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HPLC analysis of artificial preservatives, stimulants and sweeteners in carbonated beverages in Bangladesh

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ABSTRACT

Different (n=21) commercial carbonated beverages, available in Bangladesh, were analysed by HPLC coupled with a photodiode array detector (UV-PDA). The main objective of this study was to quantify the amount of different artificial sweeteners, stimulant and preservatives and to validate the two methods used in this purpose. The two methods are simultaneous determination of sodium benzoate, caffeine, and saccharin using the sodium acetate buffer with pH 3.0 at 254 nm and simultaneous determination of artificial sweeteners acesulfame-K and aspartame using the potassium phosphate monobasic buffer. Correlation coefficient (r^2) obtained were 0.9977, 0.9976, 0.9975, 0.9993 and 0.9956 in the range of 1-100 µ/L, recoveries were 95.88-97.10, 96.53-97.20, 94.53-96.05, 91.15-93.31 and 92.88-94.13%, LODs were 30.06, 15.46, 31.61, 0.33 and 0.83 mg/L, and LOQs were 100.18, 51.54,105.37, 1.11 and 2.77 mg/L for saccharin, caffeine, sodium benzoate, acesulfame-K and aspartame, respectively. The present analysis shows that amount of saccharin, caffeine, sodium benzoate, acesulfame-K and aspartame in the range of ND-377.60, ND-462.36, ND-217.60, ND-48.09 and ND- 295.20 mg/L, respectively. The total carbohydrate found as 102.81-147.16 g/L using the UV-visible spectrophotometric method which implies that sample containing more amount of carbohydrate has less artificial sweeteners value.

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1. Introduction

Food acts like fuels in animal body. Different food components like carbohydrates, proteins, vitamins, fats etc. produce energy, develop growth, and meet the demand of nourishment after ingestion and assimilation of food by living body (Sanjay, 2015). To increase the shelf-life, taste, and food quality, different artificial sweeteners and preservatives are used in commercially marketed food items like carbonated beverages. In Bangladesh, the consumption rate of carbonated beverages is increasing day by day since it is easily available, cheap, tastes good, and helps to quench thirst, refreshing, and convenient. The commercial beverage companies advertise their products in different media to attract the consumers of different level, particularly the teenagers. Due to the excessive consumption of the beverages, their general and oral health face harmful effect as there is so much lacking of essential nutrients in beverages for human body (Damle et al., 2011). During preparation of different ingredients like sodium benzoate as preservatives, caffeine as stimulant, sugar, aspartame as artificial sweeteners, antioxidant, and others, additives are added to the carbonated beverage (Jacobson, 2005). Beverage companies used additives, like sweeteners and preservatives, to the carbonated beverages to maintain the quality, taste, pH, extend shelf life, appearance and consistency to make the product economical (Chua et al., 2017; Seyinde et al., 2019). These artificially synthetic chemical additives may exert adverse physiological effect and for this reason several authoritarian bodies set their permissible standard limit that varies place to another (Pressman et al., 2017).

Benzoic acid is used in beverages as its different salts/benzoates of Na, Ca and K, and phenyl acetic acid (Chaleshtori et al., 2018) due to high solubility of benzoates compared to benzoic acid, although, benzoate produce benzoic acid under acidic condition (Dolezal, 2004; Khosrokhavar et al., 2010). The use of sodium benzoate as bacteriostatic and fungistatic in soft drinks under acidic condition is very common practice to the beverage companies. However, daily consumption may impact to adverse side effects, such as skin rash, non-immunological contact urticarial, hyperpnoea, metabolic acidosis, and asthma (Sultana et al., 2016). Naturally produced caffeine, a methylxanthine alkaloid found over 63 plant species globally, which is most frequently used worldwide as psychoactive substance (Kapil et al., 2011) in most of the energy drinks due to its stimulating action on central nervous systems (Bispo et al., 2002; Gerald et al., 2014; Pennay et al., 2011; Wanyika et al., 2010). Moreover, it can promote alertness, attention and mind reaction time (Cysneiros et al., 2007), sleep disorder, breathing problems, renal system dysfunction, and irregular heartbeat as a physiological side-effect (Mendelson, 2009; Nehlig et al., 1992).

