Collaborative Trial of a Method for the Determination of Sucralose in Foods

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Summary

The collaborative trial of a liquid chromatography with refractive index detection method for the determination of sucralose in yoghurt, jam, carbonated beverage, low carbohydrate chocolate confectionery and still beverages has successfully been undertaken.

Statistical evaluation of the results from the collaborative trial indicated that the method generally gave acceptable HorRat values (<2.0) for still and carbonated beverages and jam. A poorer level of precision was seen for results from yoghurt (HorRat = 2.2) which may be attributable to greater variability at the low end of the calibration range. This issue has been addressed by amending the method to include the option to either reduce the sample dilution factor or to allow for method calibration at lower concentrations. The most complex matrix, chocolate confectionery, gave the poorest level of precision with a HorRat of 3.2.

The trial also established typical performance characteristics for the method. These performance characteristics are a useful quality control guide that can be used when the method is applied to new sample matrices.

Introduction

Sucralose (1,6-dichloro-1,6-dideoxy-beta-D-fructofuranosyl-4-chloro-4-deoxy-alpha-D galacto-pyranoside, E955, CAS RN 56038-13-2) is formed by the chlorination of sucrose. During this reaction the stereo-chemistry of the glucose ring is changed to produce a "galacto-sucrose". Sucralose is a permitted sweetener for use in foods in the UK and the rest of the European Union (EU). It is controlled by Regulation (EC) No. 1333/2008[(1)] of the European Parliament and of the Council on food additives implemented in the UK by The Food Additives (England) Regulations 2009 (No. 3238)[(2)], The Food Additives Regulations (Northern Ireland) 2009, (No. 416)[(3)], The Food Additives (Scotland) Regulations 2009 (No. 436)[(4)] and The Food Additives (Wales) Regulations 2009 (No. 3378 W.300) [(5)]. These regulations revoke the Sweeteners in Food Regulations 1995 and re-enact with changes and on a transitional basis certain of their provisions.

Additionally, sucralose, like all approved sweeteners must comply with specific purity criteria as detailed in Commission Directive 2008/60/EC [(6)] laying down specific purity criteria concerning sweeteners for use in foodstuffs.

Various techniques have been used to determine sucralose, for example high-performance anion-exchange chromatography with pulsed amperometric detection[(7)], reverse-phase liquid chromatography with UV detection[(8)] and capillary electrophoresis[(9)].

This paper reports results obtained from a collaborative trial, funded by the Food Standards Agency (FSA), of a reversed-phase liquid chromatography method with refractive index detection for the determination of sucralose in various food products. The method fully validated through this collaborative trial was single-laboratory validated by LGC as part of Food Standards Agency project E01067 (www.food.gov.uk). The collaborative

trial reported in this paper was carried out with 15 laboratories analysing five sample types (i.e. yoghurt, jam, carbonated beverage, chocolate and still beverages). The still beverages were analysed at three concentrations.

Collaborative Trial Organisation

Participants

Fifteen UK Public Analyst laboratories participated in this study which was undertaken according to the International Union of Pure and Applied Chemistry (IUPAC) Harmonised protocol for the design, conduct and interpretation of method-performance studies[10].

Selection of Test Materials

All samples used as test materials were commercial foods that contained sucralose:

- Still beverage – Fruit flavoured still drink
- Carbonated beverage – Carbonated ginger beer flavoured drink
- Jam – High fruit content raspberry spread
- Yoghurt – Low calorie strawberry yoghurt
- Chocolate confectionery – Low carbohydrate chocolate confectionery

Preparation of Test Materials

Bulk quantities of the chosen samples were purchased and either homogenised in a food processor or, in the case of beverages, mixed thoroughly. One still beverage matrix was purchased and diluted to two different concentrations to provide blind duplicates.

Blank Samples

Retail samples of strawberry yoghurt, raspberry jam, ginger beer and fruit flavoured still drink were purchased to use as blank samples. Sucralose was not a listed ingredient in any of these products. These samples were analysed to confirm that they did not contain sucralose or produce any interfering peaks.

Several brands of low carbohydrate chocolate bars, with sucralose not listed as an ingredient, were analysed to establish whether they would be suitable as a blank material. As interfering peaks were detected at similar retention times to sucralose in these products several brands of "normal" chocolate i.e. did not contain sucralose, were analysed. The analysis of the potential blank samples indicated that the method may require additional development prior to its application to different types of chocolate confectionery.

Storage of Samples

After preparation, approximately 40 g aliquots of all samples were transferred to 60 ml plastic bottles, capped tightly and stored in a freezer until required.

Sucralose Standard

Sucralose (certified as 98.0 – 102.0 %) to be used as a reference standard was obtained from Tate & Lyle. This material was stored at LGC at room temperature in an air-tight sealed container. To check whether the bulk standard may have absorbed water during storage at LGC, approximately 10 g was weighed into a container and left at room temperature for 6 days with the lid loosely covered. The sample was weighed periodically over this time and no significant change of weight was detected demonstrating that no significant amount of water had been gained or lost.

Approximately 3 g of this standard was sent to each participant with the collaborative trial samples. The participants were instructed to use this material for the preparation of calibration standards and for spiking. This was to ensure that there were no discrepancies in the results due to the use of sucralose standards of varying purity.

Homogeneity Testing

The homogeneity testing procedure employed was based on that given within the IUPAC proficiency testing protocol[11]. Ten samples of each matrix, pre-homogenised and placed in sample pots in 40 g quantities, were taken at random and analysed in duplicate (A and B) in a randomised order, under repeatability conditions. The results are presented in Table 1.

