

RESEARCH ARTICLE

DETERMINATION OF ARTIFICIAL SWEETENERS IN LIQUID FOODS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

DOUSHI D. RAMSURN, SABINA JHAUMEER

LAULLOO, MINU G. BHOWON*

Department of Chemistry, Faculty of Science, University of Mauritius, Reduit, Mauritius

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Email: mbhowon@uom.ac.mu

Abstract: Artificial sweeteners are widely used nowadays in order to substitute sugar. The analysis for various foods is very important for food quality control and regulation enforcement. In this study, the presence of five different artificial sweeteners aspartame, acesulfame k, saccharin, sucralose and rebaudioside A were analysed in 38 liquid food items including soft drinks, fruit juices, energy drinks and dairy products using high performance liquid chromatography (HPLC). The sweeteners aspartame, acesulfame k and saccharin were separated on a C18 column and were identified using a diode array detector. Sucralose was analysed using a refractive index detector while a UV detector was used for rebaudioside A. The validation of the analysis methods was determined in terms of sensitivity, linearity range, precision, repeatability, analytical recovery and residual analysis. Out of the thirty eight samples analysed, artificial sweeteners were detected in twenty samples. Therefore it can be concluded that there is a tendency by manufacturers to add mixture of artificial sweeteners in a product.

Key words: acesulfame k, aspartame, saccharin, Sucralose, rebaudioside A, HPLC determination

INTRODUCTION:

Artificial sweeteners are substances which are significantly sweeter than the common carbohydrate sweeteners such as sucrose. Their sweetness range from about 30 times to several thousand times that of sucrose. The use of artificial sweeteners in food industry has been growing rapidly in recent years whereas in the past, they were mainly used in diabetic products. They have gained popularity as substitute sweeteners in many food products especially in soft drinks and other beverages, as consumers tend to control their sugar intake. Artificial sweeteners provide little caloric value to the normal diet and they do not affect insulin or glucose levels. Hence, they may help in weight management and provide sweet-tasting foods for people with diabetes. Examples of commonly used artificial sweeteners are aspartame, acesulfame k, saccharin and sodium cyclamate.

However, there had been arguments based on studies^[1] which had stated that artificial sweeteners might be hazardous to human health when consumed at certain concentrations. Many experiments have associated cancers with the intake of sweeteners but there was not enough evidence to prove it. Chronic exposure to these artificial sweeteners has been reported to cause the following symptoms; headaches, blurred vision, epileptic fits, brain tumor, insomnia, nausea and memory loss.^[2-8] Hence, the use of artificial sweeteners in food has to be regulated by using a reliable, accurate, precise and robust method to monitor the concentration of added sweeteners. A variety of methods^[9] such as UV spectroscopy^[10], capillary electrophoresis^[11], high performance liquid chromatography^[12-- 15], high performance thin layer chromatography^[16] and ion chromatography^[17] have been used to determine the concentration of sweeteners in foods and beverages. The purpose of this study was to determine and quantify 5 different sweeteners namely acesulfame k, aspartame, saccharin, sucralose and rebaudioside A by using HPLC techniques in 38 different liquid foods marketed in Mauritius. The methods used were validated using several parameters such as limit of detection, limit of quantification, calculation of residuals for the standards and the recovery obtained from spiked samples. The results obtained were compared with the Food Regulation 1999 of Mauritius to see whether the concentrations of the studied sweeteners were within the limit set^[18].

Materials and Methods

Reagents and Materials

Reference compounds, acesulfame k, aspartame, saccharin, and rebaudioside A were purchased from Sigma-Aldrich (Steinheim, Germany) and sucralose was from BDH Chemicals LTD (UK). All standards were 98 or greater % purity. HPLC-grade methanol, orthophosphoric acid (85%) and acetonitrile were obtained from Sd-Fine Chem. Limited (Mumbai, Maharashtra). Sodium acetate was purchased from Fisher Chemical (UK) and acetic acid was purchased from Techno pharmchem (India) and was of HPLC grade. Water was purified using a SG instrument Ultra-pure water purification system (Hyde Cheshire).

Preparation of standard solutions

Stock solutions of a ternary mixture of acesulfame k, aspartame and saccharin (500 ppm), sucralose (300 ppm) and that of rebaudioside A (600 ppm) were prepared by dissolving the reference compounds in distilled water. A series of calibration standard solutions were prepared by diluting stock solutions in a concentration range of 50-500, 50-300 and 100- 600 ppm. Dilutions of the standards solutions were done directly in 1.5 mL vials by using an electronic pipettor of range 75 μ L -1000 μ L.