Some of artificial sweetener, saccharine (1,2-benzisothiazol-3(2H)-one-1,1-dioxide) and aspartame now take the position of natural sugar because of its obesity problems, increasing numbers of diabetic population, and health awareness of the people. Sodium or calcium salts of saccharine are used in most of the beverages due to their low-calorific value and extreme sweetening power as compared to the natural sugar. Sometimes they are mixed with another artificial sweetener like aspartame (Sik, 2012). Due to the excretion of saccharine through kidney in greater portion without being metabolized in human body, remnant is hydrolysed, thus no energy is gained from it (Lino and Pena, 2010; O'Donnell and Kearsley, 2012).European Scientific Committee on Food (SFC) established the acceptable daily intake (ADI) of saccharine and its sodium, potassium and calcium salts that is about 0–2.5 mg/kg body weight (BW) (Lino and Pena, 2009). Now the most widely used aspartame (N-L-α-aspartyl-L-phenylalanine-methyl ester) was first validated by U.S. Food and Drug Administration (FDA) in 1981 for limited use in solid food and but its authorization used in soft drinks extended in 1983 (FDA, 1981; FDA, 1983). Aspartame is used in foods and beverages worldwide as a low calorific and non-nutritive artificial sweetener which contains two amino acids, aspartic and phenylalanine and about 200 times sweeter than sucrose (Cantarelli et al., 2009;Serdar and Knezevic, 2011). Its consumption may damage brain, creates adverse neurological problems, such as insomnia, headache and seizure (Gimba et al., 2014). Acesulfame-K (also known as E950) is also about 200 times sweeter than sucrose, have a long shelf life, easily water soluble and stable at high temperature that enables it suitable for use in baking (Zygler et al., 2009). It is used in many of products especially in low-calorie products, diabetic foods, and sugarless products and excreted from our body without metabolized. It is also was found safe in use from a large number of pharmacological and toxicological studies (Sardesai and Waldshan, 1991).

Table 1. Collected carbonated beverages (CB) samples and dilution factor (DF)

Sample Code	Batch No.	Method 1 (DF)	Method 2 (DF)	Total Carbohydrate (DF)
CB_1	513	10	10	10
CB_2	697	2	10	10
CB_3	453	2	10	10
CB_4	924	10	10	10
CB_5	213	2	10	10
CB_6	611	2	10	10
CB_7	517	2	10	10
CB_8	457	2	10	10
CB_9	918	2	10	10
CB_{10}	812	2	10	10
CB_{11}	367	2	10	10
CB_{12}	895	2	10	10
CB_{13}	435	2	10	10
CB_{14}	685	2	10	10
CB_{15}	967	2	10	10
CB_{16}	643	2	10	10
CB_{17}	968	2	10	10
CB_{18}	210	2	10	10
CB_{19}	647	2	10	10
CB_{20}	357	2	10	10
CB_{21}	869	2	10	10

Chronic exposure of this sweetener produces some physiological disorder like headaches, depression, nausea, mental confusion and loss of appetite (Agarwal et al., 2016; Amer et al., 2017). Therefore, in this study, we developed suitable methods for the analysis of these artificial preservatives, stimulants, and sweeteners, also their quantitative determination in popular carbonated beverages which are commercially available in Bangladesh. Artificial sweeteners that are used as sugar substituent in beverages and preservatives are also used to inhibit microbial growth in the beverages. As both of them are causing different health hazards in excessive consumption in human body, they were targeted to quantify the present status of both artificial sweeteners and preservatives used in the commercial carbonated beverages available in Bangladesh. High performance liquid chromatography (HPLC) coupled with UV-PDA detector is frequently used globally for the analysis of artificial sweeteners, stimulants and preservatives due to its high sensitivity and high accuracy. Due to the different nature and UV-Visible absorbance of the sweeteners, stimulants, and preservatives, they can be determined individually by using HPLC-PDA (Divis et al., 2020).

2. Materials and methods

2.1. Sample collection

Twenty-one different batches of commercial carbonated beverages (CB) samples (Table 1) were collected from different markets of Dhaka city during seventh months. All the collected samples were stored at 5^{O}C in a refrigerator until analysis.

2.2. Chemicals and reagents

The chemicals and reagents used in this analysis purposes were anhydrous sodium acetate, HPLC grade methanol, glacial acetic acid, isopropyl alcohol, potassium phosphate monobasic, 85% Phosphoric acid, extra pure D-(+)-glucose (Sigma-Aldrich), acetonitrile (E. Merck Germany), deionized water, analytical standard compounds of saccharin, caffeine and sodium benzoate, acesulfame-K, aspartame (Sigma Aldrich), sulfuric acid (98%, w/w, BDH, U.K.), phenol (Merck, Mumbai, India).

