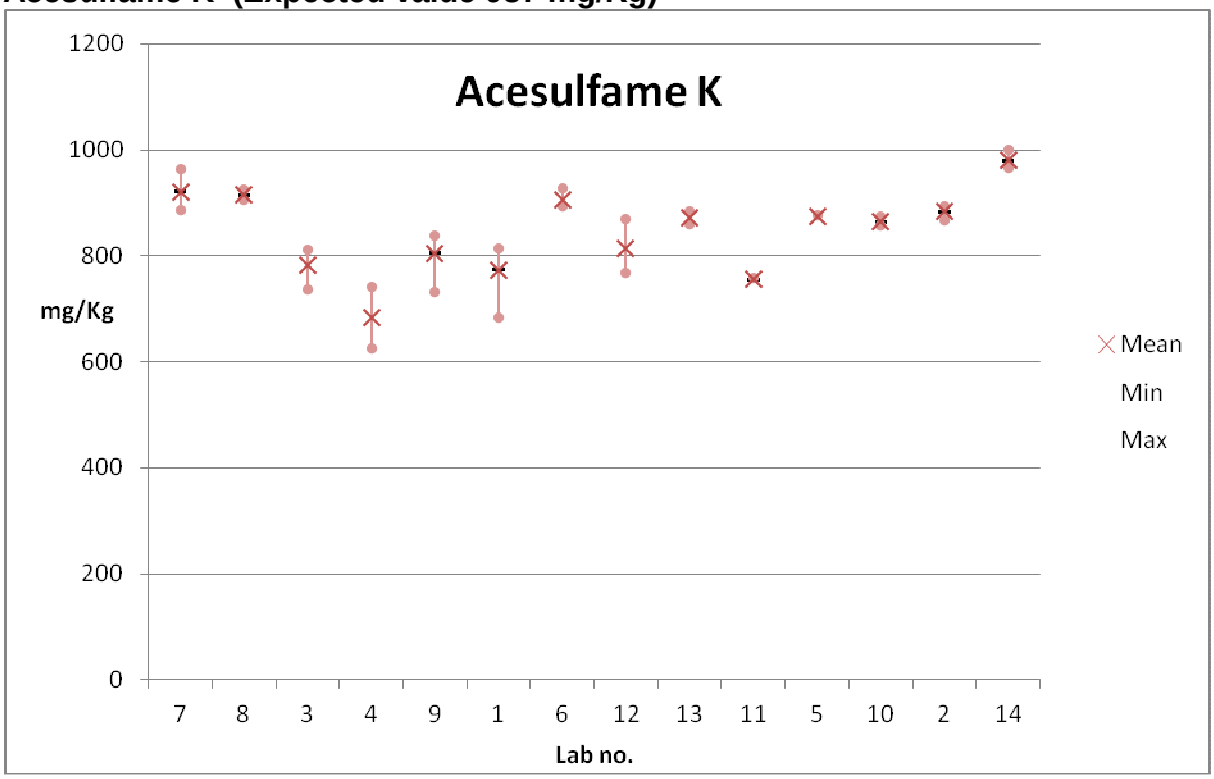
4. Evaluation of the results for the Sweeteners pre-trial

The results obtained for the pre-trial sample are summarised in the following graphs:
The mean result is the mean obtained for the four test sample measurements.
The range shown is the minimum and maximum result obtained for the four results.
Lab 14 is LGC and shows the results obtained during homogeneity testing (Mean ± 2SD n=20)

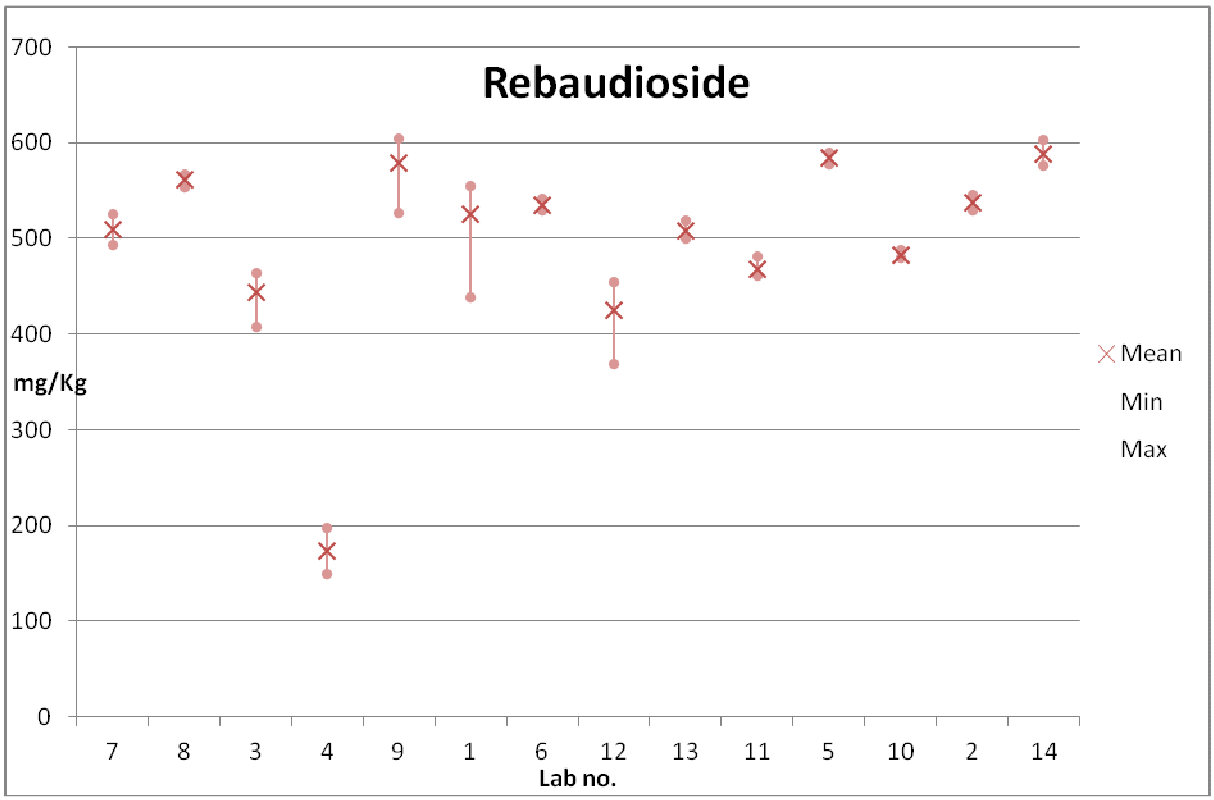
1. Aspartame (Expected Value 982mg/kg)