Preparation of Samples

A set of 38 commercially available liquid foods were purchased from the local markets. The samples consisted of 13 soft drinks, 14 fruits juices, 6 energy drinks and 5 dairy products. The samples were prepared by modified reported procedures.^[19] All the samples were first homogenised to ensure uniformity. Samples of soft drinks and energy drinks were degassed for 15 minutes in an ultrasonic bath. For dairy products, 5.0 g of the sample was dissolved in 100 mL of methanol, sonicated for 15 minutes and centrifuged at 4,000 rpm for 10 minutes. The samples were filtered through a 0.45 μ m membrane filter before being injected into the HPLC system. If concentration of the sweeteners in samples exceeded the range of the calibration curve, the sample was diluted with distilled water in order for the concentration of the sample to fit in the calibration curve. Selected category of each variety of food samples was spiked with the standards to a concentration of 100 ppm to calculate the percentage recovery of the test analysis.

Chromatographic conditions

Acesulfame k, saccharin and aspartame were separated using a Dionex HPLC system of model Ultimate 3000 series with an Agilent C18 column (150 x 2.1 mm, 5

μ m). The mobile phase was prepared by mixing methanol and the buffer solution (0.681 g of sodium acetate and 3 mL of acetic acid) in a ratio of 1:3. The mobile phase flow rate was set at 0.365 mL/min and injection volume was 2 μ L. The column temperature was set at 30 °C. The UV wavelength was set to 205 nm for aspartame and 229 nm for acesulfame k and saccharin. Each standard of the sweetener was first run separately to determine the retention time for each analyte. The retention time of acesulfame k was found to be 1.626 minutes, for saccharin 2.592 minutes and 31.017 minutes for aspartame and hence, the total run time was set to 36.00 minutes for each run of mixed standards. For sucralose, the analysis was performed using a Shimadzu HPLC system equipped with a refractive index detector. A Shim-pack VP-ODS C18 column (150 x 4.6 mm, 5 μ m) was used. The mobile phase consisted of a mixture of water/ acetonitrile (15:85) and was pumped with the flow rate of 1.00 mL/min with an injection volume of 10 μ L. The column temperature was set at 40 °C. The retention time of sucralose standard being 7.884 minutes, hence the total run time was thus set to 9.00 minutes.

The chromatographic analysis of rebaudioside A was performed on a Shimadzu HPLC system equipped with a UV detector using a Hilic packing column (150 x 5 mm, 4.6 μ m). The pH of the mobile phase (water:acetonitrile, 2:3) was adjusted to 3 by adding orthophosphoric acid. Elution flow rate was 0.300 mL/min with an injection volume of 10 μ L and compartment column was set to 40 °C. Rebaudioside A was eluted at a retention time 7.522 minutes and the total run time was set to 10.00 minutes and the UV wavelength was adjusted to 190 nm. Quantification of each sweetener was performed by measuring peak areas at the corresponding retention time and comparing them with their corresponding calibration curve.

Determination of limit of detection (LOD) and quantification (LOQ).

The EPA sw-846 protocol was used to determine the lod and loq. a visual evaluation of the lod was determined by the analysis of known concentrations of standards until the minimum level of the analyte can be reliably detected. the minimum quantifiable standard was run 7 times and the standard deviation(s) were calculated. the lod and loq were calculated using the formulae:

$$\text{LOD} = t_{0.99} \times s$$

$$\text{LOQ} = 3 \times \text{LOD}$$

Where, $t_{0.99}$ is the one-tailed t-statistic at the 99% confidence level for n-1 replicates, n = 7 and 's' is

standard deviation of n sample spikes at the estimated LOQ

In addition to the calculation method, evaluation of LOD and LOQ was based on signal to noise ratio to confirm the values. The limit of detection was estimated with concentrations giving a signal-to-noise ratio of 3 to 1.

