Quantitative Analysis of artificial sweeteners in soft drink samples

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by:

Yashna Tahjib Meghla

Student Id: 13136004

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Biotechnology Program

Department of Mathematics and Natural Sciences

BRAC University

DECLARATION

I declare that the thesis work titled “Quantitative analysis of artificial sweeteners in soft drinks.” has been written and submitted by me, Yashna Tahjib Meghla, without the use of other sources than those mentioned. It is further asserted that this Bachelor’s Thesis has never been submitted in the same or substantially similar version to any other examinations office. All explanations that have been adopted literally or analogously are marked as such. Any reference to work done by any other person or institution or any material obtained from other sources has been duly cited and referenced.

(Yashna Tahjib Meghla)

Candidate

CERTIFIED BY

Dr. Mahboob Hossain

Supervisor

Professor

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University, Dhaka.

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Abstract

Carbonated drinks are the biggest soft drinks sector around the globe and have grown significantly over the last few decades. It has become part of the urban lifestyle and is consumed by people regardless of age, religion, gender, race or culture. With this increasing popularity came the use of synthetic sweeteners, also known as Non-nutritive sweeteners (NAS), which are low caloric substances used to replace sugar or corn syrup and other caloric ones. Higher concentration of some of these sweeteners leads to various side-effects such as physical weakness, poor night vision, insomnia, mental depression, anxiety, feeling aggressive, weight gain and so on. Hence, the detection of these sweeteners is important to ensure consistency in product quality. In this study, a simple, selective, precise and low-cost procedure using the spectrophotometric method was performed to determine the concentration of three artificial (aspartame, saccharin, cyclamate) and one natural sweetener (sucrose) in eight brands of regularly consumed soft drinks. For this purpose, 12 samples from each brand were analyzed, collected from different supermarkets in Dhaka. Artificial sweeteners were found in all analyzed products. Mean concentration of aspartame, saccharine, cyclamate and sucrose were found to lie in the range between 11-104, 1-9, 45-165 and 1800-2000 µg/ml, respectively.

The correlation coefficient of the calibration curve was better than 0.99 (n> 6). The method can be validated with respect to sensitivity, linearity range, reproducibility, repeatability, recovery, and robustness, except for saccharine which showed higher %RSD.

Table of contents

List of figures

List of Tables

Chapter: ONE

Introduction

Artificial sweeteners are non-caloric food additives which enhance the sweetness of food without the same calories as sugar. As a result, they are also known as non-nutritive sweeteners (NAS). They are many times sweeter than sugar, almost 30 to 8000 times. Thus they have fewer calories than table sugar (Chang and Yeh, 2014). Being cheaper and beneficial to people having problems with sugar metabolism, their consumption has amplified significantly over the last few decades. Health conscious people rely on the use of synthetic sugars as they have higher intense sweetness per gram than natural, caloric sugars which include sucrose, corn syrups or fruit concentrates (Tandel, 2011). Many consumers, obese or lean, are inclined to use NAS based foods, drinks or finished products containing these man-made sugars (Johnson et al., 2009). Looking at our current food supply, it is very much visible. NAS is being used in thousands of food products like soft drinks, chocolates, sweets, desserts, yogurts, gums etc.

With the increase in popularity of the use of artificial sweeteners, controversies have also arisen related to the ill effects of its consumption in adults and young children. They have been considered as non-toxic, but different research made recently proved their oncogenic potential, leading to bladder cancer by mechanisms that have not been cleared up, which led to a restraint of their usage (James, Heywood and Crook, 1981). Their usage has been repeatedly associated with obesity and obesity-related diseases which include diabetes, cardiovascular diseases, hypertension, a metabolic syndrome which include increased blood cholesterol level (Johnson et al., 2009). Hence, people have mixed feeling when using artificial sweeteners today. Although several studies have been made and are being researched thoroughly, yet one of them cleared the fear of the onsets of their side-effects. Most of them, even the ones published in reliable medical publications lack the strong scientific background and did not maintain statistical analysis and in consequence, were abandoned.

Despite all the controversy related to artificial sugars and products containing them, one of the most popular and widely consumed is a soft drink, also known as soda in some countries. These

carbonated drinks are inexpensive, refreshing, convenient, a drink with “feel good” factor, tasting good and easily accessible to all people in the world. They are non-alcoholic water-based flavored drinks that are optionally sweetened, acidulated and carbonated (Abdullah and Asngari, 2011). Some carbonated soft drinks also contain caffeine; mainly the brown-colored cola drinks. It has become a regular part of the diet of urban people, regardless of the age group.

After independence, the food habit of Bangladeshi people has been changed a lot. Besides our traditional food, consumers of Bangladesh developed an inclination towards western food. Different foreign food companies were established in Bangladesh. The beverage industry is one of them. Carbonated beverage entered our market in the latter part of 1980. At that time there were a few companies in Bangladesh. But by the change of time and western culture influences, it has become very popular in Bangladesh. By the year 2000, more than 12 Beverage company operating business in Bangladesh and most of them are foreign companies (Islam and Fatema, 2014). Interestingly we don’t know that beverage is also our cultural food because beverage doesn’t mean only carbonated drinks. Yoghurt, soup, and lacchi are also beverage of our own tradition which has been consumed for the last 100 years in Bangladesh.

With the advancement of food technology, food additives have been widely used in the beverage productions. Food manufacturers often use a blend of NAS to improve the flavor acceptability of NAS. Hence food additives are utilized in the food industry in order to maintain quality, texture, consistency, appearance, taste, alkalinity and acidity of food products. Thus, the large-scale production of good wholesome food and beverage at economical prices can be made possible with the use of food additives (Brown, 2014). In the case of sweetener blends manufacturing, the control of sweeteners proportion in blends is essential to precisely reproduce the texture, sweetness profile and also reduce the adverse health effects (Schiffman et al., 2007).

In spite of all the claims cited over the past few decades, studies have suggested alternately that sugar-substitutes may be 'potentially helpful,' 'potentially harmful,' or have 'unclear effects' with regard to human health. The negative effects associated with the regular consumption of artificial sweeteners is yet unidentified but some of the side-effects reported repeatedly by consumers remained constant. Thus, an acceptable daily intake (ADI) has been established by Food and Drug Administrative (FDA) which is considered safe. However, some sweeteners like cyclamate

are banned by FDA in the USA while its consumption is considered safe within ADI level of 11 mg/kg body weight by JECFA[1] and at 7 mg/kg body weight by the SCF.[2]

Table1 below enlists some of the commonly used artificial sweeteners. [8, 9]

Table: 1 Artificial sweetener

Sweetener	Common Brand name	ADI*/JECFA toxicology monograph no. (Year)	Year FDA approved	Representative amount of sweetener in 350 ml soda, ĭ mg	Potency (Times sweeter than sucrose)
Aspartame	Equal NutraSweet	40mg/kg bw 15(1980)	1981	187	160-200
Saccharine	Sweet'N Low	5mg/kg bw 32 (1993)	Before 1958	8 (blended with aspartame)	300
Sucralose	Splenda	15mg/kg bw 28 (1991)	1999	68	600
Acesulfume -K	Sweet One	15mg/kg bw28 (1991)	1988	40(blended with aspartame)	200
Neotame	Neotame	2mg/kg bw 52 (2004)	2002	Not in carbonated beverages	8000
Cyclamate	--	--	Banned in 1969	--	30

*ADI or Acceptable Daily Intake is a measure of the amount of specific substance in food or drink that is considered safe to consume each day over the course of a person's lifetime, without any appreciable health implications. Measurement is usually expressed in milligrams of sweetener per kilogram of body weight (mg/kg bw)

ĭ The amount of sweetener if the product is exclusively sweetened with one sweetener. Very frequently it is >1 sweetener used in soda or other products. Usually this information is proprietary and is not available to public, the exception being saccharin

Source: FAO Nutrition Meetings Report Series, WHO/Food Additives; http://www.inchem.org/pages/jecfa.html

Since the beginning of the discovery of artificial sweeteners, several analytical methods have been applied to quantify the amount being used in different samples accurately. Although there are many techniques available for the estimation, including high-performance liquid chromatography (HPLC), Fourier-transform infrared spectroscopy (FTIR) technique, capillary electrophoresis, reverse-phase chromatography, ion exchange chromatography etc. being the latest technique. The present work will focus on the estimation of three artificial sweeteners

[1] *The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is an international expert scientific committee that is administered jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO).*

[2] *Scientific Committee on Food*

(aspartame, saccharine and cyclamate) and one natural sugar (sucrose) which are commonly used in soft drinks available in the markets of Bangladesh. UV-Vis spectrophotometry has been used for the assessment of these sugars in eight different brands of soft drinks. This method has the advantages of significant accuracy, precision, low cost, easy handling and most importantly, its availability in most laboratories. However, there are some implications for the analysis of different real samples which are limited to poor sensitivity and selectivity. The combination of spectrophotometry with some microextraction procedure, using chemicals for maximum extraction of sugars from samples and keeping other parameters constant, depending on the sugar being analyzed, to overcome these problems. Different reference papers were used as a guideline for the analysis procedure.

