A Cross-border Method Validation for the Determination of Sweeteners in Soft Drinks

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Summary

The validated procedure deals with the identification and determination of the sweeteners Acesulfame K, aspartame and saccharin in soft drinks and similar beverages, using reverse phase high performance liquid chromatography with a buffered mobile phase and detection by ultra violet absorption detection at 220nm. The acceptance criteria required for the method performance characteristics were:

- *Linearity, a goodness of fit, expressed as a coefficient of determination (r^2), of greater or equal to 0.995*
- *Recovery of 90-110% of theoretical value*
- *Repeatability, a relative standard deviation (RSD), to be less than 5.0%.*

The method meets these criteria which are based on guidance on procedure validation given by the European Medicines Agency.

The method is given in full in Appendix 1.

Introduction

Northern Ireland is the only part of the United Kingdom that shares a land border with another member state of the European Union. In order to avoid potential analytical disputes on items which are subject to large volumes of cross-border, wholesale as well as retail, trade it was decided to develop and validate methods for a number of such items. Soft drinks were selected as the first example because problems had been noted in a previous survey for which the analyses had been carried out in a laboratory in England [1]. The Public Analyst's Laboratory in Galway, in the Republic of Ireland, agreed to act as the cross-border partner and supplied the initial draft method protocol. During the method refinement both data and staff were exchanged between the two laboratories and the method finally validated in Belfast, Northern Ireland.

The validated procedure deals with the identification and determination of the sweeteners Acesulfame K, aspartame and saccharin in soft drinks and similar beverages, using reverse phase high performance liquid chromatography with a buffered mobile phase and detection by ultra violet absorption at 220nm. These sweeteners, of those permitted by the Food

Regulations in Northern Ireland [2], were at the time of the work the only ones currently in widespread use on the island. In addition to the maximum permitted levels of the sweeteners, their use is prohibited for infants (under 12 months) and young children (between 0 and 3 years), this and the guidance for the use of terms "with no added sugar" and "energy reduced" [2] can give rise to the possibility of offences by the mislabelling of products.

The experiments were designed following the guidance on procedure validation given by the European Medicines Agency [3]. The acceptance criteria required for the method performance characteristics were: Linearity, a goodness of fit, expressed as a coefficient of determination (r^2), of greater or equal to 0.995; a recovery of 90-110% of theoretical value; repeatability, a relative standard deviation (RSD), to be less than 5.0%.

Experimental and Results

Linearity of responses

Duplicate, 10 µL samples of six mixed standard solutions of Acesulfame K, aspartame and saccharin with analyte concentrations covering the ranges 0-800 mgL^{-1} of Acesulfame, 0-1200 mgL^{-1} of aspartame and 0-160 mgL^{-1} of saccharin were prepared and chromatographed on a single day under the conditions specified in the developed procedure (see Appendix 1). Each compound gave a good peak shape with clear baseline separations for each compound at reproducible retention times. The goodness of fit, the retention times and their repeatability standard deviations are given in Table 1. Thus the observed linearity of response for each compound meet the required acceptance criteria, that of having a goodness of fit of greater or equal to 0.995 over the range tested.

Table 1
Goodness of fit for calibration data and retention times of the analytes

	Acesulfame K	Aspartame	Saccharin
Goodness of fit (r^2)	0.99974	0.99967	0.99972
Retention time (min) ± s	11.11 ± 0.08	13.07 ± 0.06	14.45 ± 0.06

Recoveries and Repeatabilities

The accuracy (expressed in terms of recovery) and the repeatability of the method for Acesulfame K, aspartame and saccharin were determined by one analyst, within a two hour period from chromatograms from a set of six replicate injections of a sample of known concentration, using calibration data prepared the same day against a blank and four reference standards. The data for each replicate, the means and the standard deviations are given in Table 2.

Expressing this data in terms of recoveries, for Acesulfame K, recovery = 99.0% with an RSD = 1.16%; aspartame, recovery = 102.0 % with an RSD = 1.17% and for saccharin, recovery = 99.9% with an RSD = 1.07%. Thus, all the data were well within the required acceptance criteria for recovery to be in the range 90-110 % and repeatability to be less than 5.0%.