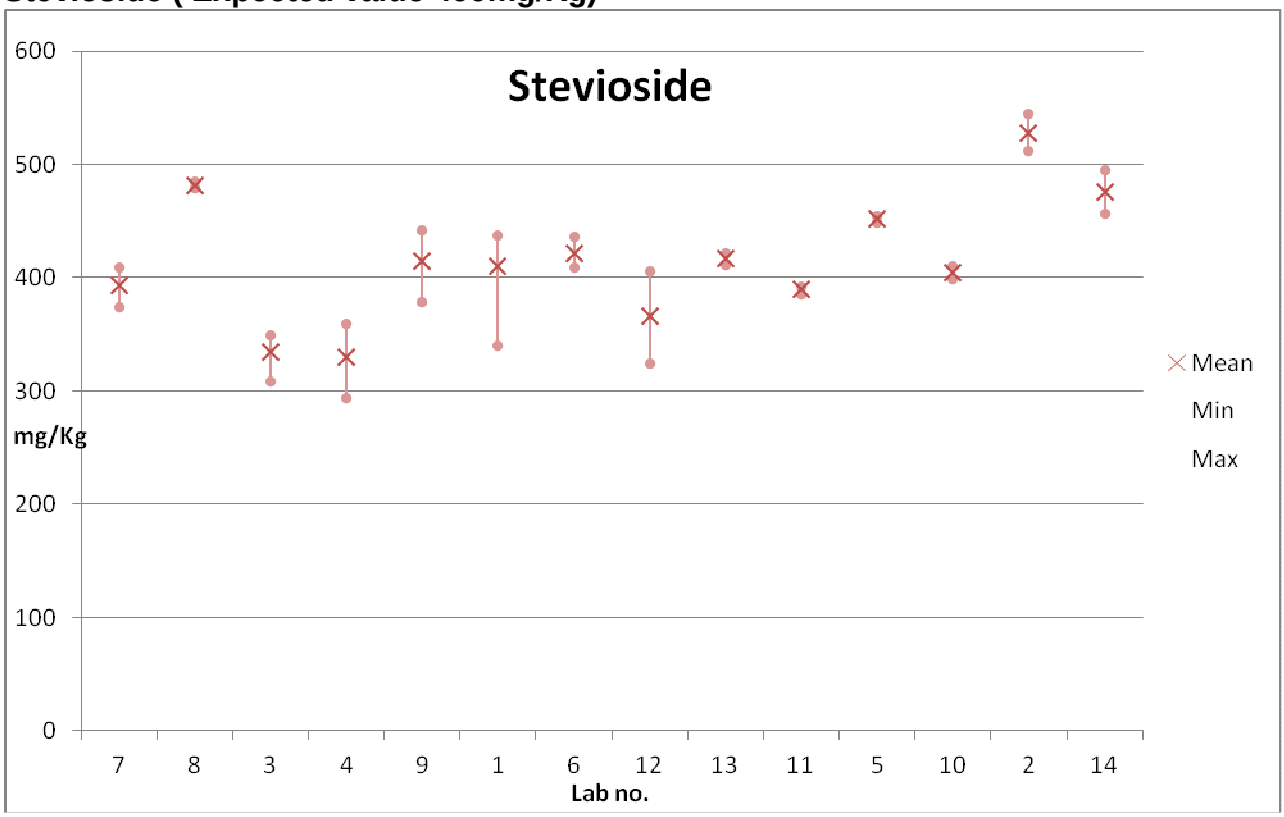
2. **Acesulfame K (Expected value 987 mg/Kg)**



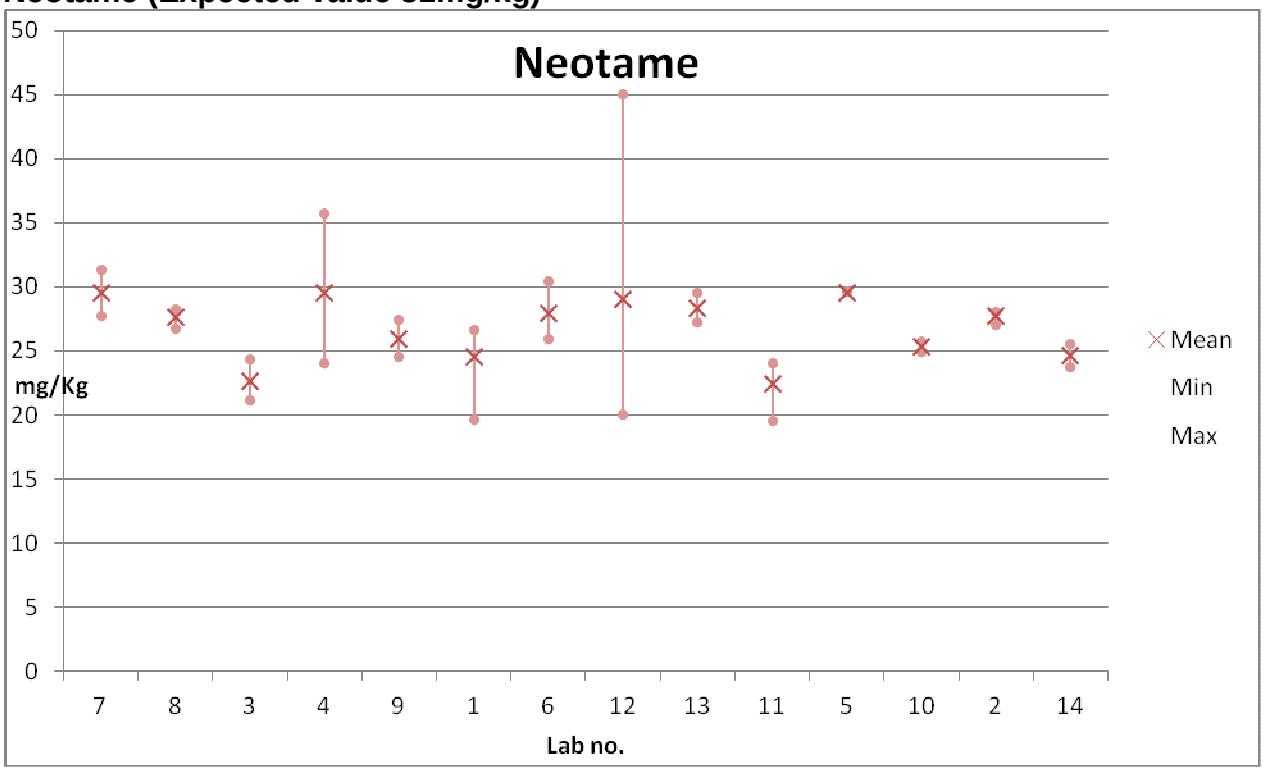
3. **Rebaudioside (Expected value 590mg/Kg)**



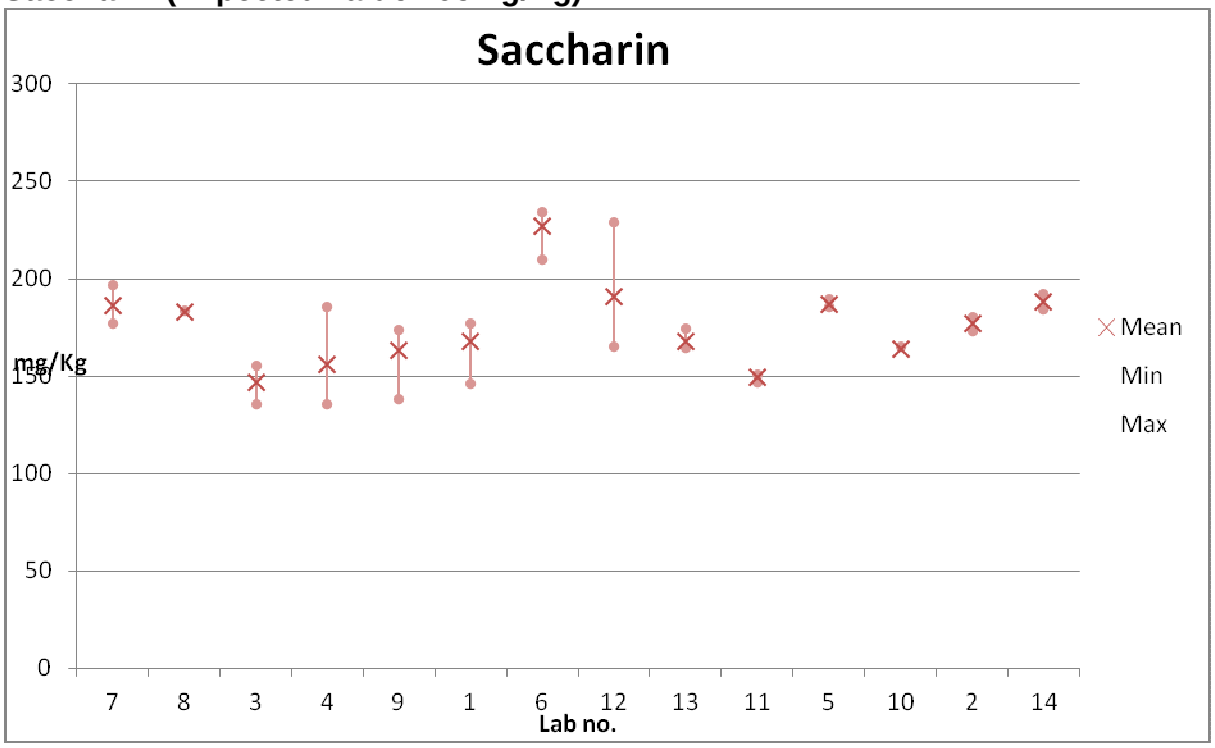
4. Stevioside (Expected value 499mg/Kg)