1.1The Market

Sugar-sweetened beverages (SSBs) include all non-diet soft drinks, fruit drinks, sports drinks, energy drinks, low-calorie drinks and other beverages that contain added caloric sweeteners, such as sweetened tea, rice drinks, bean beverages, sugar cane beverages, and non-alcoholic wines/malt beverages (Green, 2016).

Globally, carbonated soft drinks are third most consumed beverages. Thirsty people take beverage for refreshment and entertainment. Because of hot weather, at present Bangladesh has a very competitive market of beverage, even though soft drinks entered the market in the 1980s. At that time two or three soft drinks companies were available in the market. Now lots of soft drinks companies (both domestic and global) are operating in Bangladesh though some of them are very popular *(Islam and Fatema, 2014*). The mix of consumers in soft drinks market encompasses with 79% urban consumers and 21% semi-urban consumers. And the demand for soft drinks according to areas and age in semi-urban areas is 65% of total consumers and in urban areas, it is 100%. About 58% of total consumers are the age of below 15 years, 15% of them are 15 to 25 years, whereas 5% of them are 26 to 35 years and 13% of them are the age of above 35 years. So the age of below 15 years is the most targeted segment of people in the market, covering 64% of total demand of soft drinks, then the age of 15 to 25 years, 26 to 35 years, and above 35 years

cover 16.5%, 5%, and 14% of the total demand for soft drinks respectively. Then it is a noticing factor that 91% of consumers take the soft drinks and 9% doesn't (Islam and Farha, 2014).

1.2 Aim and objectives:

Interest in the analysis of soft drinks arises for the following reasons:

- The analysis of the sugar content in soft drinks using spectrophotometer or any other methodology has not been undertaken before. Any literature review which has been carried out by local researcher in this regard was not obtained, to my best knowledge.
- To estimate quantitatively the level of different widely used sugars in each sample of these soft drinks available in the market.
- From a nutritional point of view, consumption of SSB's has been linked to several adverse health effects. An ADI level has been established for each variety of artificial sugar and this work aims to find out if it is being maintained.
- This work also aims to advise the public about their intake. Reports on the level of these substances in soft drinks are scanty. Moreover, new drinks are being released into the market daily and the need for continuous monitoring of these substances in energy drinks is a necessity.

This study aimed to determine the level of cyclamate, aspartame, saccharine and sucrose in soft drinks.

1.3. History of soft drinks:

Soft drinks can trace their history back to the mineral water found in natural springs. The first marketed soft drinks (non-carbonated) appeared in the 17th century. They were made from water and lemon juice sweetened with honey (Pietka and Korab, 2017). In 1767, the first drinkable man-made glass of carbonated water was created by Englishmen Doctor Joseph Priestley. Three years later, Swedish chemist Torbern Bergman invented a generating apparatus that made

carbonated water from chalk by the use of sulfuric acid. These first carbonated waters were meant to imitate the mineral waters found in nature.

In 1810, the first United States patent was issued for the "means of mass manufacture of imitation mineral waters" to Simons and Rundell of Charleston, South Carolina. However, carbonated beverages did not achieve great popularity in America until 1832, when John Mathews invented his apparatus for the making carbonated water (Tchudi, 1986). The American pharmacists selling mineral waters, which were believed to be a healthy practice, began to add medicinal and flavorful herbs to unflavored mineral water. They used birch bark, dandelion, sarsaparilla, and fruit extracts. Some historians consider that the first flavored carbonated soft drink was that made in 1807 by Doctor Philip Syng Physick of Philadelphia (Pendergrast, 1993). The customers soon wanted to take their "health" drinks home with them and a soft drink bottling industry grew from consumer demand.

By about 1820, improvements in manufacturing processes allowed a much greater output, and bottled water became popular. Mineral salts and flavors were added—ginger about 1820, lemon in the 1830s, tonic in 1858. In 1886 John Pemberton, a pharmacist in Atlanta, Georgia, invented Coca-Cola, the first cola drink (Pietka et al., 2017)

1.4 Artificial Sweeteners

The fondness of humans for sweet foods is congenital: studies have proved a preference for sweet-tasting nutrition in newborns. Therefore, mankind has always added sweet substances to their food. The first recorded sweetener was honey, which was used in the ancient cultures of Greece and China (Maone et al., 1990). Honey was later replaced by saccharose, the common sugar, which was originally obtained from sugar cane (Bright, 1999).

Artificial high-intensity sweeteners form an important class of food additives which are commonly used in the food, beverage, confectionery and pharmaceutical industry. They provide the sensation of sweetness but a common trend is to use sweetener blends because some disseminate after tastes which is not desirable. As a result, the food and beverage industry uses a blend to overcome this. A common example of such a mixture is saccharine: cyclamate (1:10)

ratio. The bitter taste of saccharine is masked by cyclamate and the unpleasant aftertaste of cyclamate is cloaked by saccharine (Mitchell, 2006).

Sugar substitutes or NAS are categorized into three: synthetic, semi-synthetic and natural. The majority of sugar substitutes which are allowed in the food industry are synthetic, also referred to as artificial sweetener (U. S. Food and Drug Administration, 2014). As the amount of artificial sweeteners needed for inducing the same level of sweetness is only a fraction, when compared to using sugar or corn syrups, their demand in the food industry has reached new heights over the last few decades.

The basic characteristics, structures, molar mass, applications and other adverse effects or health hazards of Aspartame, Cyclamate, Saccharine, and Sucrose will be discussed with individual compounds.

1.4.1 Saccharin

1.4.1 (a) Structure:

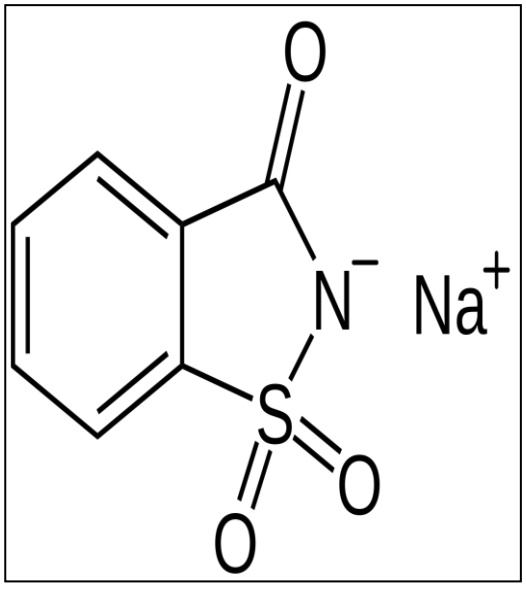


Figure: 1 Structure of saccharin

Source: Pubchem

- ✓ **IUPAC name:** 1,1-dioxo-1,2-benzothiazol-3-one
- ✓ **Full name:** Sodium Saccharine
- ✓ **Acronym:** SAC
- ✓ **Brand name:** Sweet and Low®, Sweet Twin®, Sweet'N Low®, and Necta Sweet®
- ✓ **Chemical formula:** $C_7H_5NO_3S$
- ✓ **Molar Mass:** 205.16

1.4.1 (b) Basic characteristics and applications

Saccharin is the oldest chemical sugar substitute and the best researched of all sweeteners. It was first discovered in 1879 by Constantin Fahlberg in Ira Remsen's laboratory at the Johns Hopkins University. It is 200 to 700 times sweeter than table sugar (sucrose), and it does not contain any calories. It is currently approved safe for consumption by the FDA and to be used in beverages, fruit juice drinks, and bases or mixes in accordance with directions, as a sugar substitute for cooking or table use, and in processed foods (FDA). It is an odorless white crystal and has high solubility and stability. It has a bitter metallic aftertaste (PubChem).

1.4.1 (c) Probable Health hazards:

Since the discovery of saccharine, it has been subjected to various controversies regarding the safety of the compound. According to various scientific research, the artificial sweetener saccharin has been regarded to exhibit potential toxicity in the bodies of test animals as well as humans. In 1978, an animal study on has shown that saccharin causes cancer in the urinary bladder of rats and mice. Male rats were found to be more susceptible to urinary bladder carcinogenesis than female rats. This rose concerns about the consumption of saccharin and health (Reubar, 1978).