Table 2
Data for the analysis of replicate samples of a standard solution containing 141.0 mgL^{-1} Acesulfame K; 474.7 mgL^{-1} aspartame and 20.3 mgL^{-1} saccharin

Replicate No.	Acesulfame K (mgL^{-1})	Aspartame (mgL^{-1})	Saccharin (mgL^{-1})
1	140.7	488.8	20.41
2	140.3	487.5	20.44
3	139.8	487.4	20.39
4	136.4	473.5	19.87
5	139.5	482.6	20.20
6	140.7	485.5	20.37
Mean ± s	139.56 ± 1.62	484.22 ± 5.68	20.28 ± 0.22

Within-laboratory reproducibility of the method

The within-laboratory reproducibility of the chromatographic system was examined by analysis of six quality assurance standards, each independently made up, to contain the same amounts of each of the analytes, within a four hour period, using calibration data prepared the same day using a blank and four standards (for results see Table 3).

The within-laboratory reproducibility of the method including the sample preparation in addition to that from the chromatographic system, was examined by the analyses of two drink samples carried out on different days, with fresh calibrations, each drink was sub-sampled six times and each sub-sample independently prepared for analysis (for results see Table 4).

Thus all the data in Table 3, for the variation in recovery and for within-laboratory reproducibility arising from the chromatographic system, for each compound meet the required acceptance criteria.

The data summarised in Table 4 for soft drink no.1 show the within-in laboratory RSD for the method including that which arose from the sample preparation of, for Acesulfame K of 0.53% and aspartame of 0.25% meet the required acceptance criterion. For soft drink no.2 the values of the RSD for Acesulfame K of 0.94% and for saccharin of 0.11%, also meet the required acceptance criterion.

Table 3
Data for the analysis of six, independently prepared, quality assurance standard solutions, each made to contain 400 mgL^{-1} Acesulfame; 600 mgL^{-1} aspartame and 85 mgL^{-1} saccharin

Quality Assurance Standard No	Acesulfame K (% Recovery)	Aspartame (% Recovery)	Saccharin (% Recovery)
1	100.92	97.61	102.71
2	99.63	97.01	101.22
3	100.00	97.23	101.66
4	100.44	97.19	102.16
5	100.10	97.61	101.88
6	99.82	97.88	101.39
Mean ± RSD	100.15 ± 0.46	97.42 ± 0.34	101.84 ± 0.53

Table 4
Data for the analysis of six independently prepared sub-samples of two soft drinks

Soft drink 1 Replicate sub-sample No.	Acesulfame K (mgL^{-1})	Aspartame (mgL^{-1})
1	44.27	369.66
2	43.84	368.37
3	44.43	369.20
4	44.48	367.87
5	44.43	367.93
6	44.29	367.18
Mean ± s	44.29 ± 0.23	368.37± 0.92

Soft drink 2 Replicate sub-sample No.	Acesulfame K (mgL-1)	Saccharin (mgL-1)
1	44.42	56.32
2	44.79	56.33
3	44.22	56.29
4	43.53	56.32
5	44.23	56.40
6	44.43	56.45
Mean ± s	44.27 ± 0.42	56.35 ± 0.06

Limits of Quantification

Because the base line was very steady, due to the stability of the chromatographic system, serial dilutions of a standard reference solution were carried out to determine the limit of quantification of the method for Acesulfame K, aspartame and saccharin as the concentrations for which the repeatability approached 5%. Conservative estimates were for Acesulfame K, 2.0 mg/L, for aspartame 8.0 mg/L and for saccharin 0.4 mg/L.