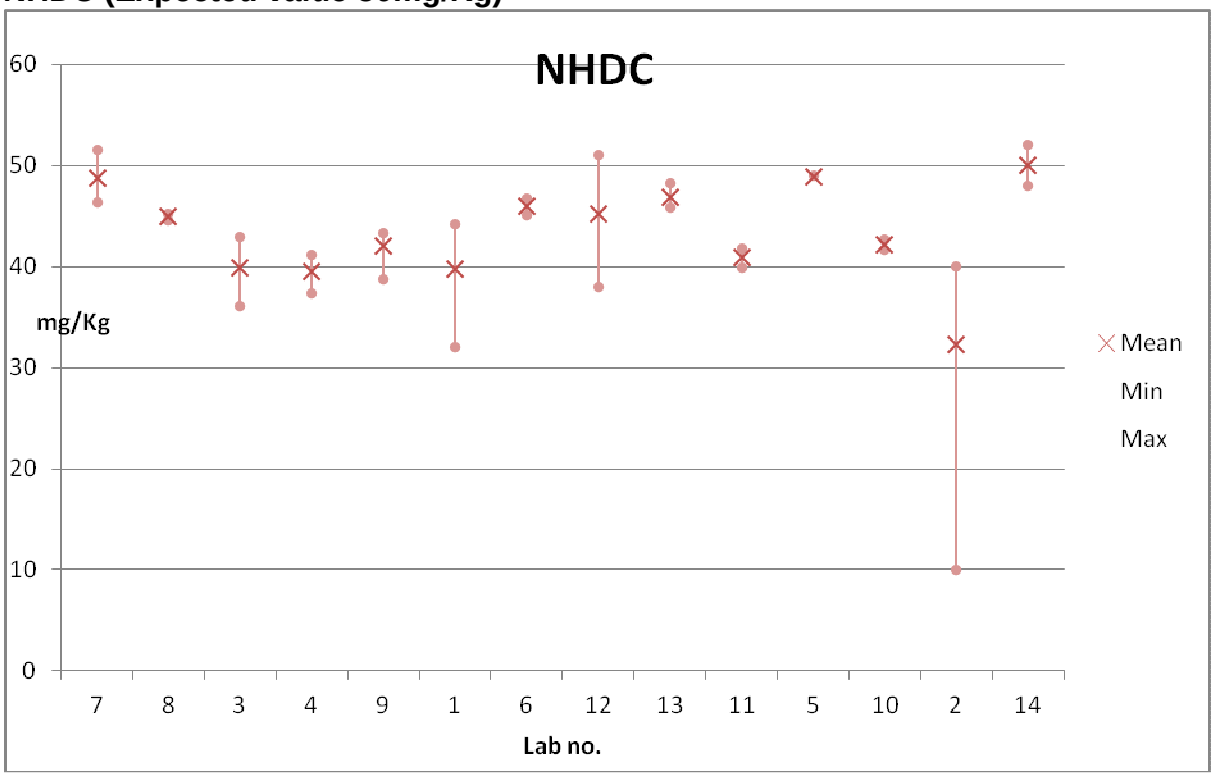
5. **Neotame (Expected value 31mg/kg)**



6. **Saccharin (Expected value 195mg/Kg)**

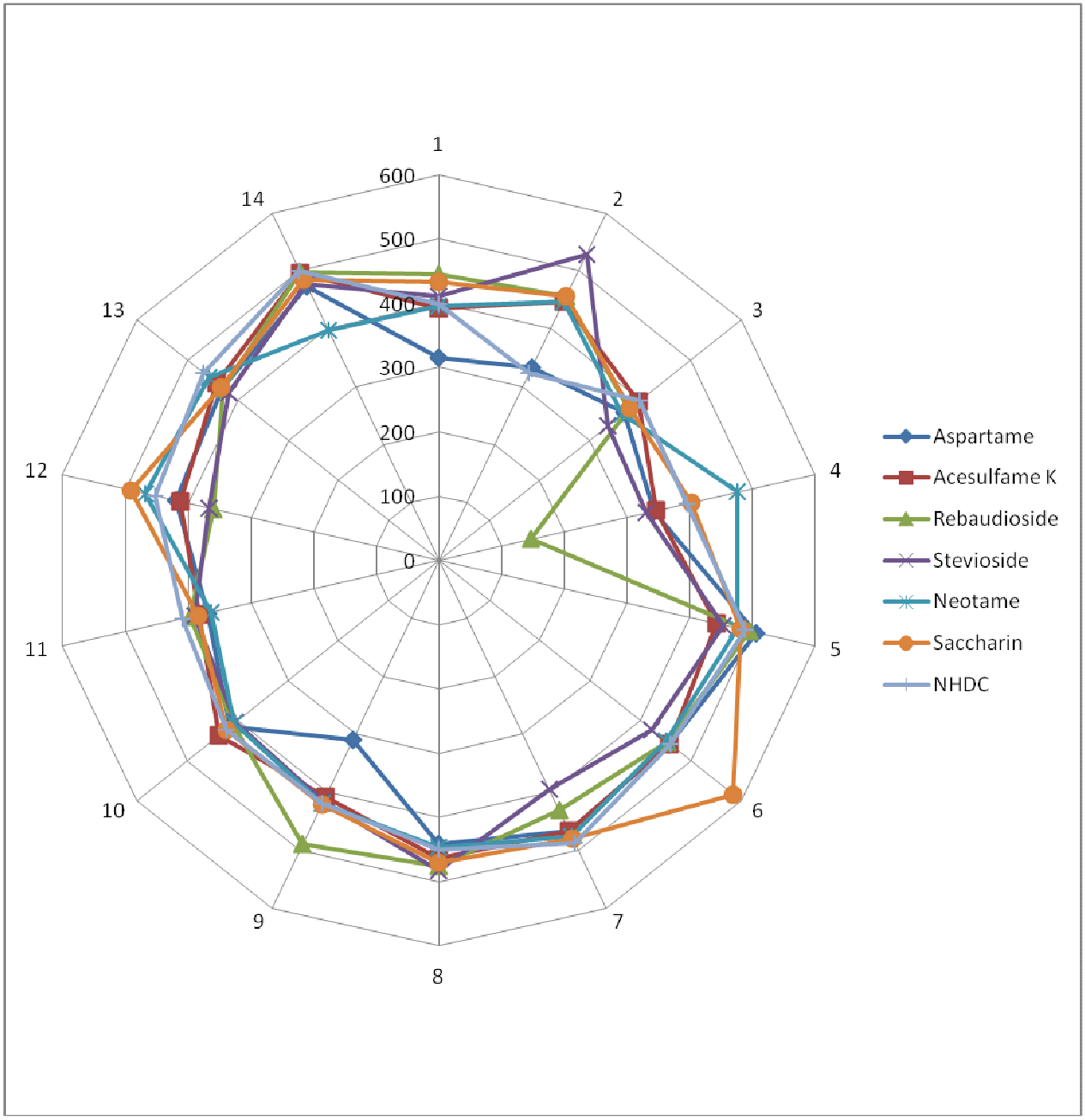


7. NHDC (Expected value 50mg/Kg)



8 Comparison of normalised results by laboratory

In the following plot, the results have been normalised to an arbitrary value of 500 by normalising the ratios of the expected amounts. This allows a comparison of laboratory performance on a similar scale for all laboratories and sweeteners.