A more recent study published in Nature in 2014 found that saccharin along with other artificial sweeteners can enhance glucose intolerance by affecting the gut micro-organisms (Suez et al., 2014). Increased glucose intolerance can result in a pre-diabetic condition known as hyperglycemia, where the blood sugar level is above the normal range, but not high enough to be classified as diabetes. Hyperglycemia is associated with insulin resistance, stroke, heart disease, and mortality risk (Mayo Clinic, 2015).

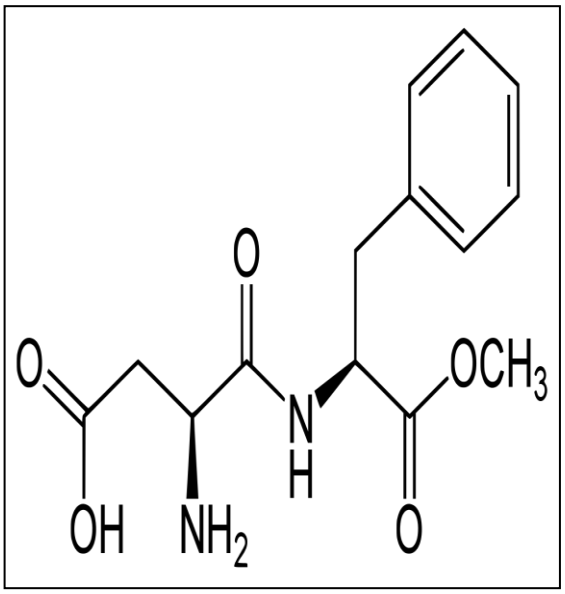
Another study published in Elsevier in 2017 suggested that saccharin promotes liver inflammation (Bian et al., 2017). An additional animal study showed that saccharin use can lead to weight gain, obesity, and increased appetite. However, most of these studies result have not

been duplicated in large, human studies. It still remains an important issue concerned with the consumption of saccharin (Ellwein and Cohen, 2008).

Since saccharin is a synthetic compound, it may induce allergic reactions in some people in high dosage with some side-effects like loss of breath, headache, and diarrhea etc.

1.4.2 Aspartame:

1.4.2(a) Structure:



- ✓ **IUPAC name:** N-L-α-aspartyl-L-phenylalanine-1- methyl ester
- ✓ **Full name:** Aspartame
- ✓ **Acronym:** ASP
- ✓ **Brand name:** Nutrasweet®, Equal®, and Sugar Twin®.
- ✓ **Chemical formula:** $C_{14}H_{18}N_2O_5$
- ✓ **Molar Mass:** 294.3

Figure: 2 Structure of Aspartame

Souce: PubChem

1.4.2 (b) Basic characteristics and applications

Aspartame was discovered in 1965 by James M. Schlatter, a chemist working for G.D. Searle & Company. It has a caloric value of 17kJ per gram, yet used as a non-nutritive sweetener. This is because it is 200 times sweeter than table sugar, so a very small amount is required to produce the same level of sweetness. It was approved by the US FDA in1981as a tabletop sweetener, in chewing gum, cold breakfast cereals, beverages, instant coffee and tea, gelatins, puddings, and

fillings, and dairy products and toppings. In 1983, FDA approved the use of aspartame in carbonated beverages and carbonated beverage syrup bases, and in 1996, FDA approved it for use as a "general purpose sweetener" (U. S. Food and Drug Administration, 2014). It is a white, odorless crystalline powder and is relatively stable in its dry form, it undergoes pH and temperature changes and this makes it undesirable as a baking sweetening agent (PubChem). It is permitted in more than 90 countries.

1.4.2 (c) Probable Health hazards:

Unlike other artificial sweeteners which are non-nutritive, aspartame is classified as nutritive. It is a low caloric sweetener. Although aspartame has been deemed safe for human consumption, a 2017 study suggested that its consumption can enhance weight gain rather than weight loss and can wane blood glucose tolerance (Choudhary, 2017).

Another study has reported plausible behavioral and cognitive problems, including depression, mood swings, headaches, anxiety, and insomnia (Choudhary and Lee, 2017). Other researchers have put forward that aspartame contributes to hunger or increases appetite psychologically (Yang, 2010).

Aspartame contains phenylalanine, a substance that people with phenylketonuria (PKU), can't metabolize. Products containing aspartame are required to indicate a warning for people with PKU on their labels. People with PKU have too much phenylalanine in their blood. People with this condition aren't able to properly process phenylalanine as they lack phenylalanine hydroxylase enzyme (Celik et al., 2014).

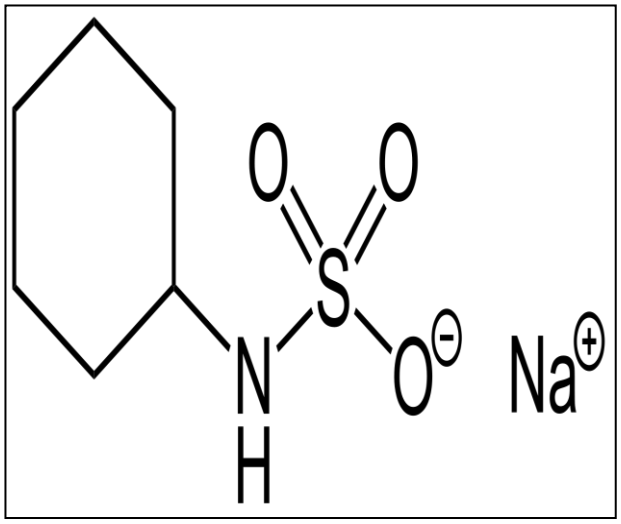
Exposure to aspartame have shown to cause headaches, blurred vision, brain tumors, eye problems, numbness, insomnia, memory loss, nausea, loss of energy, slurred speech, hearing problems, hyperactivity, and others (Choudhary and Lee, 2017).

1.4.3 Cyclamate:

Cyclamate is available in three different forms: sodium cyclamate, calcium cyclamate or cyclamic acid. The hot and cold stability and the long shelf life of Sodium and calcium cyclamate make them suitable for the manufacture of beverages and other foods. Among all the

available NAS, cyclamate has the least sweetening power and is least expensive which enhances its desirability. In addition, cyclamate has been reported to leave a weird aftertaste.

1.5.3 (a) Structure:



- ✓ **IUPAC name:** Sodium N-cyclohexylsulfamate
- ✓ **Full name:** Sodium Cyclamate
- ✓ **Acronym:** CYC
- ✓ **Chemical formula:** $C_6H_{13}NO_3S$
- ✓ **Molar Mass:** 201.2

Figure: 3 Structure of cyclamate

Source: PubChem

1.4.3 (b) Basic characteristics and applications

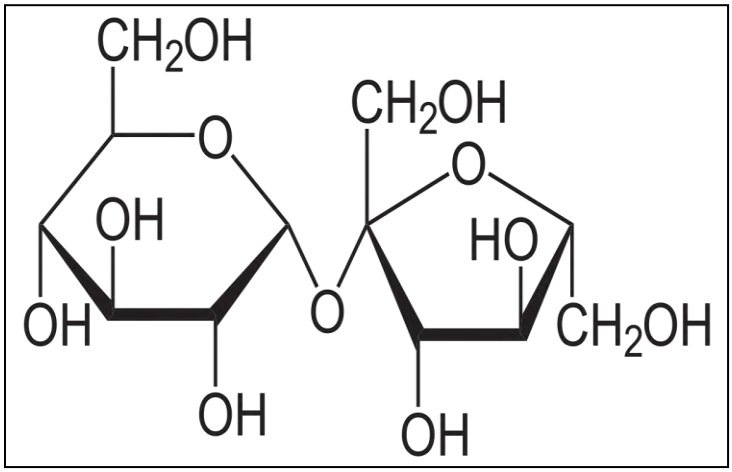
Cyclamate is the second oldest artificial sweetener in use today. It was discovered in 1937 about 50 years after saccharin by a graduate student named Michael Sveda at University of Illinois. It is almost 30 times sweeter than table sugar. It is a white, odorless crystal and often prepared as salts of Na or Ca (PubChem). It has a bitter taste but tastes better when used with sacchari (Mitchell, 2006). It is stable over a wide range of temperature and pH. It is banned in several countries, including in USA due to suspicions of toxicity and carcinogenic activity. It was approved by FDA in 1958 but removed in 1969 and been in that state since. It is approved safe by EU at levels of 250-1600 ppm in food product (PubChem). It is one of the cheapest artificial sweeteners and often mixed with saccharin and sold as a generic sweetener. Sucaryl is a common brand.