Further Verification

The performance of the method was further evaluated by participation in the Food Analysis Performance Assessment Scheme (FAPAS®) [4], Series 3, Soft Drinks, Round 48, in which a sample of Tonic Water, containing Acesulfame K, aspartame, benzoic acid and quinine, was distributed for analysis. Results were returned for Acesulfame K (48.8 mg/L) and aspartame (160.2 mg/L) using the method in Appendix 1. The values subsequently assigned to the above analytes, from the 48 and the 47 values returned by the participating laboratories in the round, were Acesulfame K, 50.0 with a range ±2z of 41.1 to 58.9 mg/L and aspartame, 172 with a range ±2z of 147 to 197 mg/L. Thus the results using the method developed in the study described herein were within the normal acceptance criteria for collaborative trials [5].

The method was audited at an annual UKAS visit and subsequently accredited to conform to BS EN ISO 17025: 2005.

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Appendix 1

Method for the Determination of Acesulfame K, Aspartame and Saccharin in Soft Drinks

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. Principle

1.1. After preparation, a filtered sub-sample is analysed for Acesulfame K (ACE), aspartame (ASP) and saccharin (SAC) by high performance liquid chromatography on a reverse phase column using ultraviolet detection. The concentrations are determined from the peak areas and a calibration graph prepared using mixed standard solutions of the analytes.

2. Scope and Field of Application

2.1 Suitable for the determination of Acesulfame K, aspartame and saccharin in commercial soft drink beverages.

3. Reagents

3.1 Acesulfame K – Sunsett or equivalent from two different sources or batches, one to be used to prepare the calibration standard solutions, the other to be used to prepare the Quality Control stock solution.

3.2 Aspartame – Sigma or equivalent from two different sources or batches, one to be used to prepare the calibration standard solutions, the other to be used to prepare the Quality Control stock solution.

3.3 Saccharin – Sigma or Aldrich from two different sources or batches, one to be used to prepare the calibration standard solutions, the other to be used to prepare the Quality Control stock solution.

3.4 Methanol – HPLC grade.

3.5 Tetrabutyl ammonium hydrogen sulphate – Sigma

3.6 Distilled Water – Double distilled or deionised filtered through a 47mm FP-Vericel membrane filter.

4. Solutions

4.1 Aqueous pH 4.55 solution – Dissolve 1.7g of tetrabutyl ammonium hydrogen sulphate and 0.7g sodium acetate in 1L of distilled water and adjust to pH 4.55

by addition of 0.1M NaOH with stirring and the aid of a pH meter. Filter this solution through a 0.45µ, 47mm Vericel membrane filter.

4.2 HPLC mobile phase – Mix methanol and aqueous pH 4.55 buffer solution in the v:v ratio, 30:70.

4.3 Confirmatory Mobile Phase – The same as the analysis mobile phase (4.2).

4.4 Washing Mobile Phase and auto-injector cleaning solvent – Mix methanol and distilled water in the v:v ratio, 30:70, filter as in 4.1.

5. Apparatus

5.1 High Performance Liquid Chromatography system – Two Shimadzu pumps (LC-10S), Auto-injector (SIL-10A), UV-visible detector (SPD-10A), System Controller (SCL-10A), or equivalents, and data handling system.

5.2 Analytical Column – Phenomonex Luna C18 (25cm x 4.6mm i.d.), 5µ particle size or equivalent.

5.3 Confirmatory Column – Phenomonex Luna C8 (25cm x 4.6 mm i.d.), 5µ particle size or equivalent.

5.4 Pre-column – Phenomonex guard column or equivalent.

5.5 Filters – Gelman Laboratory FP-450, 0.45µm, 47mm FP-Vericel membrane filter, Whatman GF/C filter papers and Millex-LCR 0.45 µm syringe filters or the equivalents.

5.6 pH Meter – Orion Model 420A or equivalent.

5.7 Glassware – Appropriate volumetric flasks and pipettes of Class A standard.

5.8 Balance – Analytical, capacity 100g, reading to 0.1mg.

5.9 Sonicator – Ultrasonik 300 or equivalent.

6. Calibration Standard, Quality Control Standard and Recovery Solutions

6.1 Mixed Stock Standard Solution of Acesulfame K, Aspartame and Saccharin

Weigh 1.000g ± 0.020g of Acesulfame K, 1.500g ± 0.020g of aspartame and 0.200g ± 0.020g of saccharin, recording the masses to ± 0.0001g, into a 1000.0mL volumetric flask. Add 250mL 50% methanol/water, swirl to dissolve, make up to volume with 50% methanol/water and mix. Store this

solution in a tightly stoppered container in a refrigerator at 2-8^{o}C. Discard after 3 months.