Discussion

With the exception of Neotame, many (but not all) of the results obtained by the participating laboratories are lower than those obtained by LGC during homogeneity testing. The cause for this is unknown but may reflect some difference between the samples or standards after distribution compared with those used at LGC, although this was not expected. We have tried to standardise the distribution as far as possible and will use a simulated postage step at LGC for the main trial.

There are some differences between the means obtained and in the spread of the individual results. These vary by laboratory and by sweetener.

The radar plot does not show that any one laboratory is getting significantly high or low results for all sweeteners. Laboratories 1, 2, 4, 6, 9 & 12 show a higher spread caused by high or low results for individual sweeteners but there is no obvious trend except that as mentioned, most results are slightly lower than originally expected.

Please identify your own laboratory and where results stand out or have a large spread, please review your procedures and calculations to see whether any reasons can be identified for these variations. Any issues identified, should be corrected before carrying out the main trial. It would be useful if you could notify the coordinator of any issues found.

Paul Lawrance
LGC

5. Main trial instructions and results sheet

Collaborative Validation of a Method for Simultaneous Determination of Seven Sweeteners by High Performance Liquid Chromatography.

Study Instructions:

Materials Supplied:

- 1 unit each of 10 test samples. (1-10)
- 1 unit each of 5 blank samples.
- 1 unit each of 7 sweetener stock standards (in water) (For calibration and spiking)
- 25 SPE Columns

The samples and standards should be stored in a fridge until required for analysis.

Standards:

The stock standards supplied are prepared as shown in Section 5.1 of the SOP and have the following concentrations:

Standard	Concentration ug/ml
Aspartame	2018
Acesulfame K	2012
Rebaudioside	2002
Stevioside	2002
Neotame	1840
Saccharin	1002

NHDC	400

Test Samples:

Samples and blanks are provided as follows**:**

Samples

Test Samples
1
2
3
4
5
6
7
8
9
10

Blanks

Blank label	Blank Number
“ For samples 5 & 10”	1
“ For samples 4 & 9”	2
“ For samples 2 & 7”	3
“ For samples 1 & 6”	4
“ For samples 3 & 8”	5

Note: There are two samples of each matrix type, however the sweetener content may vary.

Analysis required

Each of the test samples should be analysed once only using the supplied SOP. A 5g sample weight should be used.

Blank Sample :

The blank samples supplied should be analysed once only.

In-house Spiked Sample:

The blank sample should be spiked in duplicate with the supplied stock standards using the volumes shown in Section 5.4 of the SOP (Table 3- Test samples 1-10).

A 5g sample weight should be used in all cases. The analysis may be batched if required but each sample and its relevant blank and spikes should be run within a single batch. The SOP should be followed without deviation.

Information required.

- The results obtained for each sample
- The results obtained for each blank sample
- The results obtained for the spiked samples
- Calculate the recovery of each sweetener compared to the amount added

Results should be corrected for any sweetener found in the relevant blank sample but the amount found in the blank should be reported. The recoveries for the spikes should be reported but do NOT correct the samples for recovery.

- Please enter all data onto the supplied results sheet. *(Please use the excel sheet provided and submit the form as an Excel file to avoid unnecessary result transcription).*

- Please supply a copy of the chromatograms obtained for the top standard (Standard 5), and for each sample, blank and spike. Calibration lines for each sweetener should also be provided. *Preferably, these should be submitted in electronic form (e.g. as scanned pdf's) but hard copies are acceptable.*

- Please provide the HPLC conditions used and provide details of any deviations from the SOP (however small) if these are absolutely necessary.

Results should be sent by email to Paul Lawrance, Food & Consumer Safety, LGC, Queens Road, Teddington, Middx. TW11 0LY. (Email:paul.lawrance@lgcgroup.com).

The deadline for return of results is the 15th November 2013. Results submitted after this date may not be used.

Please feel free to contact me if you have any queries

Paul Lawrance

LGC – Food & Consumer Safety (Study organiser)

Results sheets

Sweetener Concentration mg/Kg										
Test Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Aspartame										
Acesulfame K										
Rebaudioside										
Stevioside										
Neotame										
Saccharin										
NHDC										
Please report results to 3 significant figures										
Analysis Date (s)										
Laboratory										
Contact										
Tel/email:										

Column	
Column dimensions	
Mobile Phase	
Oven temperature ℃	
Injection volume µL	
Gradient	
Flow-rate mL/min	
Detection wavelength. nm	
Please enter conditions used	
If identical to the SOP enter "as SOP"	

Blank 1	Sweetener Concentration mg/Kg			Spike Recovery (%)	
Sample	Blank	Spike A	Spike B	A	B
Aspartame					
Acesulfame K					
Rebaudioside					
Stevioside					
Neotame					
Saccharin					
NHDC					

Please report results to 3 significant figures

Blank 2	Sweetener Concentration mg/Kg			Spike Recovery (%)	
Sample	Blank	Spike A	Spike B	A	B
Aspartame					
Acesulfame K					
Rebaudioside					
Stevioside					
Neotame					
Saccharin					
NHDC					

Please report results to 3 significant figures

Blank 3	Sweetener Concentration mg/Kg			Spike Recovery (%)	
Sample	Blank	Spike A	Spike B	A	B
Aspartame					
Acesulfame K					
Rebaudioside					
Stevioside					
Neotame					
Saccharin					
NHDC					

Please report results to 3 significant figures

Blank 4	Sweetener Concentration mg/Kg			Spike Recovery (%)	
Sample	Blank	Spike A	Spike B	A	B
Aspartame					
Acesulfame K					
Rebaudioside					
Stevioside					
Neotame					
Saccharin					
NHDC					

Please report results to 3 significant figures

Blank 5	Sweetener Concentration mg/Kg			Spike Recovery (%)	
Sample	Blank	Spike A	Spike B	A	B
Aspartame					
Acesulfame K					
Rebaudioside					
Stevioside					
Neotame					
Saccharin					
NHDC					

Please report results to 3 significant figures

6. Data returned from Collaborative Trial

A. Results Summary per Laboratory

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	866.2	909.8	213.3	219.9	558.1	580.8	623	662	704	641
Acesulfame K	912.7	952.3	137.7	156.7	346.6	350.3	276	290	288	266
Rebaudioside	615.6	637.5	138.8	156.9	341.2	324.6	272	278	293	253
Stevioside	446.7	465.2	75.4	85.3	224.5	222.4	335	342	361	341
Neotame	26.54	27.34	6.14	7.12	19.39	19.2	18	19	21	19
Saccharin	184.6	192.4	35.1	41.6	81.9	84.5	81	84	94	86
NHDC	45.2	47.8	190.6	218.9	23.6	28.7	48	49	48	45