2.3. Instruments

The instruments used to analyse the samples were analytical balance (FR-200, NDO-450ND, Japan), oven &furnace (GSM 11/8 Hope valley, S336RB, England), high performance liquid chromatography (Model RF-20, Prominence, Shimadzu), pH meter (Hanna HI 2211), deionized water used for HPLC was obtained from a Mill-Q System (Millipore, Denmark and Mildford, MA, USA), double beam UV spectrophotometer (Model: UV-1800, Shimadzu), Sartorius vacuum pump device (pre-cut membrane with 0.45 µm pore size), Ultra sound vibration Sonicator (Miyako Chopper, Japan) and vortex machine (Model: KEBO Lab REAX-2000).

2.4. Sample preparation for analysis by HPLC

Volumetric flasks (100 mL) and round bottom flasks (250 mL) were cleaned thoroughly with clean water followed by acetone. The samples were filtered with HPLC grade filter paper and degassed by ultra sound sonicator for 30 min. The samples were then diluted by a factor according to Table 1. The solutions were homogeneously mixed by using vortex machine and shaking was carried out for two minuntes. Then, prepared samples were injected into injector loop of HPLC one by one. Operating conditions for both the methods were shown in Table 2.

Table 2. Operating condition of HPLC

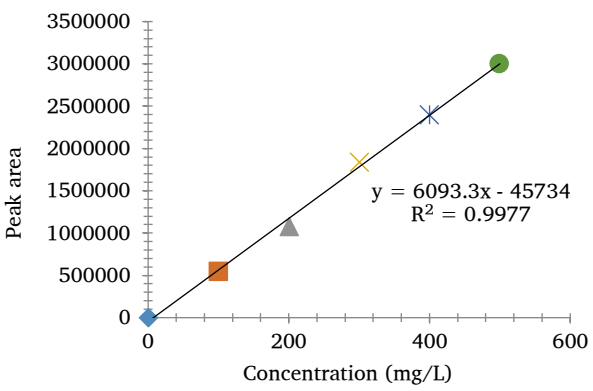
Parameter	Method 1	Method 2
Detector Wavelength	Sodium benzoate, Caffeine and Saccharine at 254 nm	Aspartame at 193 nm and Acesulfame-K at 226 nm
Column	Luna C18, 5µm, 250 x 4.60 mm, 100 Å	Luna C18, 5µm, 250 x 4.60 mm, 100 Å
Flow Rate	1.5 mL/min	1 mL/min
Gradient	Isocratic	Isocratic
Mobile Phase	Acetic acid/ isopropyl alcohol/ water (12/2/86%) (v/v/v)	Potassium phosphate monobasic/ acetonitrile (85/15%)
Oven Temperature	Ambient	Ambient
Injection Volume	20µL	20µL

2.5. Sample preparation for total carbohydrate determination

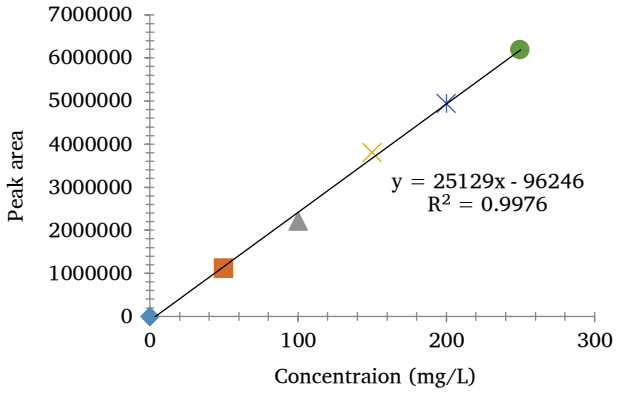
Exactly 3.0 mL of the sample and 50.0 µL of 80% aqueous phenol were mixed with 98% sulfuric acid until reddish-brown colour was developed and the solution was transferred in a 25 mL volumetric flask. The volume was adjusted with concentrated sulfuric acid (98%) and the absorbance of the solution was determined by UV-Vis Spectrophotometer.