Table 1: Results of Homogeneity Testing

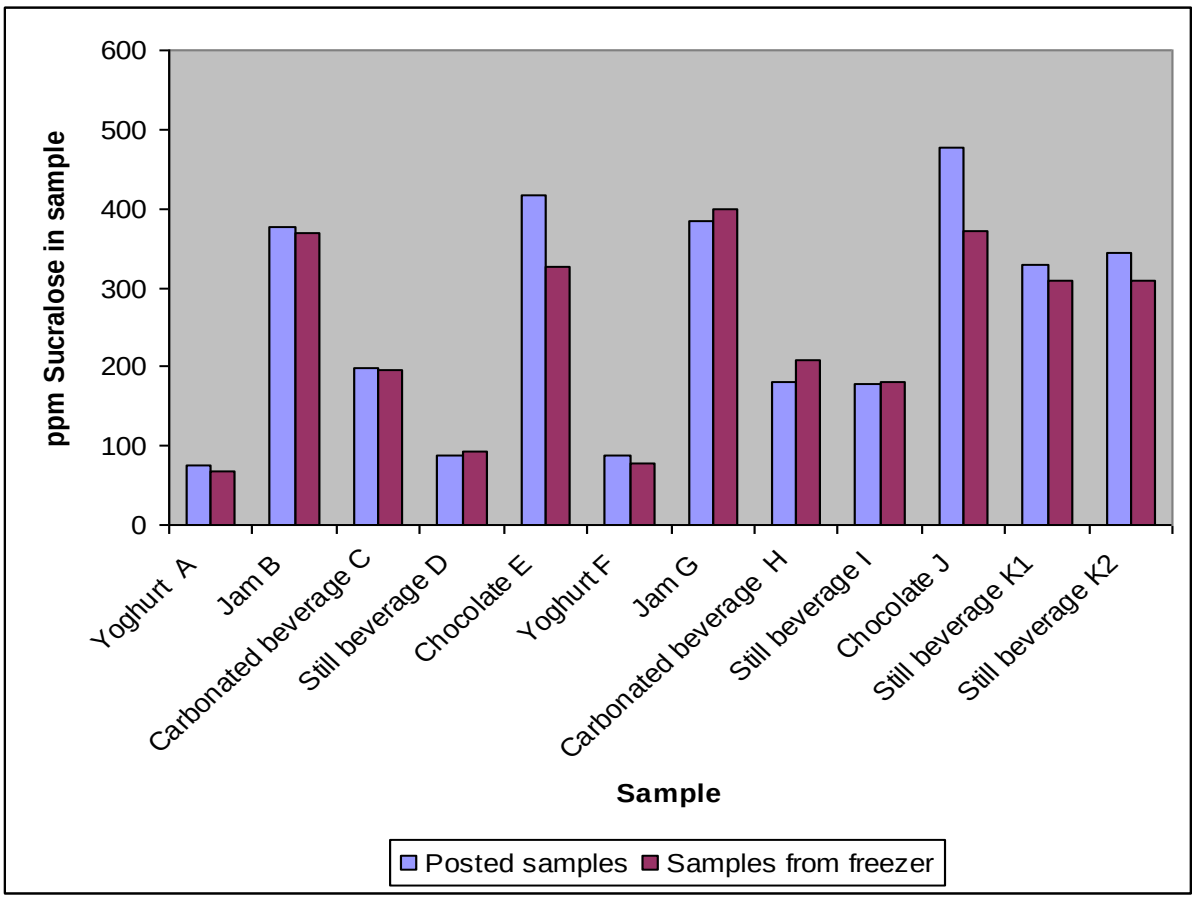
	Yoghurt (mg/kg)		Jam (mg/kg)		Carbonated Beverage (mg/l)		Chocolate (mg/kg)		Still Beverage (Sample D) (mg/l)		Still Beverage (Sample I) (mg/l)		Still Beverage (Sample K) (mg/l)	
Sample number	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
1	86	91	361	411	179	181	273	257	87	95	171	151	327	350
2	83	72	357	384	189	173	310	302	79	93	175	179	358	321
3	82	83	379	336	186	182	330	294	96	93	175	178	320	354
4	85	81	369	379	196	194	277	261	67	89	174	177	340	350
5	79	81	368	362	194	183	285	318	93	91	174	181	345	344
6	79	81	357	362	181	187	281	289	91	85	181	182	329	305
7	80	72	373	349	191	202	271	323	77	86	153	178	349	307
8	94	87	343	365	187	177	288	344	96	74	164	175	333	313
9	77	84	324	315	200	187	326	268	59	91	180	178	321	332
10	81	80	371	326	187	188	262	290	90	85	171	178	334	339

Statistical tests initially undertaken checked the data for any widely discrepant pairs using Cochran's test[10]. No outliers were identified (95% confidence). Thereafter the remaining data were subject to analysis of variance (ANOVA) to estimate the sampling and analytical variances. Throughout this process the target standard was set according the theoretical value as determined from the Horwitz function[12]. All of the test materials were deemed to be homogenous.

Stability Testing

To evaluate the stability of the collaborative trial test samples a full set of samples, as dispatched to the participants, was sent to LGC by overnight courier. On arrival the samples were booked into LGC's Laboratory Information Management System (LIMS) and then transferred to a freezer until required for analysis. This process was followed to mimic the conditions the participants' samples would be subjected to. Within a few days of the analysis of these, a second set which had not been removed from the freezer was also analysed. A comparison of the results of the two sets of samples is presented in Figure 1.