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	915	861	262	259	615	608	152	151	147	139
Acesulfame K	951	910	167	164	351	351	276	275	302	304
Rebaudioside	600	583	152	151	327	326	273	275	276	284
Stevioside	522	505	94.9	94.5	241	240	268	275	279	289
Neotame	26.7	25.9	8.50	8.62	19.2	19.8	<2.5	<2.5	<2.5	<2.5
Saccharin	180	173	42.8	42.2	78.4	78.3	69.4	71.9	91.9	87.4
NHDC	48.1	44.9	10.8	11.6	29.7	29.7	21.5	22.6	33.9	34.3

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	760	761	180	176	530	577	16.0	16.1	5.71	18.6
Acesulfame K	860	837	129	132	287	322	278.0	277	307	289
Rebaudioside	711	698	151	147	344	412	341.0	344	379	347
Stevioside	474	442	61.7	60	173	199	220.0	218	237	225
Neotame	23.4	24.7	5.81	5.8	15.7	17.7	0.0	0	0	0
Saccharin	152	157	24.9	25.9	66.9	76.9	251.0	256	35.9	35.7
NHDC	44.0	48.1	12.4	9.54	26.1	29.1	23.2	21.5	33.4	32.9

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	997	1013	228	207	581	547	494	516	587	549
Acesulfame K	874	927	177	165	345	314	274	270	309	292
Rebaudioside	553	554	112	110	344	323	251	261	296	286
Stevioside	418	420	69	69	212	196	229	237	281	272
Neotame	26.7	25.1	6.8	6.0	19.8	17.5	9.3	10.2	11.8	10.7
Saccharin	229	220	33	33	93	90	71	69	92	85
NHDC	29.7	30.6	<1	15.5	18.1	19.0	21.6	42.1	30.5	47.3

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	693	862	204	210	574	576	18	15	6	15
Acesulfame K	901	944	129	144	344	344	317	310	305	309
Rebaudioside	432	457	125	132	264	244	246	310	301	303
Stevioside	347	314	72	76	198	198	261	244	237	240
Neotame	26.0	28.0	8.0	8.0	20.0	21.0	n/d	n/d	n/d	n/d
Saccharin	184	196	48	38	89	90	257	258	225	229
NHDC	47.0	49.0	249.0	257.0	33.0	33.0	58.0	55.0	62.0	59.0

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	588	775	257	81	487	444	90	81	113	23
Acesulfame K	649	854	193	79	358	363	841	758	224	227
Rebaudioside	461	893	117	84	312	346	313	296	210	238
Stevioside	243	489	35	38	184	201	189	177	159	172
Neotame	17.3	22.7	5.1	1.5	11.2	13.0	0.0	0.0	4.8	0.2
Saccharin	127	160	41	19	82	79	72	64	64	70
NHDC	37.4	59.6	0.0	0.0	45.1	37.9	16.4	12.3	21.0	22.0

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	748	780	95	127	474	534	116	94	72	78
Acesulfame K	862	834	126	122	334	335	283	285	327	288
Rebaudioside	595	584	115	116	301	306	255	255	279	288
Stevioside	445	443	57	66	176	199	193	196	217	227
Neotame	25.3	25.5	5.8	6.1	17.8	18.6	0.0	0.0	0.0	0.0
Saccharin	176	174	33	37	79	80	76	76	92	89
NHDC	41.2	41.8	9.8	9.7	29.3	30.6	22.9	19.8	30.7	26.4

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	976	929	191	187	580	576	35	36	14	10
Acesulfame K	931	877	119	117	333	330	272	272	296	296
Rebaudioside	565	534	119	117	303	301	259	259	277	292
Stevioside	472	437	59	60	187	187	192	193	221	231
Neotame	25.4	26.1	5.8	5.1	17.9	17.6	0.0	0.0	0.0	0.0
Saccharin	169	170	25	23	85	85	86	84	106	104
NHDC	42.5	42.6	26.8	18.0	32.7	31.1	17.4	17.2	28.0	28.8

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	849	857	203	208	552	551	11	12	7	8
Acesulfame K	889	896	135	134	333	340	291	290	311	313
Rebaudioside	517	515	125	122	361	367	329	331	346	349
Stevioside	441	424	61	59	204	210	263	260	278	284
Neotame	25.1	25.1	5.7	6.0	17.9	17.4	n/d	n/d	25.2	26.7
Saccharin	162	162	47	47	94	94	95	97	121	117
NHDC	51.9	52.9	7.1	6.3	31.9	30.9	15.2	16.4	23.1	22.2

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	887	874	187	187	560	536	<1	<1	<1	<1
Acesulfame K	920	910	125	126	332	317	274	267	295	296
Rebaudioside	645	625	123	125	330	316	272	269	294	303
Stevioside	466	442	69	71	205	198	200	200	210	235
Neotame	21.8	24.4	5.8	5.8	17.7	16.9	<1	<1	<0.5	<0.5
Saccharin	159	172	23	23	84	75	271	263	286	285
NHDC	38.0	42.2	9.4	9.1	30.6	29.0	18.7	20.6	29.8	27.7

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	591	579	84	80	503	399	20	4	0	19
Acesulfame K	730	654	107	102	297	319	265	285	277	302
Rebaudioside	608	526	72	43	221	141	264	255	268	374
Stevioside	598	528	115	78	279	204	290	250	263	348
Neotame	24.0	17.4	4.7	4.6	14.1	17.5	0.0	0.0	0.0	0.0
Saccharin	142	127	25	24	67	83	68	68	78	82
NHDC	39.1	35.4	8.1	9.1	29.8	31.4	21.0	16.5	26.2	22.2

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	730	726	138	139	487	421	525	445	631	625
Acesulfame K	831	822	117	120	296	284	259	261	292	287
Rebaudioside	570	556	110	112	283	249	257	259	278	274
Stevioside	455	430	71	71	185	166	206	205	225	224
Neotame	27.4	27.0	6.5	6.7	19.0	15.6	16.0	16.5	21.3	20.4
Saccharin	184	190	31	31	77	66	79	77	92	90
NHDC	39.6	38.8	5.6	3.9	24.3	19.9	21.5	19.9	24.0	24.3

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	839	848	177	179	535	534	14	19	14	18
Acesulfame K	888	895	124	125	322	324	299	289	293	300
Rebaudioside	585	587	115	115	293	297	253	250	272	275
Stevioside	509	512	74	74	220	222	234	230	252	257
Neotame	25.4	25.5	6.5	6.5	17.8	18.3	<0.500	<0.500	<0.500	<0.500
Saccharin	172	174	28	29	74	75	75	74	86	88
NHDC	43.1	43.6	9.7	9.7	29.7	29.1	35.3	35.0	40.6	43.4

Sample Type	Jam		Juice Drink Conc		Juice Drink Dil.		Low Fat Yoghurt		High Fat Yoghurt	
Test Sample	5	10	4	9	2	7	1	6	3	8
Aspartame	802	811	216	218	566	549	3	5	0	0
Acesulfame K	849	867	158	162	349	324	280	280	303	304
Rebaudioside	841	761	179	184	517	450	310	344	377	438
Stevioside	427	472	91	97	224	214	226	236	241	246
Neotame	26.2	25.2	8.6	8.4	18.6	19.1	0.0	0.0	0.0	0.0
Saccharin	181	178	40	38	81	79	76	79	91	91
NHDC	47.9	47.4	4.2	0.0	27.8	24.9	18.8	19.2	48.9	34.1

B. Recovery Data by Laboratory (Note: for information only, statistical analysis was carried out on the non-corrected data)