Results and discussion

Method Development and Optimization of HPLC system

Method validation is a procedure used to confirm that the analytical procedure implemented for a particular test for the quality, reliability and uniformity of analytical results. Accordingly, the sensitivity, linearity range, reproducibility and recovery of the standards were evaluated. The sensitivity of each method was evaluated via the limits of detection and quantification, which were determined according to International Conference for Harmonization (ICH, 1990). The precision of our method was evaluated as analytical repeatability based on three replications of the standard solutions and calibration curves were plotted from the standard drug concentrations versus peak areas of the individual drugs. Precision is expressed as the relative standard deviation (RSD) of the replicates. Table 1 summarizes the different validation parameters used during the analytical tests.

The linearity of each standard was tested within the amount range which covers the most likely amounts injected in the column for real sample analysis. The correlation coefficients, r^2 for all the sweeteners were higher than 0.99, which revealed a good linearity in the concentration range for each sweetener. The LOD was estimated with concentration giving a signal-to-noise ratio of 3 to 1. The LOQ for saccharin was the lowest whereas that of sucralose was the highest. Hence, the analysis performance for saccharin had the best sensitivity. The % RSD values for all methods were less than 10% which confirmed the repeatability of the analysis. [20]

The accuracy of the analytical method was evaluated using the recovery test. Selected samples (J10, S9, D1, E2) were spiked with 100 ppm standards and were treated as normal samples. The developed method resulted in satisfactory recoveries for the samples, ranging

from 96.5 % to 103.5%. This revealed the reliability of the method (Table 2).

Residuals of standards

Figure 1 shows the residual plot for each sweetener standard with the residuals on the vertical axis and concentration on the horizontal axis. A residual is the vertical difference between the Y value of an individual and the regression line at the value of X corresponding to that individual, for regressing Y on X and identifies non-linearity or outliers. In general, a null linear residual plot shows that there are no observable defects in the model, a curved plot indicates non-linearity and a fan-shaped or double-bow pattern indicates non-constant variance [21]. If the residual points are randomly dispersed around the horizontal axis, the linear regression model is appropriate for the data which indicate a good fit for a linear model [22]. From Table 3 and Figure 1, the residuals are spread uniformly and are at random around the regression lines, passing the normality distribution test ($p < 0.05$). The standard error for each sweetener is very small (except for rebaudioside A) and hence it can be concluded that all the regression models are good ones

Application of the developed method to real samples

The proposed methods for the five sweeteners were applied for the analysis of the 38 liquid samples. The presence of acesulfame k, saccharin and aspartame sweeteners in the samples were determined by comparing both the retention time and spectral data generated by the diode array detector at wavelength 205 nm and 229 nm of standards with that of the samples peaks. For sucralose and rebaudioside A analysis, presence of the sweeteners in samples were determined by overlaying the chromatogram of the standards with that of the samples; that is based on retention time. Quantification of the sweeteners was based on peak areas. Table 4 summarized the average concentration of the sweeteners in the analysed samples obtained on HPLC with their standard deviation.

Table 1: Validation parameters of the analytical tests

Parameter	Acesulfame k	Saccharin	Aspartame	Sucralose	Rebaudioside A
Linearity range (ppm)	0-500	0-500	0-500	0-300	0-600
r^2	0.9999	0.9999	0.9995	0.9994	0.9995
LOD (ppm)	0.104	0.0486	1.59	2.20	0.24
LOQ (ppm)	0.312	0.146	4.77	6.99	0.718
RSD %	1.45	0.99	0.56	3.00	9.96

Table 2: Recovery of spiking samples

Sample	% Recovery				
	Acesulfame k	Saccharin	Aspartame	Sucralose	Rebaudioside A
J10	98.99	99.47	99.20	99.88	103.5
S9	98.49	97.97	99.04	99.21	102.4
D1	97.33	96.51	98.95	98.46	101.9
E2	99.58	99.13	99.51	99.84	101.1