Figure 1: Results of stability test



ppm denotes either mg/kg or mg/l

The greatest difference between results was seen for the chocolate confectionery, however all results fall within the range of concentrations submitted by the participants in the collaborative trial.

The stability data was assessed by ANOVA. Natural concentration and log10(concentration) data was processed to check for the effect of concentration-dependent variance. The split-level sample was excluded from the analysis to avoid inflating the within-group term. The effect of the different stability treatments (Posted, Frozen) was not significant at the 95% level of confidence, either in the concentration or in the log10(concentration) domain ($p=0.09$ and $p=0.07$ respectively). The change in mean value for chocolate appeared particularly large and was tested separately; no significant difference was found ($p=0.12$). It was concluded that the samples had not degraded significantly during transportation.

Pre-Trial

The following samples were sent to 15 participating laboratories, including LGC:

- Still Beverage – Sucralose
- Still Beverage – Blank (i.e. did not contain sucralose)
- Sucralose standard material

A summary of the results is presented in Table 2.

Table 2: Statistical Evaluation of Pre-Trial Results Employing the IUPAC Harmonised Protocol

Lab Number	Sucralose (mg/l) Replicate A	Sucralose (mg/l) Replicate B
1	347	347
2	330	342
3	333	361
4	341	334
5	347	349
6	325	332
7	310	330
8	398	368
9	346	342
10	341	336
11	370	373
12	343	322
13	256	255
14	340	342
15	297	299
mean	340.9	
n	15	
nc	0	
outliers	1	
n_1	14	
r	28.2	
s_r	10.08	
RSD_r	3.0	
Ho_r	0.7	
R	60.5	
s_R	21.59	
RSD_R	6.3	
Ho_R	1.0	

See Glossary for definition of terms.

The pre-trial results demonstrated that the modified method could be successfully transferred to fourteen other laboratories for the determination of sucralose in one matrix giving a Horwitz ratio of <2.0. It was agreed with the FSA to proceed to the main collaborative trial with 15 laboratories analysing all five matrices.

Collaborative Trial

Details of the samples sent to the 15 participant laboratories for the collaborative trial are presented in Table 3.

The yoghurt, jam, carbonated beverage and chocolate bar were dispatched as blind duplicates. The still beverage samples D and I were single but different concentration samples (split level duplicates), whereas the still beverage (concentrate) sample K was a known duplicate.

Table 3: Samples Used in Main Collaborative Trial

Sample Codes	Matrix	Replicate Type
A & F	Yoghurt	Blind duplicates
B & G	Jam	Blind duplicates
C & H	Carbonated beverage	Blind duplicates
D & I	Still beverage	Split level duplicates
E & J	Chocolate bar	Blind duplicates
K	Still beverage (concentrate)	Known duplicates

The participants in the trial were asked to analyse all samples once only according to the method detailed in Appendix 1. Blank samples for each matrix were also sent and the laboratories were requested to also analyse these samples once only with duplicate spiked recoveries at the legislative limit relevant for each matrix.

Results and Statistical Analysis

The results submitted by participants for the determination of sucralose in each sample were statistically analysed according to procedures laid down within the IUPAC Harmonised Protocol[10] . A number of statistical outliers were identified but no causes were identified for the aberrant results.

Repeatability and Reproducibility

Calculations for repeatability, r, and Reproducibility, R, as defined by the Harmonised IUPAC Protocol[12] were carried out on the remaining data following removal of outliers.

Horwitz Predicted Precision Parameters

There is often no validated reference or statutory method with which to compare precision criteria when assessing a new method. In such cases it is useful to compare the precision data obtained from a collaborative trial with predicted acceptable levels of precision. These levels, as predicted by the Horwitz equation, give an indication as to whether the method is sufficiently precise for the concentration of analyte being measured[13].

The Horwitz predicted value is calculated from the Horwitz equation[14]:

$$RSD_R = 2^{(1-0.5\log C)}$$

Where C is the measured concentration of analyte expressed as a decimal, i.e. 1g/100g = 0.01.

Thompson[15] has described the use of a modified Horwitz function to predict levels of precision at µg/kg and sub µg/kg levels up to 120 µg/kg. The use of this function is shown to give an improved statistical representation at these levels.

Horrat Value (Ho)

The Horrat[15] value gives a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method measuring at that particular level of analyte. It is calculated as follows:

$$Ho_R = \frac{RSD_R(measured)}{RSD_R(Horwitz)}$$

An Ho_R value of 1 usually indicates satisfactory inter-laboratory precision, whilst a value of >2 indicates unsatisfactory precision i.e. one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method employed. Similarly, Ho_r is calculated, and used to assess intra-laboratory precision, using the approximation $RSD_{r,}$(Horwitz) = 0.66RSD_R (Horwitz). This assumes the approximation that r = 0.66R. Table 4 presents a summary of the statistical evaluation of the results from the collaborative trial including the calculated Horwitz values. The full set of data from the collaborative trial is presented in Appendix 2.