Matrix	Jam						Juice Drink Conc.						Juice Drink Dil.					
Sweetener	Sweetener Concentration mg/Kg			Spike Recovery (%)		Mean Recovery	Sweetener Concentration mg/Kg			Spike Recovery (%)		Mean Recovery	Sweetener Concentration mg/Kg			Spike Recovery (%)		Mean Recovery
	Blank	Spike A	Spike B	A	B	%	Blank	Spike A	Spike B	A	B	%	Blank	Spike A	Spike B	A	B	%
Aspartame	0	767	964	78.1	98.3	88	0	577	529	95.3	87.4	91	0	522	578	86.1	95.4	91
Acesulfame K	22.1	794	1007	81.7	103	93	0	395	368	113	105	109	0	328	374	93.6	107	100
Rebaudioside	0	494	637	101	131	116	0	277	259	139	130	134	0	248	261	99.1	104	102
Stevioside	0	398	489	67.4	82.9	75	0	218	225	89.9	92.8	91	0	207	222	85.5	91.7	89
Neotame	0	23.0	29.8	74.2	96.2	85	0	23.2	19.7	116	98.3	107	0	17.2	19.2	86.2	96.1	91
Saccharin	0	163	211	83.4	108	96	0	90.6	83.4	113	104	109	0	87.1	89.0	109	111	110
NHDC	0	40.4	51.8	138	178	158	0	272	251	905	836	870	0	24.7	25.3	82.3	84.3	83
Aspartame	<5	989	1010	98.0	99.9	99	<5	629	605	104	99.9	102	<5	607	608	100	100	100
Acesulfame K	<5	1030	1050	103	105	104	<5	345	349	98.1	99	99	<5	349	350	99.1	99.3	99
Rebaudioside	<5	587	613	96.7	101	99	<5	242	244	99.8	101	100	<5	236	235	97.2	96.9	97
Stevioside	<5	515	532	103	106	105	<5	193	193	96.4	96.5	96	<5	190	189	95.1	94.7	95
Neotame	<2.5	29.3	30.2	99.4	103	101	<2.5	19.4	19.0	106	103	105	<2.5	18.3	18.2	99.5	99.0	99
Saccharin	<5	202	205	101	102	102	<5	85.2	85.8	106	107	107	<5	80.0	80.1	99.9	99.9	100
NHDC	<2.5	52.0	53.1	104	106	105	<2.5	29.7	29.7	99.2	99.1	99	<2.5	28.9	28.2	96.3	94.0	95
Aspartame	0	832	871	87.0	87.4	87	0	542	545	94.0	94.2	94	0	599	591	101	99.4	100
Acesulfame K	0	910	949	95.5	95.5	96	0	312	322	92.9	95.4	94	0	312	311	90.4	89.8	90
Rebaudioside	0	508	548	88.5	91.4	90	0	214	223	92.8	96.3	95	0	219	217	92.1	91.3	92
Stevioside	0	465	497	97.0	99.3	98	0	181	198	93.8	102	98	0	183	182	92.2	91.7	92
Neotame	0	26.0	27.5	93.4	94.4	94	0	16.4	16.5	93.4	93.6	94	0	18.8	18.3	104	101	103
Saccharin	0	170	180	89.8	90.9	90	0	66.0	69.2	86.5	90.2	88	0	76.1	75.6	96.8	96.0	96
NHDC	0	47.3	49.1	99.8	99.3	100	0	28.7	29.8	101	104	103	0	26.6	26.9	90.3	91.3	91
Aspartame	86.8	1017	1037	100	103	101	75.3	431	405	77.9	80.0	79	8.95	502	562	85.51	96.7	91
Acesulfame K	32.7	917	969	94	96	95	137	153	146	47.7	49.6	49	27.0	318	361	93.5	106	100
Rebaudioside	<1	529	512	86	88	87	<1	183	172	85.113	86.976	86	<1	192	214	84.7	93.5	89
Stevioside	32.6	425	437	86	89	88	<1	170	162	95.671	100.289	98	<1	171	190	90.8	101	96
Neotame	1.7	29.3	27.0	98.9	93.8	96	1.37	14.1	13.6	87.439	91.428	89	1.16	14.3	15.8	82.5	92.0	87
Saccharin	9.6	253	262	125	130	127	32.2	45.2	39.5	62.148	59.639	61	3.69	90.8	98.1	118	128	123
NHDC	4.3	31.4	30.7	61.6	60.4	61	199	<1	<1	0	0	0	45.5	23.0	28.8	81.3	98.5	90
Aspartame	22.9	727	950	71	98	85	0	582	443	96	73	85	24.2	448	441	77	72	75
Acesulfame K	26.8	783	1023	77	105	91	0	345	282	98	80	89	0	346	325	100	96	98
Rebaudioside	0	453	654	74	110	92	0	177	166	73	69	71	0	205	196	87	84	86
Stevioside	0	246	483	49	100	75	0	139	142	70	71	71	0	182	170	93	88	91
Neotame	0	23.6	29.6	79	105	92	0	9.5	14.2	52	77	65	0	15.7	11	78	62	70
Saccharin	2.35	166	211	82	107	95	0	81.1	63.4	101	82	92	0	79	73.5	101	95	98
NHDC	0	31.8	39.3	63	81	72	437	0	0	0	0	0	45.65	52.5	41.2	181	142	162
Aspartame	7.7	727	893	71.3	87.7	80	0.0	556	547	91.8	90.3	91	0.0	860	861	85.3	85.3	85
Acesulfame K	4.9	330	318	91.8	88.3	90	0.0	330	330	93.3	93.1	93	3.7	320	319	89.2	89.0	89
Rebaudioside	0	277	266	90.9	87.5	89	0.0	211	212	86.2	86.9	87	0.0	268	276	88.1	90.6	89
Stevioside	0	218	213	86.6	84.3	85	0.0	162	165	80.8	82.7	82	0.0	212	217	84.0	86.1	85
Neotame	0	26.6	29.7	90.4	101.0	96	0.0	15.9	16.7	86.2	90.7	88	0.0	26.5	29.7	90.1	101	95
Saccharin	0	93.1	89.2	92.9	89.1	91	0.0	79.5	78.7	99.2	98.2	99	0.0	90.4	91.7	90.2	91.5	91
NHDC	0	31.4	29.8	62.4	59.2	61	0.0	26.9	28.0	88.3	92.2	90	0.0	30.0	23.2	59.4	46.0	53
Aspartame	11.2	982	871	101	89.4	95	24.3	590	591	101	101	101	1	598	578	99	96.2	98
Acesulfame K	16.1	931	959	95.7	98.8	97	8.5	340	341	100	100	100	2.35	346	337	98.4	96.4	97
Rebaudioside	0	497	511	84.7	87.3	86	0	229	231	94.8	95.4	95	0	230	226	95.3	93.9	95