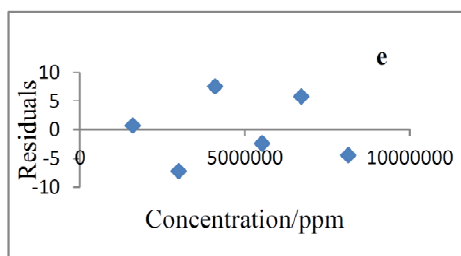
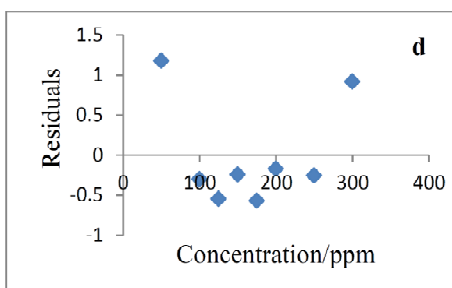
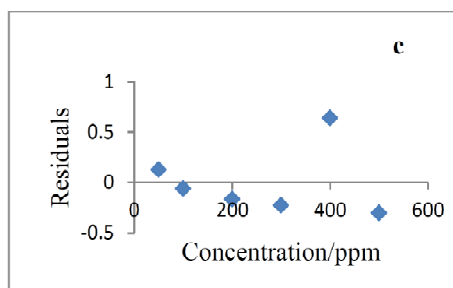
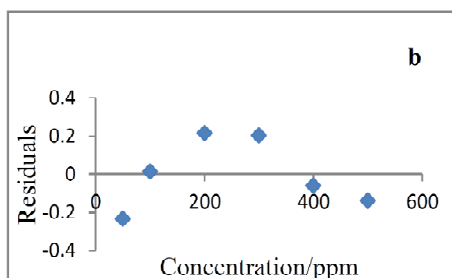
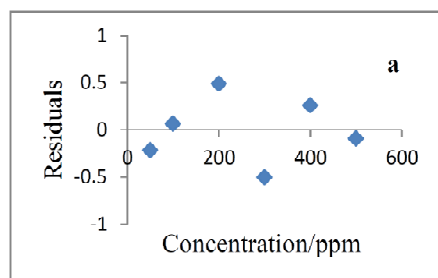


Table 3: Summary output of the standards

	Acesulfame k	Saccharin	Aspartame	Sucralose	Rebaudioside A
residuals	-0.502–0.487	-0.062–0.217	0.061 – 0.640	-0.260 – 1.770	-7.24 – 5.80
Standard error	0.392	0.159	0.388	0.720	6.51
p-value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Table 4: Concentration of Sweeteners in food samples

Sample	Acesulfame k/ ppm	Saccharin/ ppm	Aspartame/ ppm	Sucralose	Rebaudioside A
J1	-	-	15.03± 1.10	-	232.70± 0.86
J2	2.93± 0.06	14.95± 0.02	-	-	-
J3	2950± 89	-	-	-	-
J4	-	9.46± 0.41	-	-	16.13± 0.71
J5	422.20± 0.65	-	844.00 ± 0.42	-	-
J6	165.60± 0.75	-	-	96.46±	-
J7	-	145.20± 0.14	-	-	-
J8	-	-	-	-	-
J9	73.52± 0.12	-	1336± 37	-	-
J10	-	-	-	-	-
J11	-	-	-	-	-
J12	-	-	-	-	-
J13	-	-	-	-	-
J14	-	-	-	69.32±	-
E1	-	-	-	-	-
E2	-	-	-	-	-
E3	-	-	-	-	-
E4	163± 1	-	74.68± 1.25	-	-
E5	22.46± 1.60	-	-	218.30±	-
E6	-	-	-	-	-
S1	136.70± 2.76	60.23± 1.47	-	-	-
S2	-	-	-	-	142.90± 0.08
S3	-	-	-	-	-
S4	-	-	-	-	-
S5	61.11± 1.09	-	-	-	-
S6	197.00± 0.06	-	-	-	-
S7	82.20± 0.55	-	377.50±	-	-
S8	-	-	-	-	-
S9	-	-	-	-	-
S10	190.20± 16.75	-	318.80±	-	-
S11	-	-	-	-	-
S12	-	-	-	-	144.90± 1.39
S13	-	-	-	-	-
D1	-	-	-	-	-
D2	-	-	-	-	-
D3	17.61± 0.46	-	-	-	-
D4	-	-	-	-	-
D5	24.54± 0.08	-	24.13± 0.45	-	-