1.4.3 (c) Probable Health hazards:

Cyclamate has been subjected to several controversies associated with health implications. A report in 1970 stated increased cases of bladder tumor in rats when sodium cyclamate was used with sodium saccharine in 10:1 ratio for up to 105 weeks (Price et al., 1970). Cyclamate is converted to cyclohexylamine (CHA), a toxic metabolite, by the bacterial flora of the gastrointestinal tract. It has been reported that CHA is harmful to the male reproductive organ in rats. Testicular atrophy, a condition where the size of the testicle is reduced, was reported in rats (Gaunt et al., 1974) and dogs (James, Heywood and Crook, 1981). When tested on monkeys, the side effect of high dosage of cyclamate consumption might have on the health found out that the test monkeys developed different types of cancer more than the placebo group (Takayama et al., 1999). However, it has also been established that there is a large inter-and intra-individual variation in conversion rates carried by the gut microflora (*Roberts and Renwick, 1989*). In humans, as well, some peoples are recognized as non-converters and some as high converters. This makes the exclusive conclusion of the negative health impact of cyclamate consumption in humans to remain unresolved. As a result, usage of cyclamate has been banned FDA while approved in more than 55 countries.

1.4.4 Sucrose:

1.4.4 (a) Structure:



- **Full name:** Sucrose
- **Brand name:**
- **Chemical formula:** $C_{12}H_{22}O_{11}$
- **Molar Mass:**342.3

Figure:04 Structure of sucrose

Source: PubChem

IUPAC name:

(2R,3R,4S,5S,6R)-2-[(2S,3S,4S,5R)-3,4-dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol

1.4.4 (b) Basic characteristics and applications

Sucrose is a white odorless crystalline solid. It is a naturally occurring sugar, obtained commercially from sugarcane, sugar beet (beta vulgaris), and other plants and used extensively as a sweetener. Sucrose is derived by crushing and extraction of sugarcane (Saccharum officinarum) with water or extraction of the sugar beet (Beta vulgaris) with water, evaporating, and purifying with lime, carbon, and various liquids. Sucrose is also obtainable from sorghum. It is a non-reducing disaccharide composed of glucose and fructose. Sucrose is used as a sweetener in foods and soft drinks, in the manufacture of syrups, in invert sugar, confectionery, preserves and jams, demulcent, pharmaceutical products, and caramel. It is a caloric sweetener[26].

1.4.4 (c) Probable Health Hazards

Structurally, the sucrose is a disaccharide constituted by the union of the monosaccharides glucose and fructose. So in humans, sucrose is broken down into its constituent monosaccharides, which has been linked to serious health risks. The commercial sugar is commonly available in a refined form (containing, at least, 98% of sucrose) or in crystals (containing, at least, 99% of sucrose).

A study published in the Iranian Journal of Public Health has linked the increased consumption of sucrose to heavier liver and kidneys in male rats, over a course of 14 days (Mohammadiha, 1974). "Obesity epidemic" has seem to develop significantly in the population across the globe, irrespective of the country, over the last few decades with the increased consumption of dietary fructose from high intake of sucrose and high fructose corn syrup, a common sweetener used in the food industry (Basciano et al., 2005).

Among other ill effects of high sucrose consumption include weight gain as they are simple in structure and are readily broken down in body, the excess of which is stored as fat. In addition to it, sucrose is left to the teeth provide a suitable environment for bacteria to grow which eventually leads to tooth decay and tooth loss.

Chapter: TWO

Materials and Methods

2.1 Place of study:

The experiment was carried out in the organic synthesis lab of Analytical pharmacy research laboratory at The Department of Pharmaceutical Chemistry, Dhaka University.

2.2 Sample collection:

A total of 48 soft drink samples of eight different brands (Coke, Pepsi, Mojo, Clemon, Sprite, Mirinda, Mountain Dew and Lemu) were collected from different super shops located in Dhaka. For each brand, six samples were assembled. Two samples of three different batch numbers were collected for every brand. The samples were stored at room temperature and opened before for analysis.

Table:2 Samples and the corresponding batch numbers (Mentioned code numbers were used during the analysis)

Brand name	**Code**	**Batch 1**	**Batch 2**	**Batch 3**
Coke	Co	69	51	86
Pepsi	Pep	132	145	129
Mojo	Mo	157M517	194M517	160M517
Sprite	SP	618	149	151
Mirinda	Mir	148	166	250
Mountain Dew	MD	132	133	129
Clemon	Cl	147C517	155C517	178C517
Lemu	L	009L17	008L17	006L17

2.3. ANALYSIS OF SUCROSE

2.3.1(a) Chemicals and standards

All chemicals were of analytical grade. Sodium hydroxide was purchased from Merck (Worli, Mumbai), hydrochloric acid from Merck (Darmstadt, Germany) and 3,5-dinitrosalicyclic acid (dNSA) from Merck (Darmstadt, Germany). Double distilled water was used throughout the analysis. The solutions were prepared fresh at the beginning of each week. All the test tubes were washed with detergents, dried in an oven and cleaned with acetone.

2.3.1(b) Equipment:

A UV-Vis Spectrophotometer model EMC-61PC-UV (EMC Lab instruments, Germany)with 400µl quartz microcell was used for the spectrophotometric analysis of the sample. A 40 kHz ultrasonic water bath model SHWB-30(Human Lab Instrument Co., Korea) was used for degassing, water bath (Biobase, USA) was used for heating. A vortex mixture, SCILOGEX MX-S was used for better mixing of reagents.

2.3.2(a) Preparation of the calibration graphs

Standard stock solution (1 mg/ml) was prepared by accurately weighing 20 mg of sucrose and transferring to a 50 ml conical flask. Then distilled water of 20 ml was added to the flask and mixed well. The calibration curve was constructed by using a series of dilutions containing different concentrations of sucrose in the range 500-5000 µg/ml. The analysis was carried out in duplicate for each sample and standard.

Two milliliters of each standard was pipette into labeled test tubes and 2 ml of distilled water was taken in another test tube for blank preparation. In each of the test tube, 2 ml of 6M HCl was pipetted and they were immersed in boiling water bath at 75-80º C for exactly 10 minutes. After that 8 ml of 2.5M NaOH was then pipetted to the test tubes, after removing them from the water bath. To each of the test tube, 2 ml of 0.050M dNSA was added and mixed thoroughly, firstly, by inverting the test tubes followed by mixing in vortex machine for approximately 15-20 seconds. The test tubes were then placed in boiling water bath for 5 minutes. After 5 minutes, the

test tubes were immediately placed in the ice-water bath for 10 minutes. 1 ml of the sample was mixed with 9 ml of distilled water and mixed thoroughly. The solutions, blank and standard, were diluted before taking the readings. The blank solution was poured into clean, dry cuvette and the absorbance readings were taken at 580 nm using spectrophotometer.