2.6. Preparation of standard solutions

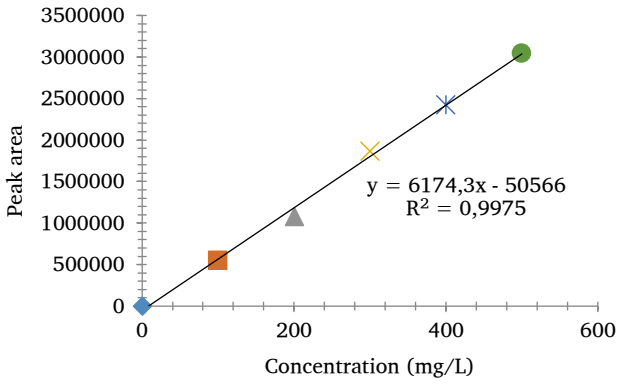
Standard solutions of 20000, 20000, 10000, 500 and 500 mg/L of saccharin, sodium benzoate, caffeine, acesulfame-K, and aspartame were prepared by weighing 0.2 g, 0.2 g, 0.1g, 0.005 g and 0.005 g of each standard respectively in five different 10.0 mL volumetric flasks and made up to the mark with deionized water. All these were primary standard solutions from which necessary working standard solution were prepared and calibration curve of each standard solution were made (Fig. 1). For total carbohydrate determination, analytical grade glucose (300g) was taken in a 1.0 L volumetric flask, following the addition of 50.0 mL 80% aqueous phenol and 30 mL of concentrated sulfuric acid (98%) which converted the glucose into furfural complex with the addition of phenol. A reddish-brown colour was formed. It was made up to the mark by 98% sulfuric acid for the preparation of 100 g/L solution from which working standard solutions were prepared. The absorbance of these solutions were measured by a double beam UV-Visible spectrophotometer at 482 nm to draw a calibration curve (Fig. 1).



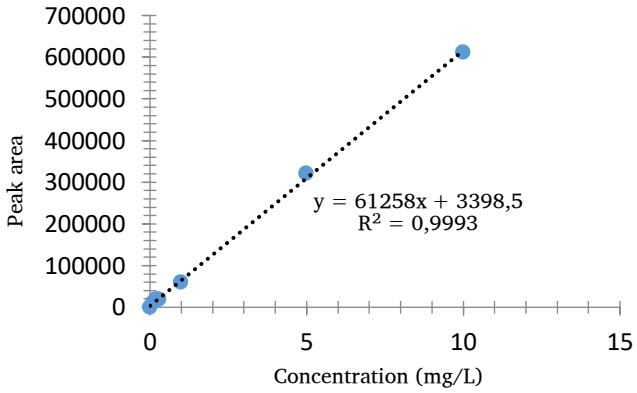
(a) Saccharin



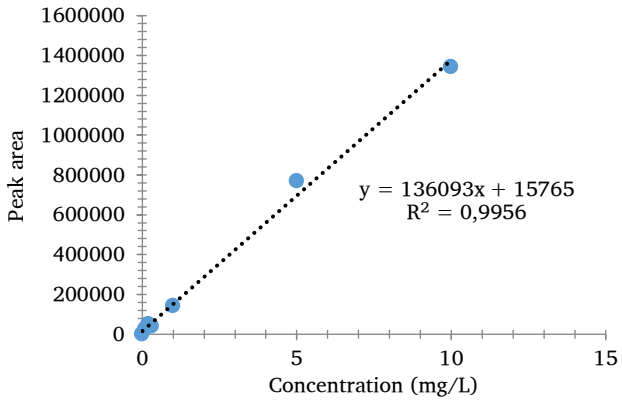
(b) Caffein



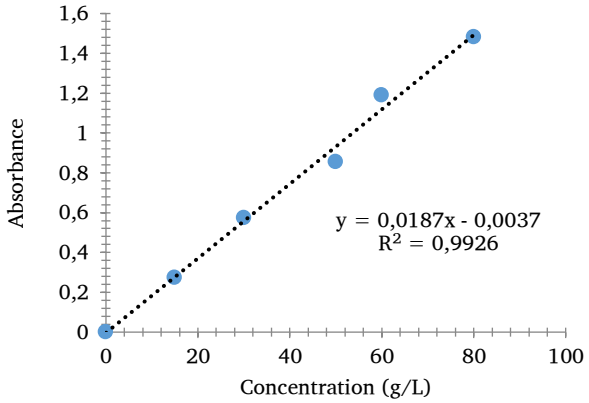
(d) Sodium Benzoate



(c) Acesulfame-K



(f) Aspartame



(e) Glucose

Fig. 1. Calibration curve followed by method-1 (a, b, c) and method-2 (d, e) and standard glucose solution (f)