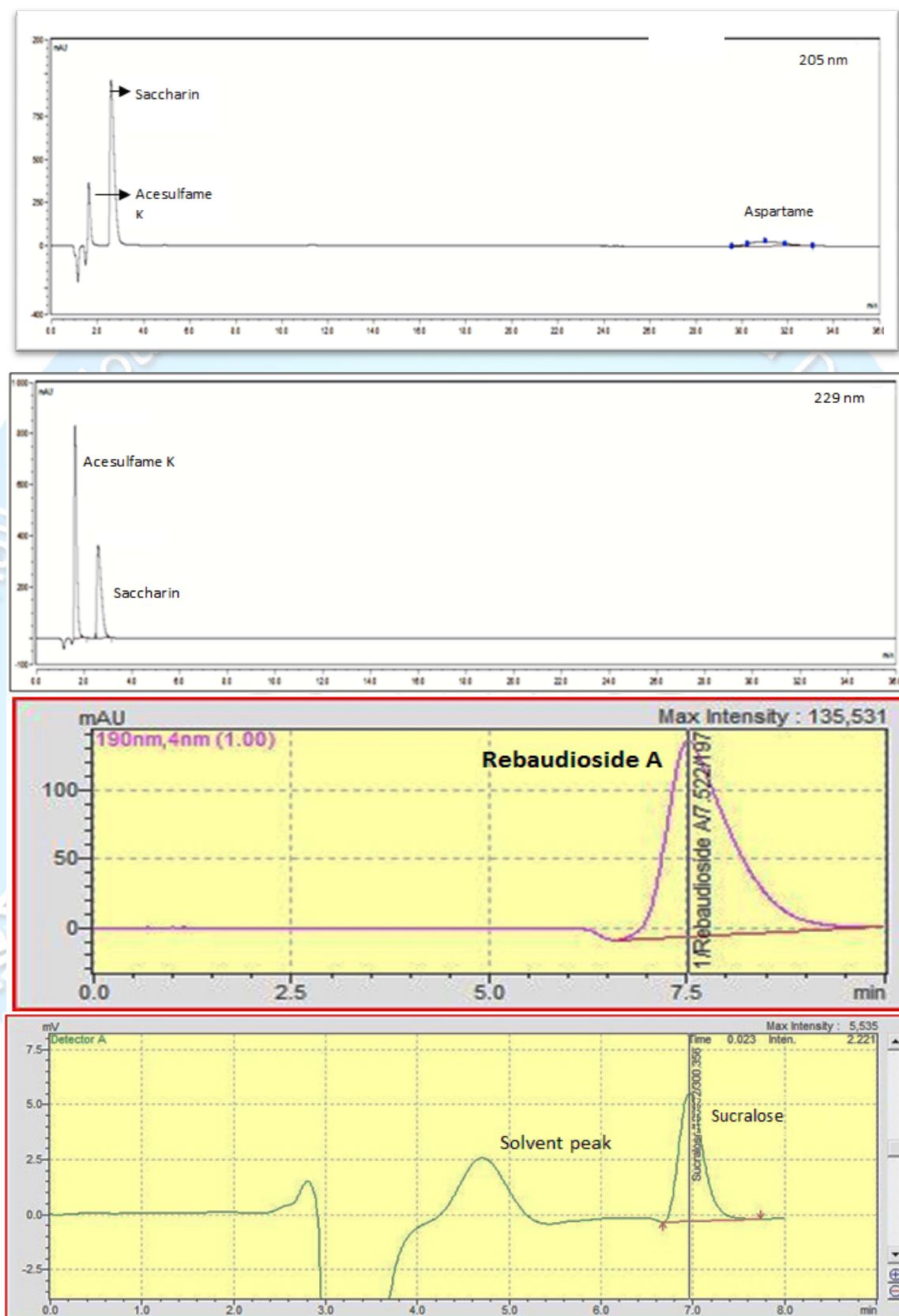