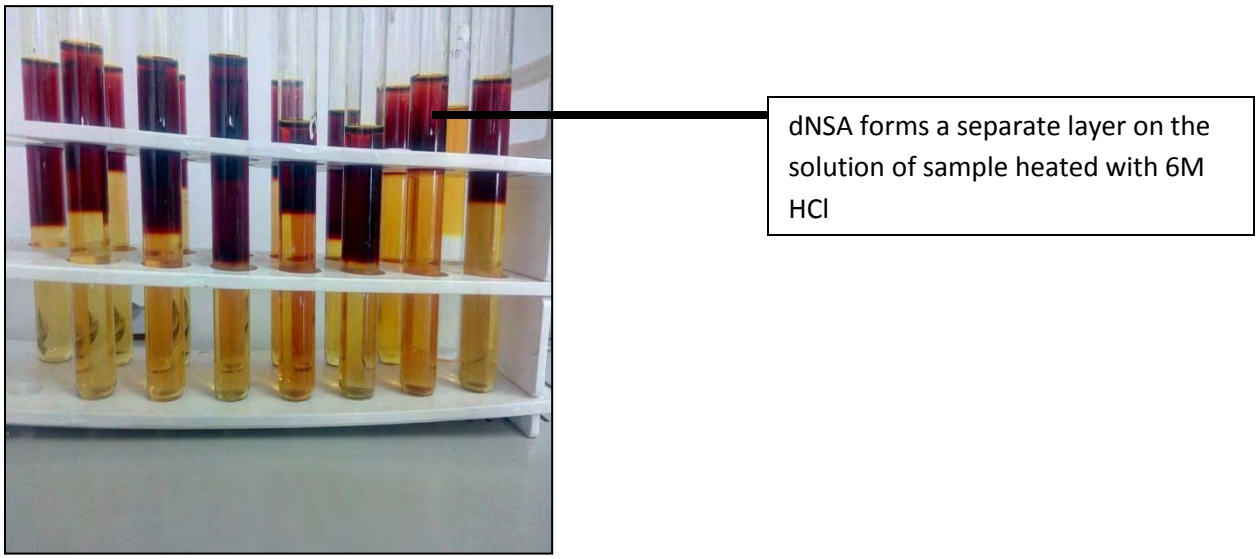


Figure 5: After immediate addition of dNSA

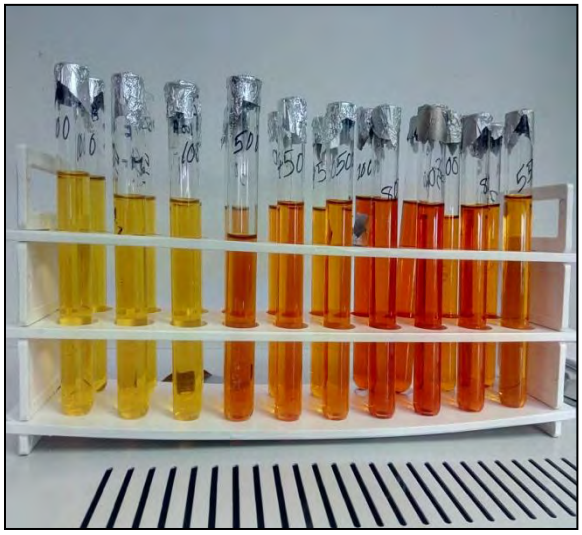


Figure 6: Vortexing the solution on addition of dNSA

Figure 7: After heating the solution for 5 minutes

2.3.2.(b) Preparation of the sample

The soft drink samples were degassed for 10 minutes in an ultrasonic water bath. 10 ml of the sample was taken in labeled test tubes and placed in 40 kHz of ultrasonic frequency at 26±2 ºC. The samples were then treated the same way as done for the standard solutions and the absorbance value was taken for each of them.

2.4 Analysis of Cyclamate

The extraction and determination procedure for analysis of samples was based on the method described by Mahdi Hashemi, Parvin Zohrabi, and Sana Abdol Hosseini (2014).

2.4.1(a) Chemicals and standards

All chemicals were of analytical grade. Cyclamate standard was purchased from Benzo Chemical Industries (Mumbai), chloroform from ACI Lab Scan Ltd. (Pathumwan, Thailand), Rhodamine B and sulphuric acid from Merck (Darmstadt, Germany). Double distilled water was used throughout the analysis. The rhodamine B solution was prepared on working day by dissolving it in chloroform in a screw flask and storing it safely by sealing the cap with masking tape followed by parafilm and covering the screw flask with aluminum foil as rhodamine is light sensitive. All the test tubes were cleaned with 0.1M nitric acid, distilled water, and acetone.

2.4.1 (b) Equipments:

A UV-Vis Spectrophotometer model EMC-61PC-UV (EMC Lab instruments, Germany) with 400 µl quartz microcell was used for the spectrophotometric analysis. A 40 kHz ultrasonic water bath model SHWB-30(Human Lab Instrument Co., Korea) was used for degassing and emulsification process. A vortex mixture, SCILOGEX MX-S was used for better mixing of reagents. For phase separation, the samples were centrifuged in a table-top centrifuge Model DSC-200T (Digisystem Laboratory Instrument Inc.,Taiwan).

2.4.2(a) Preparation of the calibration graphs

A cyclamate stock solution (1 mg/ml) was prepared by weighing exactly 20 mg of the powdered cyclamate in a conical flask and mixed it thoroughly by adding 20 ml of distilled water. 0.1M of sulphuric acid was prepared by adding 5 ml of 98% sulphuric acid to 45 ml of distilled water. Rhodamine B solution was prepared, at the beginning of each working week, by dissolving 10 mg of RhB in 104.2 ml of chlorofom as an extraction solvent. Emulsification and extraction of cyclamate was carried out in a 40 kHz ultrasonic water bath for about 20 seconds and then allowing it to reach equilibrium. After an equilibrium time of 3 minutes, the emulsion was disrupted by centrifugation at 3500 rpm for 5 minutes. This made the formation of colored organic phase at the bottom of the falcon tube. One hundred microliter of the settled sediment was pipetted out with a micropipette and simultaneously, 3 ml of chloroform was added to the quartz microcell. It was sealed with the cap and readings were taken immediately at 560 nm. The determination was carried out in duplicate for each dilution.