Table 4: Summary of the Statistical Evaluation of the Results from the Collaborative Trial

Sample Type	Yoghurt	Jam	Carbonated Beverage	Chocolate Confectionery	Still Beverage	Still Beverage	Still Beverage
Identifier	A, F	B, G	C, H	E, J	K	D[Note 1]	I [Note 1]
mean	83.2	373.2	179.7	352.0	331.9	92.3	181.3
n	15	15	15	15	15	15	15
nc	2	2	2	2	2	2	2
outliers	1	1	1	0	2	1	2
n_1	12	12	12	13	11	12	11
r	19.1	16.0	14.8	111.5	15.4	n/a	n/a
s_r	6.83	5.73	5.29	39.81	5.50	n/a	n/a
RSD_r	8.2	1.5	2.9	11	1.7	n/a	n/a
Ho_r	1.5	0.4	0.6	2.6	0.4	n/a	n/a
R	42.5	48.4	15.7	211.5	30.9	13.7	25.8
s_R	15.18	17.30	5.59	75.54	11.02	4.88	9.21
RSD_R	18.3	4.6	3.1	21.5	3.3	5.3	5.1
Ho_R	2.2	0.7	0.4	3.2	0.5	0.7	0.7

Note 1: Single material diluted twice to give significantly different concentrations; statistically analysed as separate materials with one observation per laboratory.

See Glossary for definition of terms.

In summary, the samples sent to the participants were as follows:

i) Five replicated materials; 4 blind replicate samples and 1 known replicate sample.

ii) Two separate samples, which were analysed as single observations in each laboratory. Although not replicated, preventing separation of within- and between-laboratory terms, this provided two direct estimates of reproducibility standard deviation, which provides conclusions about concentration-dependence of the reproducibility.

For the range of sucralose concentrations studied (50-400 ppm, where ppm is either mg/l or mg/kg), the repeatability standard deviation (s_r) was found to be in the range 5-7ppm except for the chocolate confectionery material with an s_r of 39.81mg/kg. The reproducibility standard deviation (s_R) was also consistent showing a range of 5-17 ppm; again, the chocolate confectionery material showed poorer reproducibility, with a s_R of 75.54 mg/kg. Horwitz ratios range from very good (under 1.0 for beverages and jam) to 3.2 for the most challenging material, the chocolate confectionery.

The effect of recovery correction was investigated by calculating s_R for recovery-corrected data. It was found that recovery correction did not improve s_R and in some cases substantially degraded performance.

Assuming that a Horwitz ratio of 2.0 represents the upper acceptable limit, the method is clearly acceptable for more simple products, which gave very good results. The relatively poor precision for the yoghurt sample may be attributable to greater variability at the low end of the calibration curve, due to over-dilution of the test material. The concentration of sucralose present in the yoghurt extract was very close to the bottom calibration standard and this may have had an influence on the overall precision. Reducing the dilution factor or recalibrating for lower concentrations is suggested. Although LGC did not experience any problems with determining sucralose at this concentration, examination of calibration lines from participants demonstrated that some did.

The participants were not provided with instructions regarding calibration line construction. To establish whether the use of an intercept value would significantly affect the calculated concentration of sucralose at the low end of the calibration line, the sucralose response for the yoghurt matrix was taken for several laboratories and the results calculated with and without a zero intercept. Table 5 presents the calculated results.

Table 5: Effect of Intercept on Calculated Results

Laboratory Number	With Intercept (% Sucralose)	Zero Intercept (% Sucralose)
6	12.3%	23.9%
7	-8.0%	-9.0%
10	20.2%	42.7%
13	10.1%	15.6%
15	10.0%	2.0%

The results presented Table 5 show that the concentration of sucralose determined in the yoghurt samples can vary greatly depending on whether or not an intercept is used. Reducing the dilution factor or recalibrating for lower concentrations is suggested to minimise this effect.

The low carbohydrate chocolate confectionery sample (E and J) gave the poorest level of precision, giving a Horwitz ratio of 3.2. Although no issues were encountered at LGC, many participants commented on having problems with this matrix. This matrix was included in the project at a late stage as a replacement for ready-to-eat desserts and perhaps would have benefitted from further method development prior to its use as a test material in the collaborative trial. Timescales however did not permit this developmental work to be conducted but this project has demonstrated that the method is matrix specific and so there are likely to be other sample matrices that have not been analysed using this method that may give rise to analytical problems. Therefore it is recommended that the method is optimised for new sample matrices to meet a set of defined performance criteria, e.g. such as the performance criteria established from the collaborative trial results. It is also recommended that the method should be validated for every substantially different matrix using a validation protocol similar to that employed in this project.

Performance Criteria for New Materials

- The chromatographic peak shape for sucralose, will exhibit some tailing on most HPLC columns, should be smooth with no obvious splits or deformities.
- The sucralose peak should be adequately resolved from any matrix component peak.
- Linearity of calibration should be established over the range 10-200 µg/ml in the final extract.
- The accuracy i.e. the recovery values determined for new blank materials should be within 80-120% of the spiking levels.
- The repeatability of the procedure was found to be within 20 ppm for the more "simple" matrices (drinks, jam & yoghurt) with sucralose contents in the range 80-380 ppm. For the most complex matrix, chocolate confectionery, a repeatability of 112 ppm was observed but this gave an unacceptably high Horrat value of 3.2 If a Horrat value of 2.0 is taken to represent the upper acceptable limit, the maximum repeatability standard deviation and maximum repeatability should be no more than:

Table 6: Maximum Theoretical Performance Characteristics

Matrix	Concentration mg/kg	Maximum s_r %	Maximum r mg/kg
Yoghurt	83.2	10.9	30.4
Jam	373	8.66	24.3
Carbonated beverage	180	9.67	27.1
Chocolate	352	8.74	24.5
Still beverage	332	8.82	24.7

i.e. an acceptable repeatability standard deviation would be in the region of 8-11% for concentrations in the range 80–380 ppm.