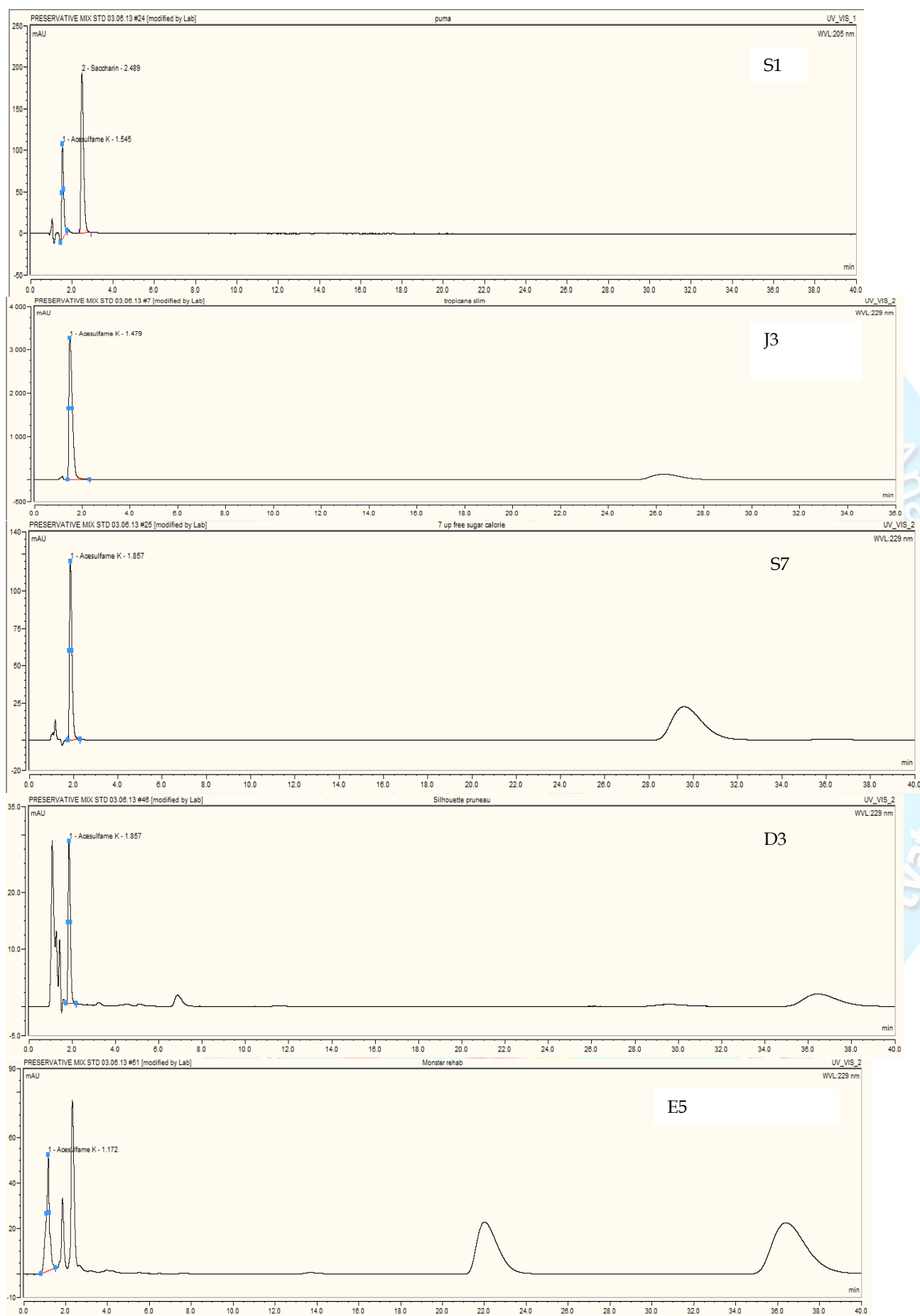