6.2 Mixed Working Standard Solutions of Acesulfame K, Aspartame and Saccharin

Pipette 0, 2.00, 4.00 and 6.00mL aliquots of the stock solution (6.1, after equilibration at room temperature) into 10.00mL volumetric flasks, make up to volume with distilled water and mix. Calculate the exact concentrations of Acesulfame K, aspartame and saccharin in each of the solutions. Prepare fresh Mixed Working Standards daily.

	Standard 1 (mgL^{-1})	**Standard 2 (mgL^{-1})**	**Standard 3 (mgL^{-1})**	**Standard 4 (mgL^{-1})**
Acesulfame K	0	200	400	600
Aspartame	0	300	600	900
Saccharin	0	40	80	120

6.3 Mixed Quality Control Stock Standard Solution of Acesulfame K, Aspartame and Saccharin

Weigh out 1.000g ± 0.020g of Acesulfame K, 1.500g ± 0.020g of aspartame and 0.200g ± 0.020g of saccharin, recording the masses to ± 0.0001g, into a 1000.0mL volumetric flask. Add 250mL of 50% methanol/water, swirl to dissolve, dilute to volume with 50% methanol/water and mix. Store this solution in a tightly stoppered container in a refrigerator. Discard after 3 months.

Note: Use different batches of the analytes from those used to prepare the Mixed Stock Standard Solution of Acesulfame K, aspartame and saccharin (6.1)

6.4 Mixed Quality Control Standard Working Solution of Acesulfame K, Aspartame and Saccharin

Pipette a 4.00mL aliquot of the quality control solution (6.3, after equilibration to room temperature) into a 10.00ml volumetric flask, make up to volume with distilled water and mix. Calculate the exact concentrations of Acesulfame K, aspartame and saccharin in each solution. Prepare a fresh Mixed Quality Control Working Standard solution daily.

6.5 Recovery Solutions A and B

Recovery Solution A – Pipette 1.00mL of distilled water into 10.00mL volumetric flask, make up to volume with sample and mix.

Recovery Solution B – Pipette a 1.00mL aliquot of a Acesulfame K, aspartame and saccharin Stock Standard Solution (6.1) into a 10.00mL volumetric flask, make up to volume with sample.

7. Sample Preparation

7.1 Degas approximately 20mL of the sample of soft drink at ambient temperature, if necessary, by sonification.

7.2 Filter the sample through a glass micro-fiber filter and then, if the solution is not clear, filter again, through a 0.45m PVDF membrane syringe filter. Use this filtrate for the analysis.

8. Analytical Procedure

8.1 Set-up the liquid chromatograph (5.1) according to the manufacturer's instructions. Fit an appropriate reverse phase column (5.2). The operating conditions must be set so as to achieve baseline separations of Acesulfame K, aspartame and saccharin

8.2 Normal Operating Conditions:

Injection volume:	10mL
Flow rate:	1.00mL/minute
Detector:	UV set at 220nm
Column Temperature:	ambient

8.3 Before proceeding to the analyses ensure the instrument response is within the normal response range by injection of 10µL of Standard Solution 3 (6.2), areas of response should be as follows, for the system as listed above:

Acesulfame K	between 10800000 and 1320000 counts
Aspartame	between 2070000 and 2530000 counts
Saccharin	between 3330000 and 4070000 counts

8.4 Preparation of calibration graphs:

Inject 10mL of each mixed Working Standard Solution of Acesulfame K, aspartame and saccharin (6.2) and prepare a calibration graph of the integrated peak area versus concentration for each analyte. (This may be carried out manually or automatically using a data handling system). The goodness of fit, coefficient of determination, r^2**,** should be greater than 0.995.