2.7. Limit of detection (LOD), limit of quantification (LOQ) and recovery experiment

To determine the limit of detection (LOD), working standard solutions were serially diluted to get desired concentration and injected one by one giving interval of solvent blank until the peak heights of the standards were same to the noise level. LOD was calculated by taking peak height three times of noise level (S/N ratio: 3:1). For limit of Quantification (LOQ), the peak height of each compound was considered about nine times higher than the baseline noise (S/N ratio, 9:1). Replicates of each sample were processed followed by respective procedure to determine the matrix effect under analysis method. Reagent blank was carried out by the procedure using only solvent and reagents (in the absence of sample) to make the analysis realistic. In both cases, no peak was observed at the retention time of standard. Recovery experiments for each standard in respective matrix individually were carried out. Known number of standard compounds were added drop by drop over to the controlled samples and allowed the sample to stand for 30 min to be absorbed into the samples. Then the samples were extracted and processed following same procedure. The recovery of each analyte was calculated by using the formula as follows:

$$\textbf{Recovery}\ (\%) = \frac{\text{Area}_{\text{Sample}} \times \text{Conc}_{\text{Std}}}{\text{Area}_{\text{Std}} \times \text{Conc}_{\text{Matrix}}} \times \frac{100}{\text{Known amount of Std}}$$

2.8. Identification and Quantification by HPLC

The reference standard solutions were injected into the instrument HPLC using PDA detector and under the same conditions where processed carbonated beverages samples were also injected. By comparing the retention time of the different peaks of the sample with standard compounds, residues present in the samples were identified. Amount of unknown analyte in the respective samples were found out using the following formula.

$$\textbf{Amount of unknown sample} = \frac{\text{Peak Area}_{\text{Sample}} \times \text{Conc.}_{\text{Std}}}{\text{Peak Area}_{\text{Std}} . \times \text{Conc.}_{\text{Matrix}}}$$

The data obtained by calculation for the identification and quantification of saccharine, caffeine, sodium benzoate, acesulfame-K, and aspartame is recorded in Table 4.