Conclusions

A liquid chromatography with refractive index detection method single-laboratory validation by LGC has been fully validated through collaborative trial according to the IUPAC Harmonised Protocol. The method has been successfully applied to the determination of sucralose in still and carbonated beverages, jam and yoghurt. The trial has also been successful in establishing typical performance characteristics for the proposed procedure. The mean repeatability for the more "simple" sample matrices (yogurt, jam and carbonated beverages) was determined as 18 ppm, which compares favourably with the theoretical mean maximum repeatability of 28 ppm for a concentration range of 80-380 ppm.

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Glossary of terms

Experimental Design	Design used for the particular set of data. May be one of: • Blind: Blind replication by provision of two or more identical test items • Split: Provision of two closely similar test items known to have a (possibly significant) difference in analyte level. • Single: Treated as single replicates
mean: n1	The mean value for all retained data
n	The number of participants submitting data
nc	Number of laboratories removed as noncompliant prior to statistical analysis
outliers	Number of laboratories removed as outliers
n_1	Number of valid laboratories
r	Repeatability: The critical value for the difference between two results obtained under repeatability conditions; calculated as 2.8 x s_r.
s_r	Repeatability standard deviation
RSD_r	The repeatability relative standard deviation
Ho_r	Horwitz ratio for the repeatability data: $Ho_r=RSD_r/RSD_rHo$, where $RSD_rHo = 0.66 \times Ho_R$
R	Reproducibility: The critical value for the difference between two results obtained under reproducibility conditions; calculated as 2.8 x s_R
s_R	Reproducibility standard deviation
RSD_R	The reproducibility relative standard deviation
Ho_R	"Horwitz ratio" for the reproducibility data: $Ho_R=RSD_R/RSD_RHo$
PPM/ppm	Parts per million, denotes either mg/kg or mg/l
G	Results rejected as a Grubbs test outlier
C	Discrepant pairs of results determined using Cochran's test

Appendix 1

Method for the Determination of Sucralose in Foods

Note: Before applying the method carry out a risk assessment and insert a section on Health & Safety in any written protocol based on this method.

1. Scope and Field of Application

1.1 This method defines the procedure for the extraction and quantification of sucralose from still beverages, carbonated beverages, yoghurt, jam and chocolate "low carbohydrate" bars.

Note: Outcomes of the trial indicates that chocolate confectionery should not be stated in the scope of the method. See discussion in body of text. The scope should be amended to read:

1. Scope and Field of Application

1.1 This method defines the procedure for the extraction and quantification of sucralose from still beverages, carbonated beverages, yoghurt and jam. The method gives indicative values for chocolate "low carbohydrate" bars.

2. Principle

2.1 Sucralose is extracted from the sample matrix into deionised water for all matrices except ready to drink beverages where no extraction is required. Interferences are then removed by passing the sample solution through either an Alumina A or ENV+ solid phase extraction cartridge in conjunction with a C18 solid phase extraction cartridge. The resulting extract is then analysed by HPLC with refractive index detection.

3. Reagents

All reagents must be a minimum of Analytical Reagent grade.

3.1 **Sucralose Standard**
For example, Tate and Lyle, Purity 98 – 102% or Sigma product code 69293

3.2 **Deionised Water**

3.3 **Methanol**
HPLC grade

3.4 **HPLC Mobile Phase**
70:30 (v/v) water (3.2): methanol (3.3)

3.5 **Sucralose Stock Standard –** 5 mg/ml.

Weigh accurately to the nearest 0.0001g 0.25g ±0.05*g sucralose standard (3.1) into a 50 ml volumetric flask and dilute to volume with water (3.2). This will give a stock solution of approximately 5 mg/ml sucralose.

**As a result of a reviewer's comments it is now considered preferable to state this as 0.25g ± 0.01g so that the top calibration standard only varies between 192µg/ml and 208µg/ml.*

3.6 **Calibration Standards**

A range of aqueous calibration standards should be prepared to cover the expected concentrations in the sample extracts. Determining concentrations very close to either end of the calibration line should be avoided. Examples of suitable calibration standards are given in Table A1.

Table A1

Volume of 5 mg/ml Stock Solution (ml)	Final Volume (ml)	Concentration (µg/ml)
0	50	0
0.10	50	10
0.25	50	25
0.50	50	50
1.00	50	100
2.00	50	200

The appropriate volume of stock solution (3.5) should be pipetted into a volumetric flask and the solution diluted to volume with water (3.2).

4. Apparatus

4.1 **Balance**
Capable of weighing to 2 decimal places.

4.2 **General Laboratory Glassware**
Including 50 ml volumetric flasks, grade A.

4.3 **Membrane Filters 0.2 µm**
Alltech Associates Ltd, Part No 2045, or equivalent.

4.4 **Plastic Syringes**
BD Plastipak, 50 mL Luer, Ref 300866, or equivalent.

4.5 **Thermometer**
Calibrated to within a 0.5°C tolerance over the range 38 – 42°C.

4.6 **Rotary Evaporator**

Turbovap, or equivalent, with a water bath capable of achieving a temperature of 40°C ± 2°C when read against a calibrated thermometer (4.5). In practice this, allowing for tolerances of the thermometers, will give a bath temperature of 40°C ± 2.5°C which is acceptable for this method.