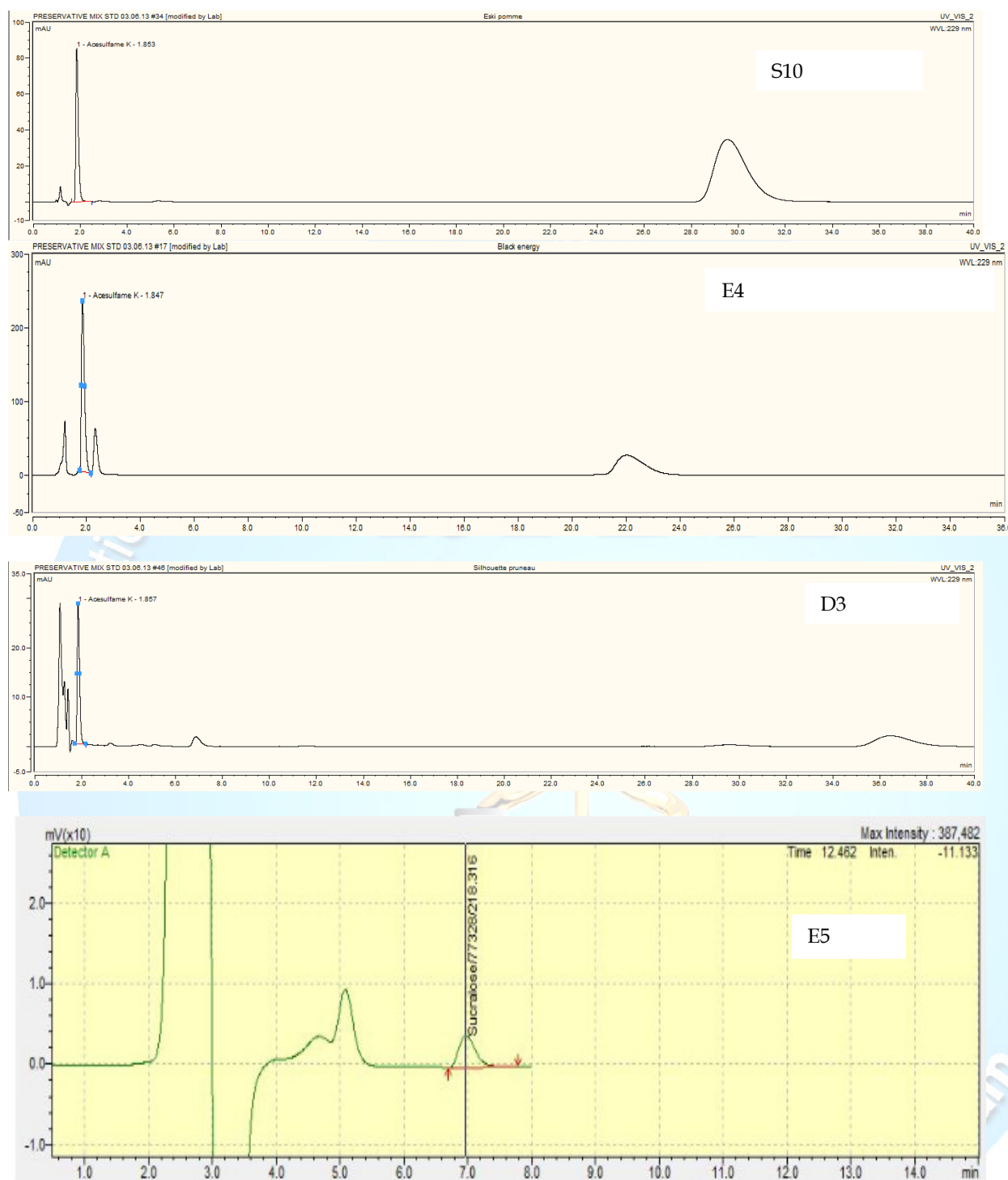