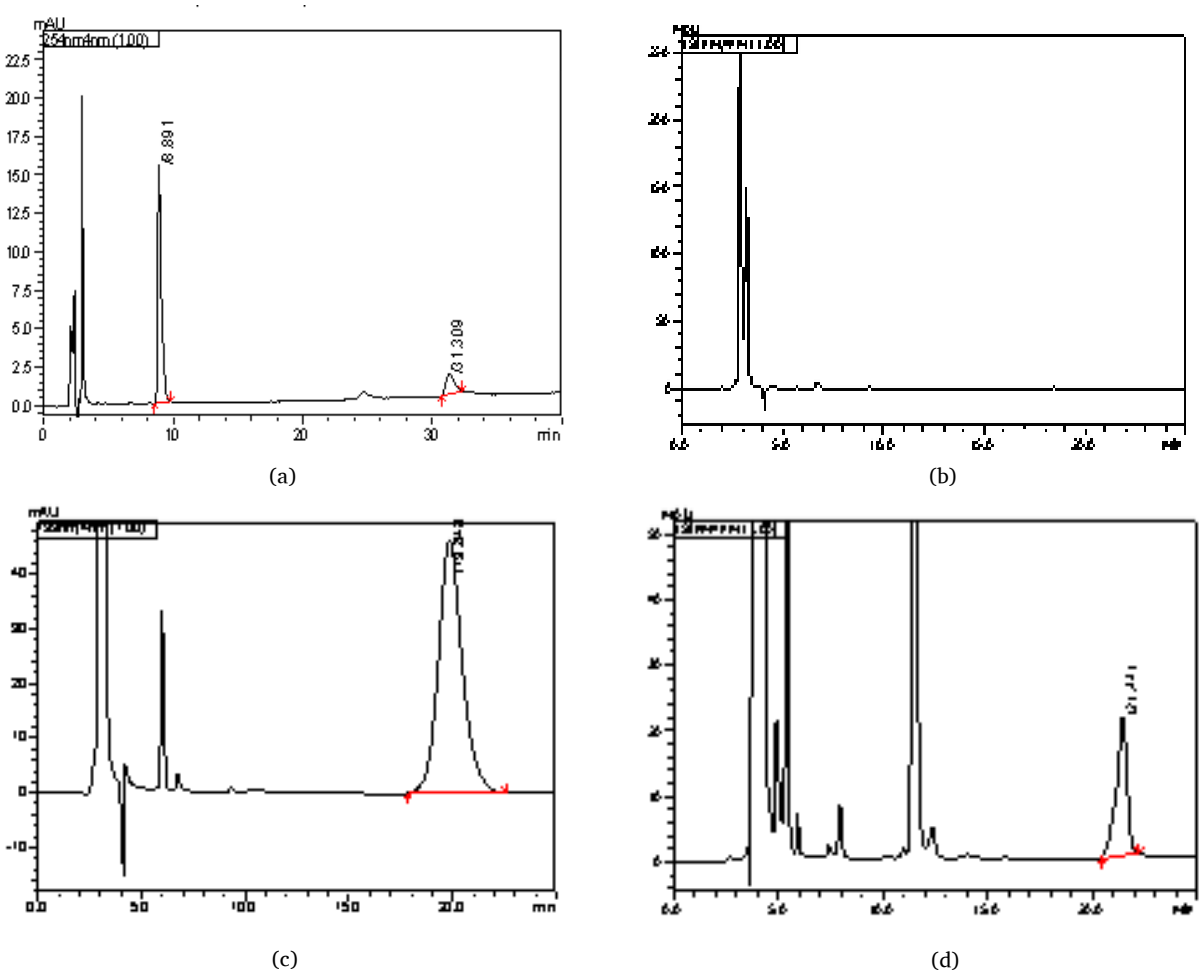


Fig. 2. Chromatogram of (a) sodium benzoate, caffeine and saccharine at 30.38, 8.72 and 3.72 min, respectively in CB_2 (b) acesulfame-K at 5.09 min in CB_6 (c) aspartame at 17.64 min in CB_{13} and (d) aspartame at 17.64 min in CB_{14}

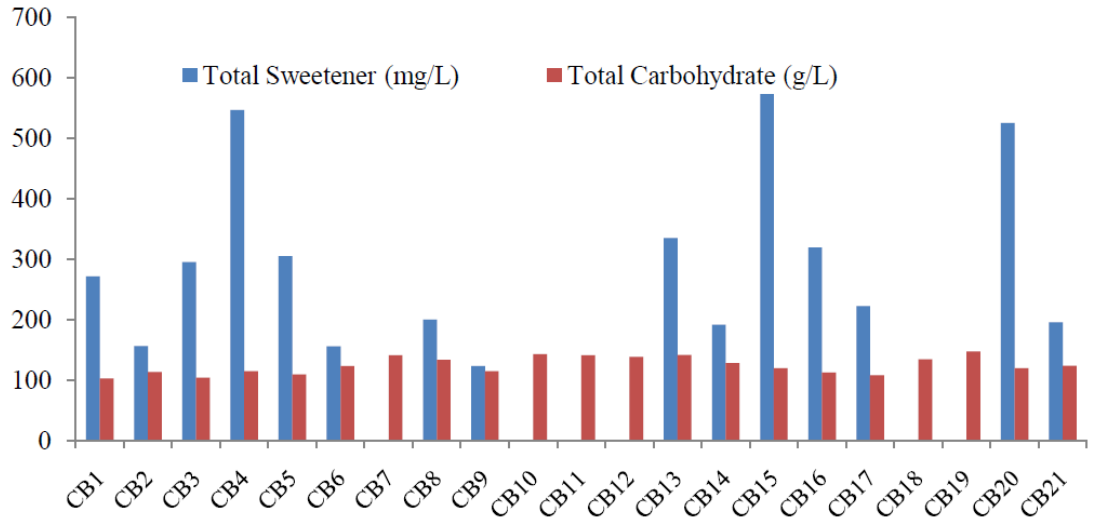


Fig. 3. Comparison between total carbohydrate and total sweetener

3. Results and discussion

Two HPLC methods with photodiode array detection have been developed for the quantitative determination of saccharin, caffeine, sodium benzoate, acesulfame-K, and aspartame in 21 different commercial carbonated beverages (CB) available in Bangladesh. These methods provided stable retention time and validated in terms of sensitivity, linearity ranges reproducibility, analytical recoveries and robustness. Some chromatograms of samples are shown in **Fig. 2**.