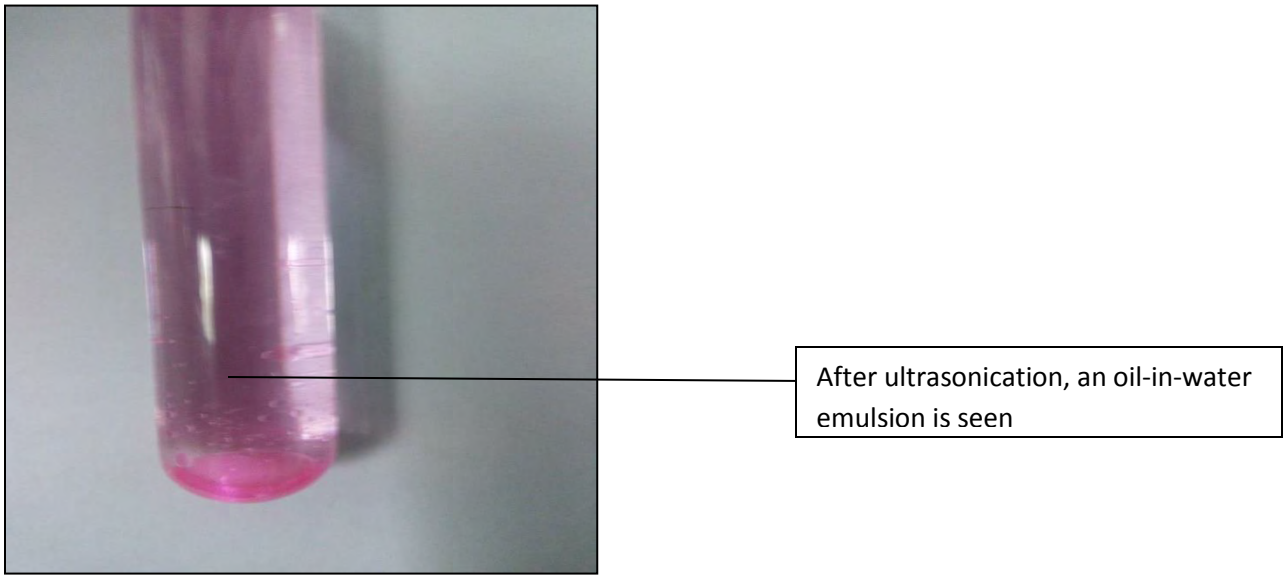


Figure 8: After ultrasonication, the oil in water emulsion

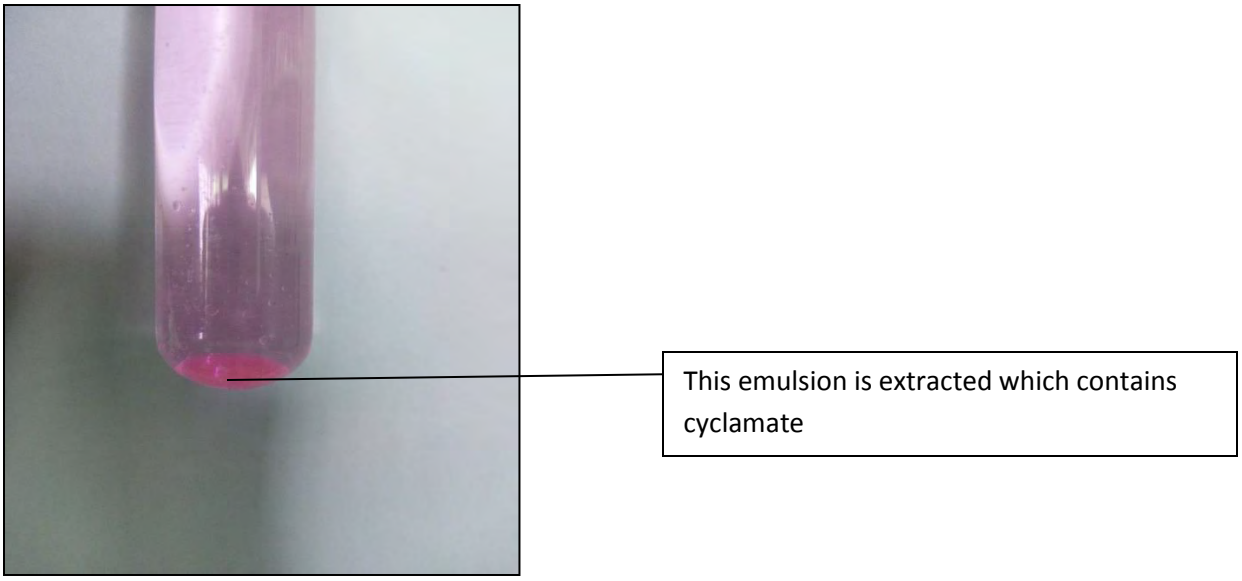


Figure 9: After centrifugation, the sediments settle at the bottom

2.4.2(b) Preparation of the sample

Ten milliliters of soft drink samples were pipetted into labeled test tubes and degassed for 10 minutes in ultrasonic water bath. Exactly 2.5 ml of the degassed sample was pipetted to labeled falcon tube and 2.5 ml of distilled water was added to each sample. Five milliliters of distilled water was poured into the blank solution. Then 2.5 ml of 0.1M of H_2SO_4 was added to the tubes and then they were immersed in an ultrasonic water bath in such a way so that the level of liquid in the tube and water-bath are same. Two hundred microliters of RhB ($2X10^{-4}M$) was added to the tube and sealed immediately. The water bath was turned on at 40 kHz at 25 ± 2^{o} C for exactly 20 seconds. As a result of this, an emulsion was formed at the bottom of the tube. The tubes were allowed to reach equilibrium for about 3 minutes before centrifuging them at 3500 rpm for 5 minutes. After the centrifugation, a pink colored sedimentation was observed at the bottom of the tube. One hundred microliters of the settled sediment was pipetted out with a micropipette and simultaneously, 3 ml of chloroform was added to the quartz microcell. It was sealed with the cap and readings were taken immediately at 560 nm.

2.5 Analysis of Saccharin

The analysis procedure for saccharin and its determination in the sample and to plot standard curve was based on the method described by Sunita B. Mathew, A.K.Pillai and V.K.Gupta (2006).

2.5.1(a) Chemicals and standards

All the chemicals used for the determination was of analytical grade and distilled water was used throughout the procedure. The chemicals were prepared freshly at the beginning of the working week. The analysis of the samples were carried out in duplicate for each standard and sample.

For the analysis of the sample, the chemicals H_2SO_4, sodium hydrogen carbonate, HCl were purchased from Merck (Darmstadt, Germany). Diethyl ether, potassium bromide, and Bromine water were purchased from Merck (Worli, Mumbai). Isoamyl alcohol which was used as the extraction solvent was purchased from aMRESCO® (Solon, Ohio). Formic acid and cetyl trimethyl ammonium bromide was purchased. Saccharin standard was purchased from Benzo Chemical Industries (Mumbai)

2.5.1(b) Equipment:

A UV-Vis Spectrophotometer model EMC-61PC-UV (EMC Lab instruments, Germany) with 400 µl quartz microcell was used for the spectrophotometric analysis. A 40 kHz ultrasonic water bath model SHWB-30(Human Lab Instrument Co., Korea) was used for degassing. The addition of bromine water and rest of the steps associated with the preparation of sample and standard was carried out in a fume hood from LabTech (Calhoun Road, New Berlin).

2.5.2(a) Preparation of standard curve:

Saccharin stock solution was prepared by measuring 50 mg of saccharin powder and mixing it in 50 ml of distilled water and a 1 mg/ml of solution was obtained. The calibration curve was produced by using a series of dilutions containing different levels (2-34 µl/ml) of saccharin.

Five milliliters of the prepared solution was pipetted into the labeled conical flask and 0.5 ml of the saturated solution of bromine water was added to it and the flask was swirled continuously

for 2 minutes. 50% v/v of formic acid was prepared by mixing equal amount of formic acid with distilled water and added drop-wise, in order to remove the excess bromine. Potassium iodide of 0.5 mg was dissolved in 50 ml of distilled water to prepare 1% w/v of potassium iodide solution. Then 1 ml of this solution was added to the conical flask immediately after removing the excess bromine. The flasks were swirled for about 20 seconds between each addition. Finally, 1 mM CTAB solution was added to the flask and was made up to 50 ml mark by adding distilled water. It was then transferred to a separating funnel and the yellow solution was extracted by adding 3 ml of isoamyl alcohol and discarding the orange layer. This step was repeated again. The solution was poured into quartz microcell and the readings were measured at 400 nm against reagent blank. The test tubes were covered with aluminum foil and the caps were sealed tightly, before the readings were taken.