8.5 Inject, in turn, 10µL of the Quality Control Standard Solution, each prepared sample (in duplicate) and each recovery solution and record the chromatograms.

If the response obtained for any of the three analytes in a sample is outside the range of the calibration graph obtained (8.4), then the sample should be diluted

appropriately quantitatively with distilled water to bring the concentration(s) within the calibration graph(s) range.

Inject 10µL of Calibration Working Standard Solution 3 (6.2), at least, after every 10 injections, and at the end of each run to confirm the stability of response. The calculated concentrations should be within ± 10% of the known concentrations in this standard.

9. Expression of Results

9.1 The concentration of Acesulfame K, aspartame and saccharin in the samples and from each injection of the Calibration Working Standard Solution 3 (made for quality control purposes and to be entered on the method quality control chart)) are obtained, either manually or automatically, from the calibration graphs.

9.2 The method, as set up, gives the concentrations of the analytes for the test samples as injected, hence any dilution factors that occurred in stage 8.5 must be taken into account when expressing the final results.

9.3 The results are expressed as mgL^{-1}.

9.4 Record the sample description, batch number, laboratory sample identification number, calculations and results on a record sheet to which are appended the chromatograms.

10. Confirmation of Analyte's Identities

10.1 General

Identify the Acesulfame K, aspartame and saccharin peaks on the sample chromatograms by comparison of their retention times with those of the standards. The difference between the retention times for each analyte in the chromatograms for the standard and sample for each analyte should normally differ by no more than ±1.0% of the retention times in the reference chromatogram (see Table 1).

10.2 Use of an alternative stationary chromatographic phase

Where confirmation of a sample result is required re-analyse the sample in duplicate using a C8 (25cm x 4.6mm i.d.), 5µ particle size, analytical HPLC column using the mobile phase and conditions as described in 8.2.

10.3 Examination of chromatogram from a sample spiked with a calibration standard

To a volume of the filtered sample add an equal volume of a calibration standard of similar concentrations of Acesulfame K, aspartame and saccharin.

Inject this "spiked" sample using the same chromatographic conditions as described in 10.2 above. This confirmation is positive when no splitting or shoulders are visible in the relevant peak(s) of the new chromatogram. Record the sample volume taken, the volume of standard solution added and the concentrations of analytes in the standard solution used for "spiking".

11. Quality Assurance

11.1 Performance Characteristics

11.1.1. The limits of quantitation observed under routine operating conditions are given in Table A1.

Table A1
Limits of Determination

	Limit of Determination (mgL^{-1})
Acesulfame K	2.0
Aspartame	8.0
Saccharin	0.4

11.1.2. Bias – The method shows no bias at or near to the maximum permitted levels for Acesulfame of 350mg/L, for aspartame of 600mg/L and for saccharin of 80mg/L [1]. Recoveries being in the range ± 2.5%, well within the acceptance criterion of ± 10.0%.

11.1.3. Precision – The method gives repeatability's at or near the maximum permitted levels [1] in the range ± 1.0%, well within the acceptance criterion of ± 5.0%.

11.2 Quality Control

11.2.1 Internal Quality Control – Plot the results of the quality control analyses of Working Standard Solution 3 on the Quality Control Chart stored in the method validation file.

11.2.2 Recovery – Within each batch of analyses carry out a recovery or "spiking" test. Add a volume of a mixed standard calibration solution to a portion of the sample sufficient to give an additional 100.0mgL^{-1} Acesulfame K, 150 mgL^{-1} aspartame and 20.0 mgL^{-1} saccharin to the injection solution above that in the "unspiked" sample, as follows:

Recovery Sample A = 9.00mL sample + 1.00mL distilled water.

Recovery Sample B = 9.00mL sample + 1.00mL of Mixed Stock Standard Solution of Acesulfame K, aspartame and saccharin (6.3).

Analyse these samples as described in Section 8. Calculate the recovery, plot the results on the Recovery Chart in the method validation file.

11.2.3 Repeatability for out of specification samples – Analyse all positive (out of specification) samples in duplicate and ensure that the precision is met.