From this study we obtained the amount of sodium benzoate content was in the ranges of ND-217.60 mg/L, bythe highest amount of sodium benzoate was observed in CB_4 drinks (217.60 mg/L) and the lowest amount that is not detectable was CB_7, CB_9 and CB_{11}. All the drinks are in the safe level of consumption in the context of sodium benzoate according the FAO/WHO Expert Committee on Food Additives (JECFA) which the limit of acceptable level is 300 mg/L (Vivek et al., 2015). Khosrokhavar R et al. reported that sodium benzoate in different carbonated beverages was in the range of 3.90 to 164 mg/L (Khosrokhavar et al., 2010), whereas Lakshmi Narayanan Venu reported that sodium benzoate in CB_5 and CB_{12} as 172.35 and 396.44 mg/L, respectively (Venu, 2019). However, in our studies it was found as 139.05 and 139.64 mg/L, respectively.

Caffeine, another additive used in most of the analysed samples as stimulant was found in the ranges of ND-426.36 mg/L. The maximum limit of caffeine in soft drinks is 200 mg/L set by the US food and Drug Administration (FDA, 1983). Only CB_{21} contained excess amount of caffeine (462.36 mg/L) among the analysed drinks. Other samples that contain more than 150 mg/L of caffeine are CB_4 and CB_8. On the other hand, caffeine was not in detectable ranges of CB_1, CB_3, CB_5, CB_{12}, CB_{13}, CB_{14}, CB_{16}, CB_{17}, CB_{18} and CB_{19} drinks. Shatha Hammad et al. found the caffeine content in CB_{21}, CB_{20}, CB_2 and CB_{10}as 101.50, 141.30, 124.10, 162.40 and 314.20 mg/L, respectively (Hammad et al., 2015). These values are very close to our study in most case (Table 4). Mei Musa Ali et al. reported that CB_{21} and CB_9 contained caffeine as 102.8 and 106.2 mg/L, respectively (Ali et al., 2012), yet it was as 462.36 and 111.99 mg/L, respectively in our study. Thus, the caffeine content in commercial carbonated beverages varies globally according to the types of the brand. Khalid A et al. calculate the amount of caffeine in different beverages as 61.72-210.85 mg/L (Khalid et al., 2016). From the Table 4, it is clear that about 50 percent of the sample is in between this range and remaining 50 percent is in below detection limit.

Artificial sweeteners such as saccharine, acesulfame-K, and aspartame are found in the ranges of ND-377.60, ND-48.09, and ND-295.20 mg/L, respectively. The Codex GSFA (General Standard for Food Additives) limits the permissible level of saccharine as artificial sweetener in carbonated beverages as 300 mg/L which is adopted in 2008. About 77 percent of the total analysed sample has saccharine content below the detection level (Table 4). Saccharine present in excess level in CB_4 (315.10 mg/L), CB_{13} (335.08 mg/L), CB_{15} (377.60 mg/L) and CB_{20} (341.99 mg/L) are not very high compared to the standard values. The maximum tolerable limit of acesulfame-K and aspartame are in the level of 600 and 750 mg/L, respectively, set by the Codex Alimentarius Commission. About 81 percent of the analysed samples are in below detection limit for acesulfame-K, whereas about 19 percent samples contain it but they are also in the below of standard value. On the other hand, aspartame present in about 62 percent sample but they are also in the below of standard level. Thus, all the samples are in the below of maximum limit of set by authority in the context of acesulfame-K and aspartame contains. This is because of their high sweetening power compare to natural sugar or sucrose. Mircea Oroian reported that acesulfame K, aspartame and saccharine occurrence in carbonated beverages as mean value 28.87, 509.91 and 9.72 mg/L, respectively, and the ranges are 0-268.51, 41.94-881.98, and 0-83.75 mg/L, respectively, from the Romanian market (Oroian et al., 2013). In many of the analysed samples, the results showed non-detectable (ND) which indicate that either the targeted substances were not used in the beverages or it might be below the sensitivity level of HPLC detector due to the presence of very minute level.

From the analysis, it is shown that all samples contained total carbohydrate greater than 100 g/L of the drinks. The recoveries were found to be 95.88-97.10, 96.53-97.20,94.53-96.05, 91.15-93.31 and 92.88-94.13% for saccharine, caffeine, sodium benzoate, acesulfame-K and aspartame in carbonated beverages, respectively which were in the range 80-120% and acceptable for carbonated beverages samples according to standard methodology.