Stevioside	0	462	480	95.4	99.3	97	0	184	183	92.1	91.8	92	0	186	182	93.2	91.7	92
Neotame	0	28.5	28.7	100	101	101	0	17.3	17.5	94.4	95.3	95	0	18	17.5	98	95.6	97
Saccharin	4.4	190	186	98.1	98.2	98	17.1	78	76.3	97.7	95.3	97	3.2	85.3	83.8	107	109	108
NHDC	1.9	44.7	45.1	92.4	97.4	95	195	48	44.1	165	152	159	0	30.5	29	102	97.5	100
Aspartame	0	956	967	94.7	95.8	95	0	607	564	100	93.2	97	0	563	583	93	96.3	95
Acesulfame K	0	972	984	96.6	97.8	97	0	356	354	101	101	101	0	353	356	100	101	101
Rebaudioside	0	492	532	81.1	87.7	84	0	244	238	101	98.1	100	0	240	244	98.0	101	100
Stevioside	0	433	485	85.5	95.8	91	0	193	190	95.1	93.9	95	0	199	204	98.5	101	100
Neotame	0	26.5	28.3	89.9	96.1	93	0	17.7	18.2	96	98.8	97	0	18.4	18.5	100	100	100
Saccharin	0	180	184	89.9	91.6	91	0	92.2	91.1	115	114	115	0	97.5	97.7	122	122	122
NHDC	0	56.9	55.9	114	112	113	0	22.4	23.5	74.7	78.2	76	0	30.6	30.8	102	103	103
Aspartame	<1.0	946	921	94.2	92.4	93	<1.0	585	580	97.8	90.0	94	<1.0	598	596	99.2	99.0	99
Acesulfame K	<1.0	1009	989	101	99.5	100	<1.0	349	345	100	98.1	99	<1.0	351	352	100	99.4	100
Rebaudioside	<1.0	601	591	99.6	98.5	99	<1.0	252	247	105	102	104	<1.0	258	257	107	107	107
Stevioside	<1.0	477	466	95.8	94.2	95	<1.0	203	200	103	102	103	<1.0	208	198	104	99.7	102
Neotame	<0.5	26.5	27.1	90.6	87.7	89	<0.5	18.3	17.8	101	96.6	99	<0.5	18.6	18.3	101	100	101
Saccharin	<1.0	195	192	97.8	96.7	97	<1.0	83.7	86.3	106	104	105	<1.0	87.0	86.6	109	109	109
NHDC	<0.5	46.3	45.6	93	92.3	93	<0.5	27.8	28.2	93.9	90.6	92	<0.5	29.5	29.7	98.8	99.7	99
Aspartame	0	774	846	86.6	86.9	87	0	475	504	86.1	86.8	86	0	532	537	87.7	87.5	88
Acesulfame K	0	834	908	93.8	93.5	94	0	296	311	92.3	92	92	0	326	278	92.4	77.8	85
Rebaudioside	0	470	472	87.7	80	84	0	111	119	50	51.2	51	0	135	126	55.6	51.2	53
Stevioside	0	487	546	116	113	115	0	192	213	105	111	108	0	222	217	110	107	109
Neotame	0	21.9	31.2	84.2	110	97	0	12.2	14.7	72.8	83.3	78	0	14.6	15.9	79.2	85	82
Saccharin	0	169	184	95.4	95.1	95	0	69	76.4	94.5	99.3	97	0	73.3	77.3	91.3	95	93
NHDC	0	40.7	45.1	92.1	93.5	93	0	24.5	25.5	89.6	88.6	89	0	26.2	27.3	87.2	89.7	88
Aspartame	4.44	958	961	96.1	96.4	96	2.13	469	468	92.5	92.4	92	<1.00	571	569	98.2	97.8	98
Acesulfame K	14.7	997	991	100.0	99.7	100	9.57	290	287	98.1	97.4	98	2.54	322	322	95.3	95.2	95
Rebaudioside	<1.00	583	583	97.3	97.4	97	<1.00	183	182	90.1	89.8	90	<1.00	211	213	90.9	91.9	91
Stevioside	<1.00	509	505	103.0	102.0	103	<1.00	160	154	95.1	91.9	93	<1.00	182	179	94.8	92.7	94
Neotame	<0.500	29.1	28.9	99.9	99.4	100	<0.500	14.8	14.7	95.8	95.6	96	<0.500	17.9	17.3	101.3	97.6	99
Saccharin	1.04	197	194	99.6	97.9	99	1.03	63.7	62.3	94.9	92.9	94	<1.00	74.1	73.5	96.2	95.4	96
NHDC	0.984	44.9	44.5	90.9	90.0	90	0.831	22.9	22.9	91.2	91.2	91	1.16	25.1	24.9	87.1	86.4	87
Aspartame	0	857	831	85	82	84	0	136	98.3	83.6	80.8	82	0	817	794	81.1	78.7	80
Acesulfame K	0	337	341	96	97	96	0	332	346	93.5	98.1	96	0	342	336	97.1	95.4	96
Rebaudioside	0	322	311	106	102	104	0	228	245	94.3	101	98	0	312	312	103	103	103
Stevioside	0	289	281	116	112	114	0	206	224	103	112	108	0	312	282	125	113	119
Neotame	0	34.7	33.5	120	116	118	0	18.3	21.6	99.6	117	108	0	33	32.7	114	113	114
Saccharin	0	105	101	105	101	103	0	80.5	87.8	101	110	106	0	101	103.5	101	103	102
NHDC	0	26.1	27.6	52	55	54	0	23.4	25.7	77.8	85.6	82	0	31	31.8	62.1	63.6	63
Aspartame		203		101		101		117		96		96		118		98		98
Acesulfame K		193		96		96		66		93		93		69		98		98
Rebaudioside		47		77		77		23		93		93		23		93		93
Stevioside		96		96		96		43		106		106		46		114		114
Neotame		5		85		85		4		111		111		3		86		86
Saccharin		37		93		93		16		102		102		15		95		95
NHDC		3		26		26		0		0		0		1		25		25
Aspartame	3	694	971	70	91	80	3	564	592	59	59	59	4	548	563	57	56	56
Acesulfame K	1	642	1080	65	102	83	n/d	339	374	35	37	36	2	343	352	35	35	35
Rebaudioside	n/d	606	648	101	101	101	n/d	248	256	43	42	43	n/d	239	244	40	40	40
Stevioside	n/d	476	494	96	94	95	n/d	192	197	40	40	40	n/d	181	151	37	30	33
Neotame	n/d	31	34	108	110	109	n/d	21	22	76	74	75	n/d	39	40	134	135	135
Saccharin	0	94	233	47	110	79	0	82	84	43	42	43	0	84	80	42	40	41
NHDC	3	48	62	98	118	108	96	295	503	*	*		25	82	103	*	*	