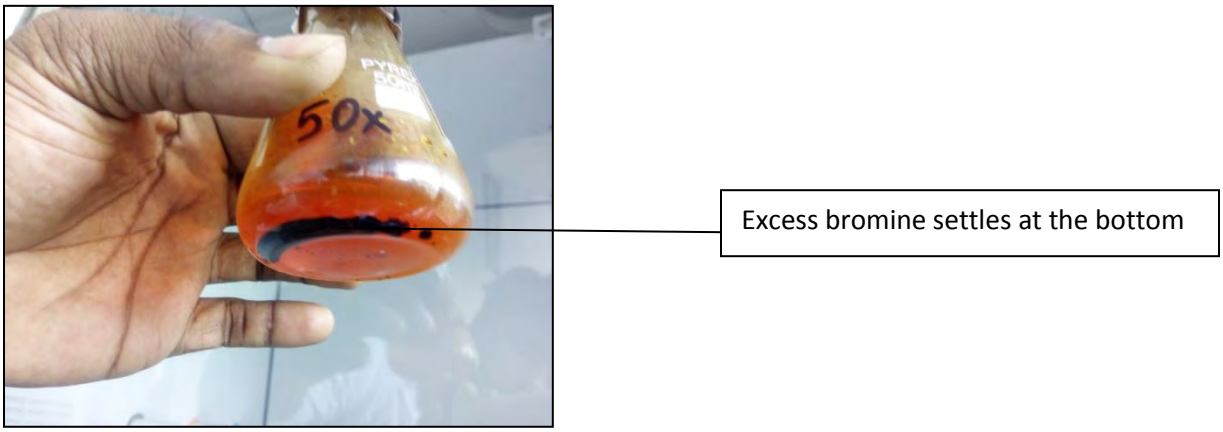


Figure 10: After the addition of Bromine water

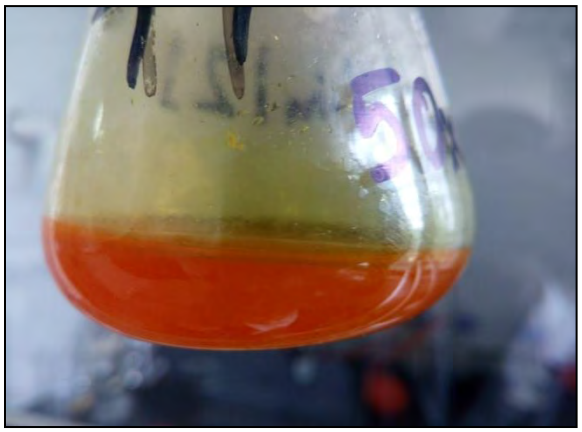


Figure 11: On drop-wise addition of formic acid

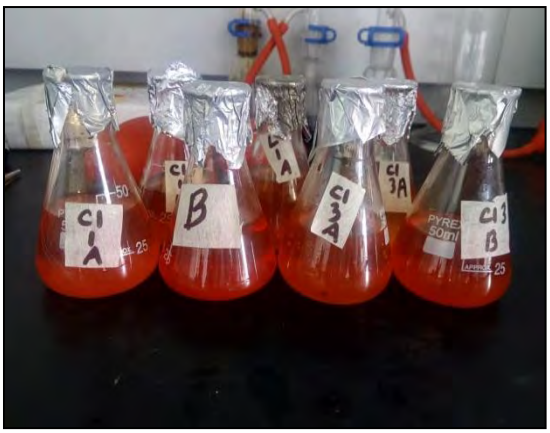


Figure 12: Diluting with distill water to 50 ml mark

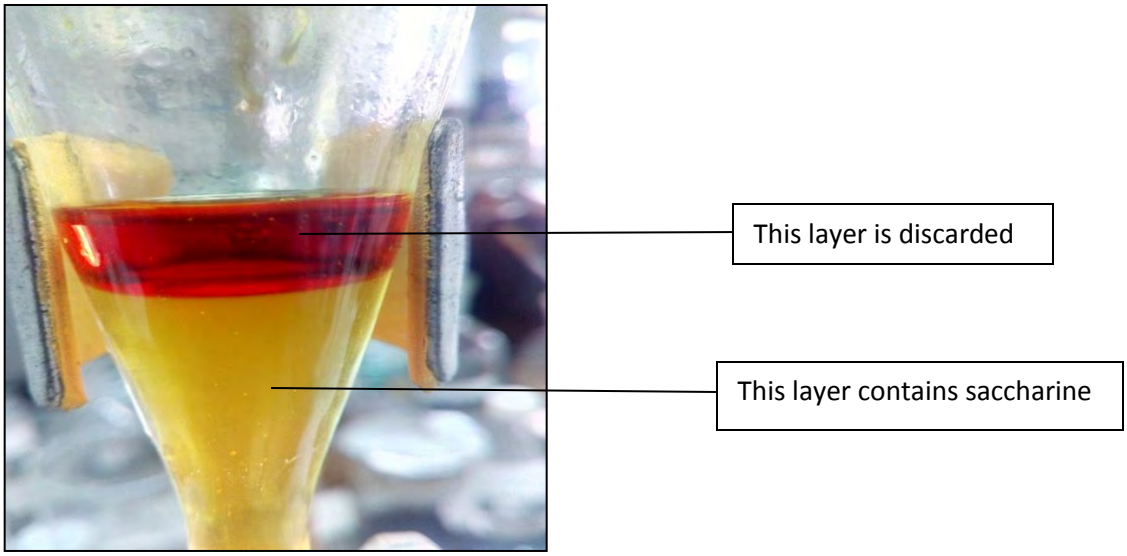


Figure 13: After addition of isoamyl alcohol, two separate layers formed

2.5.2(b) Preparation of sample:

Ten milliliters of the sample was pipetted to labeled test tubes and degassed in an ultrasonic water bath at 40 kHz and 25±2º C for 10 minutes. Five milliliters of the sample was taken to a labeled separating funnel and 1 ml of H_2SO_4 was added to it. H_2SO_4 was prepared by mixing 10 ml of the acid in 90 ml of distilled water. Then 2X6 ml of diethyl ether was added to it and mixed properly by repeated inversions which give rise to two layers. The lower aqueous layer was discarded. 2X2 ml of 2% sodium hydrogen carbonate was added to it in order to extract the ether layer. The sodium hydrogen carbonate solution was prepared by dissolving 4.08 g in 200 ml of distilled water. The ether layer was discarded and the aqueous layer was acidified with 2 ml of 5% hydrochloric acid.2X5 ml of diethyl ether was added and extracted into test tubes and evaporated in a water bath. The residue was dissolved in 10ml of distilled water and transferred to a calibrated flask and made up to 25 ml mark.

The samples were then treated the same way as the standard to determine the amount of saccharin in each sample and absorbance value was measured at 400 nm.

Figure 14: Sample before any treatment

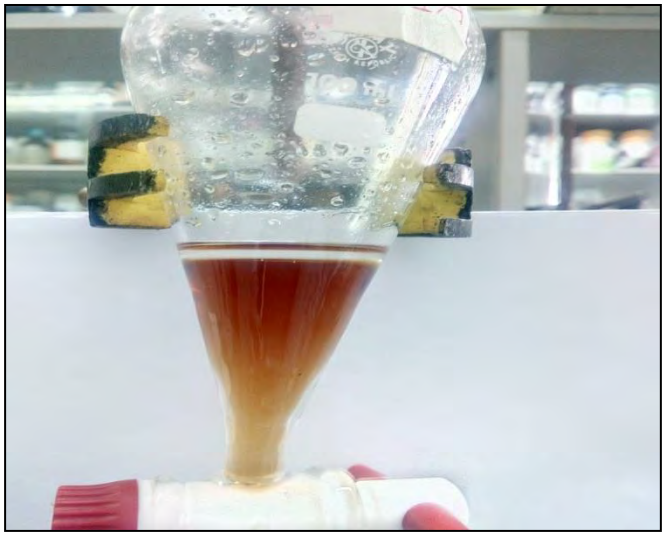


Figure 15: After addition of H_2SO_4 and diethyl ether

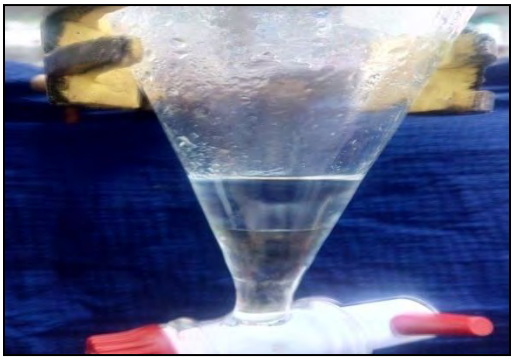


Figure 16: After final addition of diethyl ether in the last step

2.6 Analysis of Aspartame

The extraction and determination procedure for analysis of samples was based on the method described by Elif Celik, Buket Er Demirhan, Burak Demirhan and Gulderen Yentur (2014)

2.6.1(a) Chemicals and standards

All the chemicals used for the determination was of analytical grade. The chemicals were prepared freshly at the beginning of the working week. The determination was carried out in duplicate for each standard and sample.

Propylene carbonate was used as extraction media instead of water and was purchased from Wako Pure Chemical Industries Ltd. (China), anhydrous sodium sulphate as a drying agent and was bought from Scharlab (European Union), ninhydrin used as the coloring agent was **and** ethyl alcohol was purchased from Merck (Damstadt, Germany). Standard aspartame was brought from Benzo Chemicals Industries (Mumbai).

2.6.1(b) Equipment:

A UV-Vis Spectrophotometer model EMC-61PC-UV (EMC Lab instruments, Germany) with 400 µl quartz microcell was used for the spectrophotometric analysis. For phase separation, the samples were centrifuged in a table-top centrifuge Model DSC-200T (Digisystem Laboratory Instrument Inc.,Taiwan). A vortex mixture, SCILOGEX MX-S was used for better mixing of reagents and water bath from Biobase (USA) was used for heating. A 40 kHz ultrasonic water bath model SHWB-30(Human Lab Instrument Co., Korea) was used for degassing.

2.6.2(a) Preparation of standard curve:

Stock aspartame solution was prepared by weighing 10 mg of aspartame and mixing it with 10 ml of distilled water to prepare a 1 mg/ml solution. By using a series of dilutions different levels (5-40 µg/ml) of aspartame solutions were prepared.

A solution of 0.75 ml was taken in a falcon tube to which 0.25 ml of acetate buffer solution (pH 3.53) was added to it. Propylene carbonate of 3 ml and 2 ml of ethyl alcohol was added to the solution and mixed in an ultrasonic water bath at 40 kHz for 5 minutes. The tubes were then centrifuged at 5000 rpm for 5 minutes. The lower phase of 3.5 ml was pipette out and taken to a test tube to which a pinch of anhydrous sodium sulfate was added as a drying agent. The tubes were then vortexed for 10 seconds and were left to settle for 10 minutes. One and a half milliliters of the dried solution was transferred to a fresh test tube to which 2 ml of 2% ninhydrin solution was added. The test tubes were then heated in a boiling water bath at 80º C for 20 minutes. The solution was left to cool and 3ml of ethyl alcohol was added to the test tubes. The absorbance readings were taken at 585 nm wavelength against reagent blank.

2.6.2(b) Preparation of sample:

Five milliliters of the sample was pipetted to labeled test tubes and degassed in an ultrasonic water bath at 40 kHz and 25±2º C for 10 minutes. Then 0.75ml of the sample was taken into labeled falcon tubes and were treated the same way as the standard preparation. The readings were taken at 585 nm wavelength and values calculated from the calibration graph plotted.