To elucidate the sensitivity of experimental methods, LODs and LOQs were determined. LODs (S/N ratio, 3:1) were found to be 30.06, 15.46, 31.61, 0.33, and 0.83 mg/L for saccharine, caffeine, sodium benzoate, acesulfame-K and aspartame, respectively, whereas LOQs were 100.18, 51.54,105.37, 1.11, and 2.77 mg/L of saccharine, caffeine, sodium benzoate, acesulfame-K, and aspartame, respectively. A comparison was made between total artificial sweeteners and total carbohydrate in Fig. 3 which indicates that those samples contained larger amount of total carbohydrate they contained lower amount of total sweetener. Photodiode array detector can choose the most suitable wavelength

of analytes and HPLC-PDA is one of the best chromatographic techniques for the systematic analysis of analytes like artificial sweeteners, stimulants, and preservatives in different samples, such as beverages due to its sensitivity and higher separation efficiency and easy quantification.

Table 3. LOD, LOQ and results of recovery experiments

Method	Standards	Spiked level (mg/L)	Concentration found (mg/L)	Recovery (%)	Relative standard deviation (RSD) (%)	LOD (mg/L)	LOQ (mg/L)
Method 1	Sodium Benzoate	100	96.05	96.05	0.65	31.61	105.37
		200	189.06	94.53			
		300	285.51	95.17			
	Caffeine	50	48.60	97.20	0.28	15.46	51.54
		100	96.81	96.81			
		150	144.45	96.53			
	Saccharine	100	96.70	96.70	0.53	30.06	100.18
		200	194.20	97.10			
		300	287.64	95.88			
Method 2	Acesulfame-K	1	0.91	91.15	1.02	0.33	1.11
		5	4.67	93.31			
		10	9.29	92.93			
	Aspartame	1	0.94	93.53	0.55	0.83	2.77
		5	4.64	92.88			
		10	9.41	94.13			

Table 4. Identification and quantification of sodium benzoate, caffeine, saccharine, acesulfame-K and aspartame in carbonated beverages samples

Sample Code	Sodium Benzoate (mg/L)	Caffeine (mg/L)	Saccharine (mg/L)	Acesulfame-K (mg/L)	Aspartame(mg/L)	Total Carbohydrate (g/L)
CB_1	168.50	-	-	-	271.63	102.81
CB_2	176.30	32.30	-	-	156.35	113.20
CB_3	80.03	-	-	-	295.20	104.12
CB_4	217.60	180.30	315.10	-	231.42	114.66
CB_5	139.05	-	-	26.43	278.55	109.53
CB_6	148.57	123.83	-	-	156.01	123.31
CB_7	-	83.88	-	-	-	141.23
CB_8	182.69	163.14	200.14	-	-	133.34
CB_9	-	111.99	-	-	123.27	114.72
CB_{10}	144.42	128.18	-	-	-	143.12
CB_{11}	-	12.37	-	-	-	141.20
CB_{12}	139.65	-	-	-	-	138.25
CB_{13}	199.05	-	335.08	-	-	141.31
CB_{14}	141.66	-	-	30.23	161.40	128.13
CB_{15}	139.61	97.19	377.60	-	195.53	119.73
CB_{16}	125.72	-	-	48.09	271.30	112.55
CB_{17}	168.67	-	-	38.76	183.68	108.12
CB_{18}	180.60	-	-	-	-	134.34
CB_{19}	214.39	-	-	-	-	147.16
CB_{20}	204.35	143.00	341.99	-	183.32	119.32
CB_{21}	-	462.36	-	-	195.43	123.92

Note: "-" Mark indicates amounts are not detectable (ND) level

4. Conclusions

From this study it is obvious that the methods are suitable, simple, precise, sensitive, and accurate, and allowed for obtaining good results at proficiency test for the simultaneous determination of sodium benzoate, caffeine, saccharine, acesulfame-K, and aspartame using HPLC coupled with photodiode array detector. The mean recoveries were in the range of 80-120% according to standard methodology. All the carbonated beverages analysed by this study might be in the safe level of consumption in the context of sodium benzoate, caffeine, acesulfame-K, and aspartame, except CB_{21} which can contain caffeine in excess level (462.36 mg/L). Saccharine presents in little bit excess level in CB_4, CB_{13}, CB_{15} and CB_{20} drinks. Another observation is that those samples that contain more amount of total carbohydrate were containing lower amount of total sweetener. Since the average consumption of carbonated beverages increases in Bangladesh, continuous monitoring is required to maintain the standard of food safety by proper regulatory agency.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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