Matrix	Low Fat Yoghurt						High Fat Yoghurt					
Sweetener	Sweetener Concentration mg/Kg			Spike Recovery (%)		Mean Recovery	Sweetener Concentration mg/Kg			Spike Recovery (%)		Mean Recovery
	Blank	Spike A	Spike B	A	B	%	Blank	Spike A	Spike B	A	B	%
Aspartame	8.50	973	852	96.5	84.4	90	0	908	951	90.0	94.3	92
Acesulfame K	0	357	308	102	87.9	95	0	325	340	92.9	97.2	95
Rebaudioside	0	308	277	123	111	117	0	287	298	115	119	117
Stevioside	0	271	269	89.2	88.6	89	0	235	243	77.4	80.1	79
Neotame	0	30.7	27.6	95.8	86.2	91	0	28.8	29.1	89.9	91.0	90
Saccharin	0	96.2	95.7	96.0	95.5	96	0	90.6	96.2	90.4	96.0	93
NHDC	12	48.9	44.7	97.8	89.4	94	13.5	44.3	48.3	88.6	96.6	93
Aspartame	<5	1010	998	100	99.0	100	<5	1010	976	100	96.7	98
Acesulfame K	<5	350	339	99.3	96.1	98	<5	348	337	98.8	95.7	97
Rebaudioside	<5	295	290	97.3	95.7	97	<5	289	286	95.4	94.3	95
Stevioside	<5	269	264	108	106	107	<5	267	260	107	104	106
Neotame	<2.5	30.0	30.7	102	104	103	<2.5	30.1	29.4	102	99.9	101
Saccharin	<5	91.0	88.8	90.8	88.6	90	<5	93.3	90.3	93.1	90.1	92
NHDC	<2.5	38.0	38.5	75.9	76.9	76	<2.5	37.3	35.5	74.6	71.0	73
Aspartame	0	944	904	96.6	89.9	93	0	845	875	87.0	90.7	89
Acesulfame K	0	309	318	90.7	90.5	91	0	313	318	92.2	94.5	93
Rebaudioside	0	280	279	95.4	92.2	94	0	282	279	96.5	96.2	96
Stevioside	0	231	232	94.2	91.9	93	0	242	243	99.2	100	100
Neotame	0	27.4	26.3	96.3	89.7	93	0	25.2	25.3	88.9	89.9	89
Saccharin	0	95.4	97.5	98.3	97.6	98	0	96.9	98.9	100	103	102
NHDC	<1	25.8	24.2	53.3	46.6	50	<1	27.5	25.3	56.1	51.9	54
Aspartame	2.50	916	938	92.1	93.0	93	3.68	934	906	92	90	91
Acesulfame K	12.1	320	327	91.7	92.5	92	9.90	331	318	93	91	92
Rebaudioside	<1	275	292	94.2	98.7	96	<1	297	275	99	93	96
Stevioside	4.79	256	275	106	113	110	<1	290	276	118	114	116
Neotame	2.21	24.1	23.2	85.3	80.9	83	1.23	26.1	23.4	89.3	81.8	86
Saccharin	<1	86.7	90.0	88.9	90.4	90	<1	92.5	87.6	91.9	87.9	90
NHDC	<1	25.8	24.2	53.3	46.6	50	<1	27.5	25.3	56.1	51.9	54
Aspartame	32	882	981	85	98	92	7.55	194	0	4	0	2
Acesulfame K	89	258	313	73	88	81	44.3	11.3	0	4	0	2
Rebaudioside	0	254	288	84	93	89	49.3	8.9	0	3	0	2
Stevioside	0	217	242	87	95	91	36.1	11.9	0	5	0	3
Neotame	0	26.9	28.1	92	94	93	0	5.5	0	19	0	10
Saccharin	5.39	83.9	95.5	84	95	90	15.8	6.49	0	6	0	3
NHDC	34	20.6	24.8	41	49	45	22.4	0	0	0	0	0
Aspartame	0.0	478	451	79.0	74.5	77	2.0	903	879	89.3	86.9	88
Acesulfame K	0.0	302	287	85.2	81.2	83	0.0	991	950	98.6	94.5	97
Rebaudioside	0.0	189	176	77.2	72.2	75	0.0	597	594	98.0	97.6	98
Stevioside	0.0	147	135	73.5	67.6	71	0.0	497	487	99.3	97.4	98
Neotame	0.0	15.2	14.3	82.3	77.7	80	0.0	28.3	29.9	96.2	102	99
Saccharin	0.0	70.5	64.8	88.0	80.8	84	0.0	206	201	103	100	102
NHDC	0.0	24.3	22.6	79.8	74.3	77	0.0	47	49	93	96	95
Aspartame	5.3	965	997	96.1	99.1	98	0.9	933	956	93	95	94
Acesulfame K	3.7	319	326	91.1	93.8	92	2.1	322	339	91.6	96.6	94
Rebaudioside	0	281	293	93	96.8	95	0	292	295	96.4	97.6	97
Stevioside	0	210	218	84.2	87.5	86	0	232	236	93	94.7	94
Neotame	0	28.3	28.9	96.6	98.4	98	0	28.4	29.3	96.5	99.7	98
Saccharin	2.55	96.3	99.1	96.6	102	99	1.4	103	104	103	106	105
NHDC	0.35	20.9	20.1	42	41	42	0	22.6	22.9	45.2	45.9	46
Aspartame	0	935	791	92.6	78.4	86	0	894	890	88.6	88.3	88
Acesulfame K	0	337	343	95.7	97.3	97	0	341	343	96.8	97.3	97
Rebaudioside	0	344	357	113	118	116	0	348	345	115	114	115
Stevioside	0	282	294	111	116	114	0	286	281	113	111	112
Neotame	0	26.7	31.4	90.6	107	99	0	27	27	91.6	91.7	92
Saccharin	0	107	106	107	106	107	0	108	108	107	108	108
NHDC	0	22.3	19.6	44.6	39.2	42	0	24.4	28.8	48.8	57.6	53
Aspartame	<1.0	997	982	98.9	98.6	99	<1.0	994	997	99.0	99.0	99
Acesulfame K	<1.0	335	330	95.2	93.6	94	<1.0	339	339	96.8	96.5	97
Rebaudioside	<1.0	310	305	97.4	95.1	96	<1.0	302	306	100	97.5	99
Stevioside	<1.0	250	243	100	93.4	97	<1.0	248	250	99.6	100	100
Neotame	<0.5	31.7	29.2	108	100	104	<0.5	28.3	28.9	96.5	98.4	97
Saccharin	<1.0	99.4	97.6	99.3	98.7	99	<1.0	99.8	101	100	101	101
NHDC	<0.5	31.3	30.3	62.6	61.2	62	<0.5	32.5	32.5	65.4	65.0	65
Aspartame	0	949	921	93.5	93.5	94	0	656	697	70.5	72	71
Acesulfame K	0	324	314	91.5	91.4	91	0	287	299	88.4	88.5	88
Rebaudioside	0	224	245	73.4	82.7	78	0	240	251	85.6	86	86
Stevioside	0	282	268	112	110	111	0	248	255	107	106	107
Neotame	0	30.8	29.9	104	104	104	0	24.5	24.9	90.3	88.2	89
Saccharin	0	82.5	80.2	81.8	82	82	0	79.3	74.2	79.7	77.2	78
NHDC	0	11.5	12.3	22.9	25.2	24	0	19.5	20.6	42.3	42.9	43
Aspartame	<1.00	771	774	77.5	77.4	77	<1.00	587	642	58.8	64.5	62
Acesulfame K	<1.00	332	333	95.8	95.5	96	<1.00	327	311	94.0	89.7	92
Rebaudioside	<1.00	277	277	92.7	91.9	92	<1.00	268	262	89.3	87.6	88
Stevioside	<1.00	227	228	92.1	92.0	92	<1.00	222	221	89.6	89.7	90
Neotame	<0.500	21.6	21.2	74.5	72.4	73	<0.500	18.8	19.6	64.6	67.5	66
Saccharin	<1.00	89.2	89.6	90.3	90.2	90	<1.00	88.5	83.6	89.3	84.7	87
NHDC	1.80	26.8	26.8	54.4	54.0	54	1.91	27.2	24.5	55.0	49.8	52
Aspartame	0	221	446	70.3	73.6	72	0	768	767	76.1	76	76
Acesulfame K	0	329	295	93.4	83.7	89	0	937	946	93.1	94	94
Rebaudioside	0	240	225	99.2	93	96	0	601	587	99	96.8	98
Stevioside	0	231	227	115	113	114	0	608	568	121	113	117
Neotame	0	19.3	19	105	103	104	0	32.7	31.6	111	107	109
Saccharin	0	86	80.5	107	100	104	0	213	205	107	102	105
NHDC	0	19.3	19.8	64.4	66	65	0	44.9	40.8	89.9	81.6	86
Aspartame		254		126		126		190		94		94
Acesulfame K		77		110		110		68		97		97
Rebaudioside		27		89		89		26		87		87
Stevioside		54		107		107		52		103		103
Neotame		6		99		99		6		100		100
Saccharin		22		109		109		19		92		92
NHDC		0		0		0		5		52		52
Aspartame	0	886	900	88	90	89	n/d	896	857	89	86	88
Acesulfame K	0	343	350	34	35	35	n/d	361	335	36	34	35
Rebaudioside	3	307	318	51	53	52	n/d	337	318	56	53	55
Stevioside	2	228	233	46	47	46	n/d	249	232	50	47	49
Neotame	n/d	32	34	108	115	111	n/d	33	30	111	104	108
Saccharin	n/d	93	99	46	50	48	n/d	65	88	33	45	39
NHDC	6	56	51	111	103	107	4	56	53	113	106	110