4.7 **Centrifuge**
Capable of operating at 3000 rpm.

4.8 **Calibrated Positive Displacement Pipettes**
Capable of delivering volumes in the range of 25 µl-1000 µl

4.9 **Alumina A Solid Phase Extraction Cartridges**
Bond Elut LRC ALA, Part No 1211-3046, or equivalent

4.10 **C18 Solid Phase Extraction Cartridges**
Waters SepPak Plus tC18, Part No WAT036810, or equivalent.

4.11 **ENV+ Solid Phase Extraction Cartridges**
IST Isolute ENV+, 100 mg, Part No 915-0010-A or equivalent.

4.12 **HPLC System –** with suitable data acquisition system.

4.12.1 **Isocratic Pump**
Capable of maintaining a flow of 1.2 ml/min.

4.12.2 **Refractive Index Detector with Temperature Control**

4.12.3 **C18 Column**

Phenomenex Luna C18 (2), 5µ, 250 x 4.6mm, or equivalent. The following two columns have also proved to be suitable; Jones Chromatography Genesis, C18, 3µ, 150 x 4.6mm or Beckman Ultrasphere, C18, 5µ 250 x 4.6mm.

4.12.4 **Guard Cartridge**
Phenomenex C18, or equivalent.

4.12.5 **Column oven**
Capable of maintaining a temperature of 30°C.

4.13 **Ultrasonic Bath**

4.14 **Autosampler Vials**

4.15 **Calibrated Automatic Pipettes**
Delivery volumes in the range of 1 ml – 5 ml.

4.16 **Vortex Mixer**

4.17 **Filter Papers**
Whatman 541, or equivalent.

5. Procedure

5.1 **Spiking Procedure**

For each batch of analysis or matrix analysed a spiked recovery should be carried out. Suggested spiking concentrations are the maximum permitted concentrations for sucralose as stated in S.I.2009 No 3238[1]:

- Carbonated and still beverages 300 mg/l
- Jam 400 mg/kg
- Yoghurt 400 mg/kg
- Cocoa based confectionery 800 mg/kg

Examples of spiking procedures are presented in Table 2.

Table A2

Sample	Weight (g) or Volume (ml) taken for analysis	Final Volume (ml)	Volume of 5 mg/ml sucralose solution (3.5) used for spiking	Sample spiking concentration
Carbonated Beverage	10	20	600 µl	300 mg/l
Still Beverage	5	25	300 µl	300 mg/l
Jam	5	50	400 µl	400 mg/kg
Yoghurt	5	50	400 µl	400 mg/kg
Chocolate Bar	5	50	800 µl	800 mg/kg

5.2 Sample Preparation

Yoghurts, jam and chocolate bars should be homogenised thoroughly prior to analysis. Carbonated beverages should be thoroughly degassed, for example by placing in an ultrasonic bath (4.13), before analysis.

5.3 Extraction

5.3.1 Still Beverages

Accurately dilute any dilutable products to their 'ready to drink' concentration, for example for a fruit squash that is recommended to be diluted 1 part squash with 4 parts water pipette 5 ml sample into a 25 ml volumetric flask and dilute to volume with deionised water (3.2). A note of any dilutions performed. Mix well by hand.

5.3.2 Carbonated Beverages

[1] Food Additives (England) Regulations 2009 (No. 3238) or devolved equivalents

Transfer the sample to a suitable size beaker and place in an ultrasonic bath for approximately 10 minutes to degas. No sample extraction is required for carbonated beverages.

5.3.3 **Jam, Yoghurt and Chocolate Confectionery**

Accurately weigh approximately 5 g sample into a 50 ml volumetric flask. Add approximately 30 ml deionised water (3.2) to the flask and shake vigorously to disperse the sample. Place the flask in an ultrasonic bath (4.13) for 10 minutes then, after allowing the solution to come to room temperature, dilute to volume with deionised water (3.2) and mix well by hand. Transfer the solution to a suitable container and centrifuge (4.7) at ca. 3000 rpm for ca. 10 min. If the supernatant is still significantly cloudy it should be filtered, for example through a Whatman 541 filter paper (4.17).

5.4 Sample Clean Up

5.4.1 **Still Beverages, Carbonated Beverages, Jam and Yoghurts**

Pass approximately 10 ml of extract from either 5.3.1, 5.3.2 or 5.3.3 (only jam & yoghurt extracts) through an Alumina A solid phase extraction cartridge (4.9) at approximately 1 drop per sec. Using a 10 ml measuring cylinder discard the first 2 ml then collect the next 6 - 8 ml.

Condition a C18 cartridge (4.10) by slowly passing (approximately 1 drop per sec) 3 ml methanol (3.3) followed by 2 ml water (3.2) through the cartridge. Pass exactly 5 ml, measured by pipette (4.15), of sample extract from above through the cartridge, then wash the cartridge with 5 ml water (3.2). Elute the sucralose with 3 ml methanol (3.3) into a small round bottom flask. Rotary evaporate (4.6) to dryness at 40°C. The temperature of the water bath should be measured with a calibrated thermometer (4.5) and a note of the temperature made in the appropriate workbook. Alternately the extract can be taken to dryness under a stream of nitrogen, for example in a TurboVap.

Redissolve the residue in 5 ml water (3.2), measured by pipette (4.15), mix well using a vortex mixer (4.16) then transfer an aliquot to a suitable autosampler vial (4.14).