Figure 1: Chromatograms of different standards





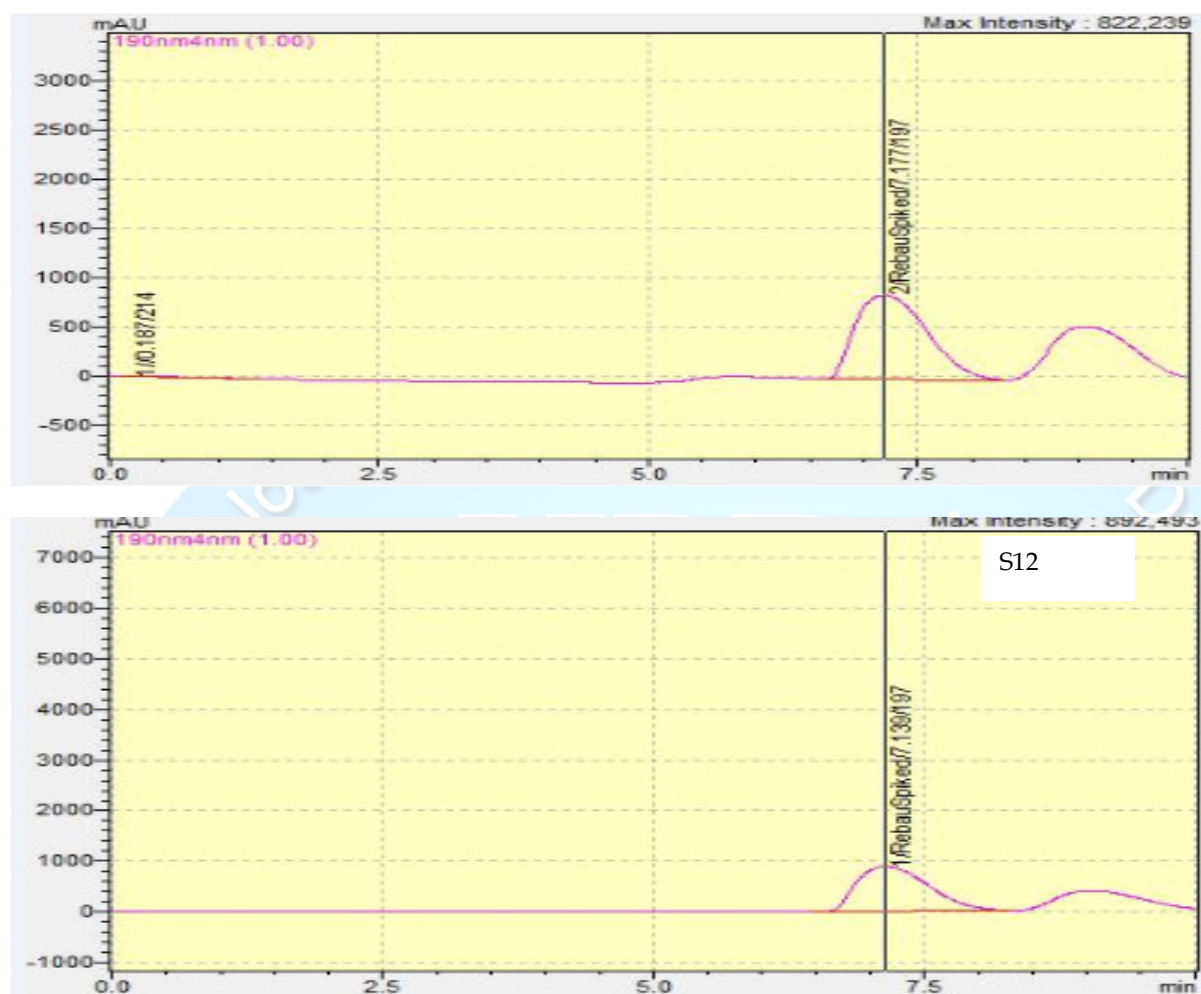


Figure 2: Chromatograms of selected samples

Application of the developed method to real samples

The proposed methods for the five sweeteners were applied for the analysis of the 38 liquid samples. The presence of acesulfame k, saccharin and aspartame sweeteners in the samples were determined by comparing both the retention time and spectral data generated by the diode array detector at wavelength 205 nm and 229 nm of standards with that of the samples peaks. For sucralose and rebaudioside A analysis, presence of the sweeteners in samples were determined by overlaying the chromatogram of the standards with that of the samples; that is based on retention time. Quantification of the sweeteners was based on peak areas. Table 4 summarized the average concentration of the sweeteners in the analysed samples obtained on HPLC with their standard deviation.

From the 38 samples analysed, artificial sweeteners were detected in 20 samples. Most samples in which sweeteners had been detected had stated to have artificial sweeteners in their ingredient lists except the juices J1 and J4. In J1, aspartame (15 ppm) and rebaudioside A (233 ppm) were detected and in J4 juice, saccharin

(9 ppm) and rebaudioside A (16 ppm) were detected. In both juices, the manufacture only labeled no sugar added but did not mention the sweeteners that were added. Moreover, most of the samples that contained sweeteners did not label the amount of sweeteners added except J3 (acesulfame k-100mg/serving) and J5 (aspartame-0.32g/L, acesulfame k-0.03g/L). For the juices J3 and J5, the amounts of sweeteners detected were beyond that stated. This is because both juices were concentrated and needed to be diluted before consumption and the data reported herein is for the concentrated juice. None of the samples analysed exceeded the maximum limits set by Food Regulation 1999. However, it should be noted that the Food Regulation 1999 only specified the concentration of aspartame and saccharin that should be added in liquid foods but no limit were specified for acesulfame k, sucralose and rebaudioside A.

CONCLUSION

Increased use of artificial sweeteners in food and beverages can present a danger to health if their concentrations exceed the acceptable daily intake. Hence, it is im-

portant to control the amount of artificial sweeteners added in food by regulating their concentration in food and beverages through legislation in order to prevent excessive intake. Sensitive, reliable and robust analytical methods to determine and quantify sweeteners are essential to meet the needs of growing markets in quality control and consumer safety. Consequently, it is important to develop analytical methods for simple, rapid and low-cost sensitive determination of sweeteners. The HPLC methods used are simple, rapid, require minimum sample preparation with high sensitivity and robustness. Hence, the method can be used routinely for regulation inspection of these different sweeteners in liquid food.

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