5.4.2 **Chocolate Confectionery**

Condition a C18 SepPak cartridge (A4.10) by slowly passing (approximately 1 drop per second) 3 ml methanol (A3.3) followed by 2 ml water (A3.2) through the cartridge. Pass exactly 5 ml, measured by pipette (A4.15), of sample extract from A5.4.3 (chocolate extract only) through the cartridge, then wash the cartridge with 5 ml water (A3.2). Elute the sucralose with 3 ml methanol (A3.3) into a small round bottom flask. Rotary evaporate (A4.6) to dryness at 40°C. The temperature of the water bath should be measured with a calibrated thermometer (A4.5) and a note of the temperature made in the appropriate workbook. Alternately the extract can be taken to dryness under a stream of nitrogen, for example in a TurboVap. Redissolve the residue in 5 ml water (A3.2), measured by pipette (A4.15), mix well using a vortex mixer (A4.16).

Condition an ENV+ SPE cartridge (A4.11) by slowly passing (approximately 1 drop per second) 3 ml methanol (A3.3) followed by 3 ml water (A3.2) through the cartridge. Pass sample extract from above through the cartridge, then wash the

cartridge with 5 ml water (A3.2). Elute the sucralose with 4 ml methanol (A3.3) into a small round bottom flask. Rotary evaporate (A4.6) to dryness at 40°C. The temperature of the water bath should be measured with a calibrated thermometer (A4.5) and a note of the temperature made in the appropriate workbook. Alternately the extract can be taken to dryness under a stream of nitrogen, for example in a TurboVap. Redissolve the residue in 5 ml water (A3.2), measured by pipette (A4.15), mix well using a vortex mixer (A4.16) then transfer an aliquot to an autosampler vial (A4.14).

6. HPLC Analysis

6.1 HPLC Conditions

The HPLC conditions, which have been found to be suitable, are detailed in A6.1.1 to A6.1.8. However, alternative conditions may be used if it can be demonstrated that they give equivalent or better chromatographic separation, sensitivity and reproducibility. Care must be taken to ensure that negative peaks seen just before the retention time of sucralose are fully separated from the sucralose peak. It is recommended that a temperature controlled refractive index detector is used and that a constant column oven temperature of 30°C is used to maintain satisfactory peak shape. Care must also be taken to avoid excessive back pressure, especially with samples such as jam. It is recommended that the guard cartridge (A4.12.4) be replaced before every run and that the overall run time is no longer than around 16 hours to avoid degradation of the peak shape. An injection volume of 100µl is recommended and should not be decreased to less than 75µl. A run time of 30 minutes is also recommended to allow sufficient time for all compounds, whether or not they are detected by RI detection, to elute from the column.

6.1.1 Mobile Phase (3.4)
6.1.2 Flow rate, 1.2 ml/min
6.1.3 Column (A4.12.3)
6.1.4 Guard cartridge (A4.12.4)
6.1.5 Temperature of column oven, 30°C
6.1.6 Injection volume, 100µl
6.1.7 Run time, ca. 30 min
6.1.8 Detector temperature control, On

6.2 HPLC Procedure

Allow the HPLC system to equilibrate by running the mobile phase, with the detector turned on, for at least 90 minutes before any injections are made. Before the sample extracts and standard solutions are injected a blank sample matrix extract must be injected 6 times to condition the column.

Inject one calibration standard prepared as described in A3.6 to check that the chromatography is satisfactory. The separation should be such that the negative peak just prior to that of sucralose is completely separated from the sucralose peak. The chromatograms should be consistent with the example with respect to peak shape and resolution.

The linearity of the system must be established by injecting all the calibrant standards. Use all the peak areas obtained for the calibration standards (beginning, during and end of the run) and the standard concentrations to plot a linear regression calibration curve. The correlation coefficient (R2) should be higher than 0.98.

When satisfactory repeatability and calibration have been obtained from repeated injections of the calibration standards, injections of the sample solutions can be made. An injection of a standard should be made at regular intervals throughout the run such that as many of the standards are incorporated into the sample extract sequence as possible. The full range of calibration standards should be injected after the last sample. When necessary the sample extract solutions should be diluted with deionised water (A3.2) to ensure that the response does not exceed the response of the top calibration standard solution or that no concentrations are determined below the bottom calibration standard response. If an extract requires dilution, the details must be recorded in the relevant workbook.

7. Expression of Results

7.1 Using a suitable spreadsheet package (Excel has been found to be suitable) construct a linear **regression** curve and determine both the slope (*m*) and intercept (c) of the curve. From the responses for the sample (*y*), determine the concentration (*x* in µg/ml) of the sucralose in the extract solution using equation I:

Equation I $$x = \frac{y - c}{m}$$

The concentration of sucralose in the sample may be determined using equation II:

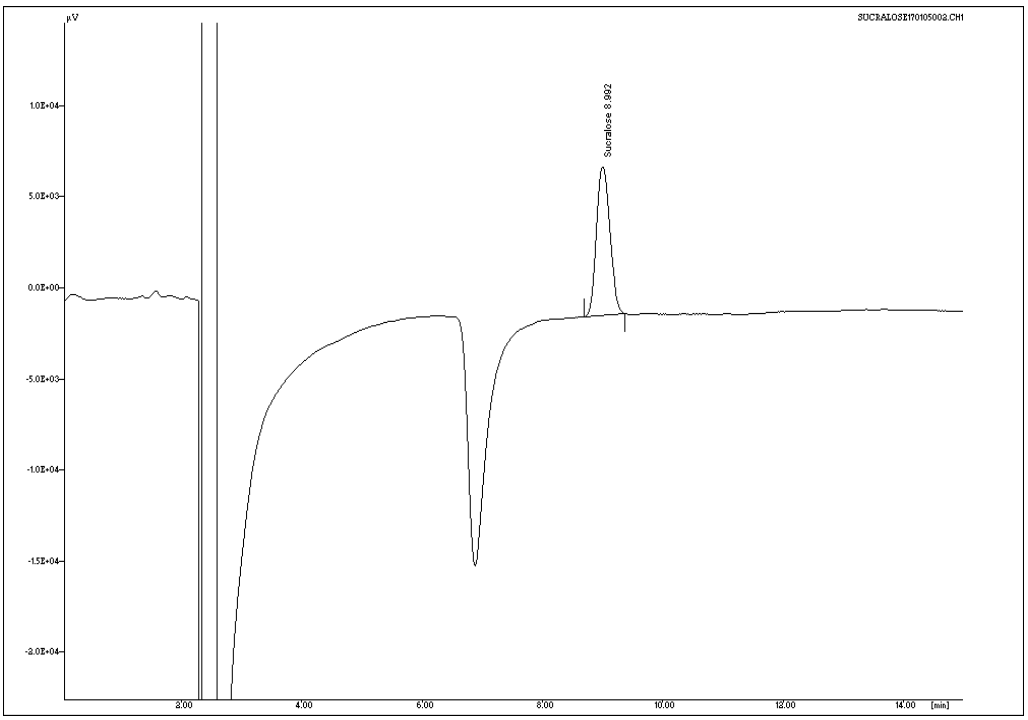
Equation II $$C = \frac{x \times V \times D}{M}$$

Where,

C	=	Concentration of sucralose in the sample (mg/kg or mg/l)
x	=	Concentration of sucralose in the final extract solution (µg/ml)
V	=	Extraction volume (ml)
D	=	Dilution factor
M	=	mass of sample taken (g)

7.2 **Example Chromatogram**

50 µg/ml Sucralose standard



Appendix 2: Main Collaborative Trial Results and Statistical Evaluation

Yoghurt (mg/kg sucralose)

Laboratory	Sample A	Sample F
1	89	89
2	88	80
3	58	58
4 (nc)	45	18
5	85	84
6	86	106
7 (nc)	0	0
8	85	98
9	85	88
10	118	103
11 (G)	0	15
12	62	65
13	65	70
14	84	89
15	76	89
mean	83.2	
n	15	
nc	2	
outliers	1	
n_1	12	
r	19.1	
s_r	6.83	
RSD_r	8.2	
Ho_r	1.5	
R	42.5	
s_R	15.18	
RSD_R	18.3	
Ho_R	2.2	

Jam (mg/kg sucralose)

Laboratory	Sample B	Sample G
1	388	384
2	350	345
3	362	349
4 (nc)	301	182
5	371	369
6	387	375
7 (nc)	456	494
8	379	382
9	376	374
10	400	401
11 (G)	169	174
12	349	350
13	355	368
14	384	399
15	376	385
mean	373.2	
n	15	
nc	2	
outliers	1	
n_1	12	
r	16.0	
s_r	5.73	
RSD_r	1.5	
Ho_r	0.4	
R	48.4	
s_R	17.30	
RSD_R	4.6	
Ho_R	0.7	

Carbonated Beverage (mg/l sucralose)

Laboratory	Sample C	Sample H
1	180	178
2	173	184
3	88	94
4 (nc)	266	286
5	175	178
6	179	181
7 (nc)	77	169
8	182	181
9	181	181
10	167	177
11 (C)	184	213
12	181	180
13	174	182
14	178	177
15	198	182
mean	179.7	
n	15	
nc	2	
outliers	1	
n_1	12	
r	14.8	
s_r	5.29	
RSD_r	2.9	
Ho_r	0.6	
R	15.7	
s_R	5.59	
RSD_R	3.1	
Ho_R	0.4	

Chocolate Confectionery (mg/kg sucralose)

Laboratory	Sample E	Sample J
1	331	208
2	383	346
3	158	259
4 (nc)	33	41
5	359	351
6	484	459
7 (nc)	0	156
8	366	449
9	319	304
10	342	346
11	320	370
12	342	322
13	405	390
14	322	324
15	416	478
mean	352.0	
n	15	
nc	2	
outliers	0	
n_1	13	
r	111.5	
s_r	39.81	
RSD_r	11.3	
Ho_r	2.6	
R	211.5	
s_R	75.54	
RSD_R	21.5	
Ho_R	3.2	

Still Beverage (mg/l sucralose)

Laboratory	Sample K - Replicate A	Sample K - Replicate B
1	336	340
2	319	328
3 [nc]	318	323
4	196	194
5	327	326
6	355	354
7 [nc]	324	313
8	340	341
9	322	337
10	322	320
11 [C]	130	460
12 [C]	288	330
13	333	340
14	325	326
15	328	344
mean	331.9	
n	15	
nc	2	
outliers	2	
n_1	11	
r	15.4	
s_r	5.50	
RSD_r	1.7	
Ho_r	0.4	
R	30.9	
s_R	11.02	
RSD_R	3.3	
Ho_R	0.5	