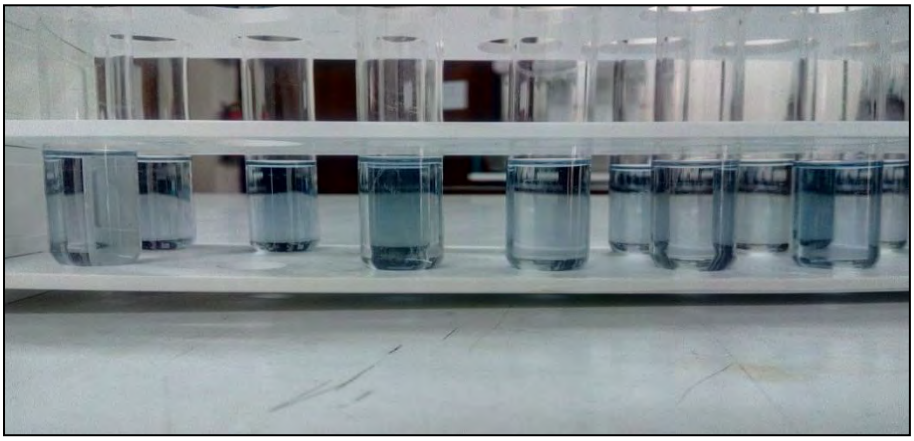


Figure 17: On adding Ninhydrin solution and heating for 20min

Chapter: THREE

Results

To test linearity, standard solutions of a series of concentrations were prepared by serial dilution. Standard solutions of Sucrose (500-5000 µg/ml), Cyclamate (25-200 µg/ml), Aspartame (5-35 µg/ml) and saccharin (2-34 µg/ml) were prepared and analyzed with three replicates and the results processed. The calibration graphs obtained are linear. The equations of the calibration graphs and the correlation coefficients (r^2) for the four analytes are presented in table 3.

Table 3: Equations of the calibration graphs and the correlation coefficients (r2) for cyclamate, aspartame, sucrose, and saccharin

ANALYTE	EQUATION OF THE CALIBRATION GRAPH	r^2
ASPARTAME	$\boldsymbol{y = 0.002x + 0.023}$	0.998
CYCLAMATE	$\boldsymbol{y = 0.001x + 0.269}$	0.989
SACCHARINE	$\boldsymbol{y = 0.004x + 0.116}$	0.999
SUCROSE	$\boldsymbol{y = 1E - 04x + 0.039}$	0.993

3.1 Validation of the method

The applicability and reproducibility of the method used in the analysis of the NAS are discussed individually. The calibration graphs showed good linearity over a range of at least six readings. Percentage recovery was calculated. A recovery near 100 % (e.g. 80-110 %) gives confidence about the analytical system being used. The standard error of the regression (SE) is also calculated which represents the average distance that the observed values fall from the regression line. Smaller values are better because it indicates that the observations are closer to the fitted line. The coefficient of determination, represented by r^2, is a statistical measure of how close the data points are to the fitted regression line. The closer is the r^2 value to 1, the better the model fits the data (Torbeck, 2010). The limit of detection (LOD) is generally evaluated for quantitative assays and impurities. The International Council for Harmonisation (2005) defines the LOD as, "The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value"

3.1.1 Aspartame

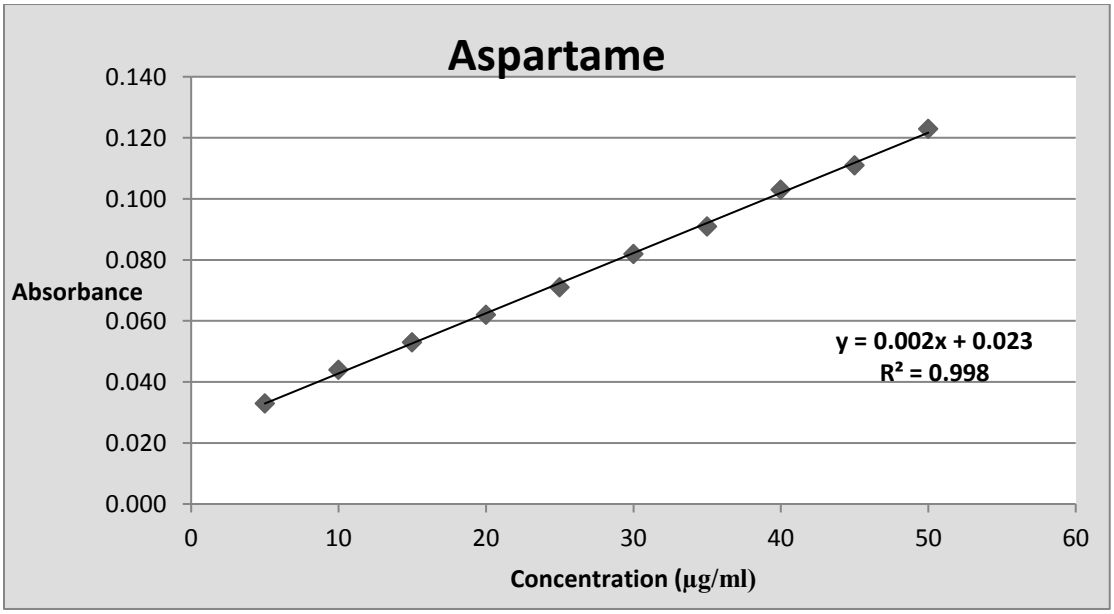
The result obtained in Table: 4 shows that the absorbance reading at 585 nm increased as the amount of aspartame in the solutions increased. Fifty micrograms of aspartame solution had the highest mean absorbance value which was 0.123 whereas 5 µg of aspartame solution had the lowest mean absorbance value which was 0.033.

Table: 4 Aspartame standard curves

Concentration (µg/ml)	mean absorbance	Observed concentration (µg/ml)	% recovery
5	0.033	5	100
10	0.044	10.5	105
15	0.053	15	100
20	0.062	19.5	97.5
25	0.071	24	96
30	0.082	29.5	98.333
35	0.091	34	97.143
40	0.103	40	100
45	0.111	44	97.778
50	0.123	50	100
Mean (n=10)			**99.175**
SD			**2.502**

The standard curve below showed a linear graph, where the mean absorbance was directly proportional to the amount of aspartame (µg/ml) [Figure 1].

Figure 18: Standard curve of mean absorbance at 585 nm against concentration of aspartame (µg/ml)



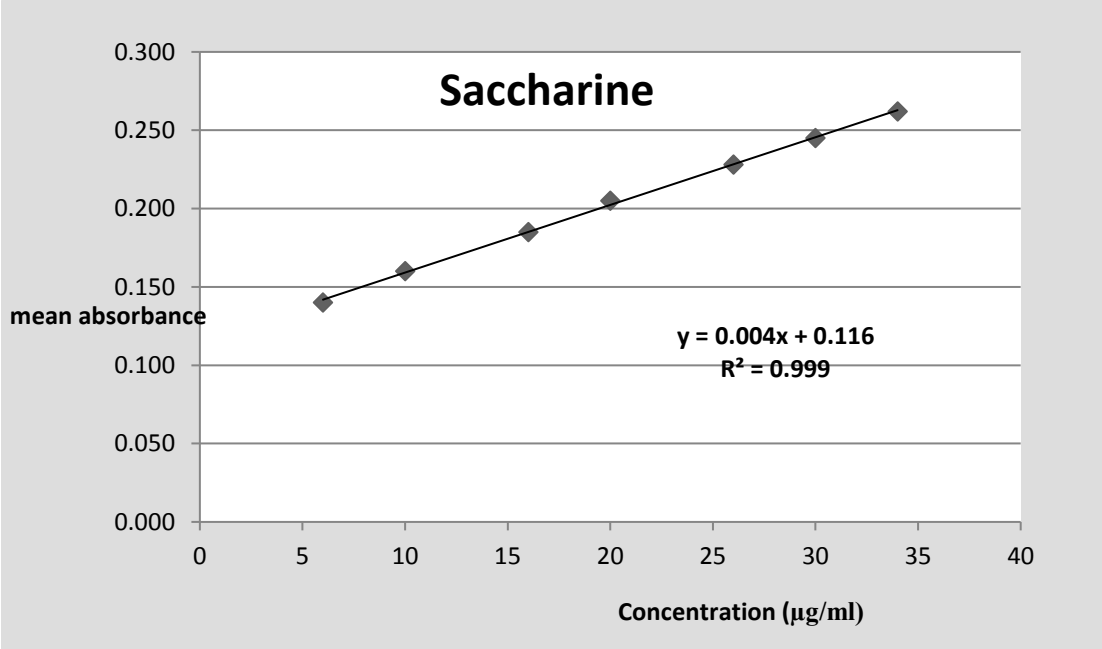
3.1.2 Validation of the method: Saccharine

The result obtained in Table: 5 shows that the absorbance reading at 400 nm increased as the amount of saccharine in the solutions increased. Thirty four microgramof saccharine solution had the highest mean absorbance value which was 0.262 whereas 6 µg solutions had the lowest mean absorbance value which was 0.14.

Table: 5 Saccharine standard curve

Concentration (µg/ml)	Mean absorbance	Observed concentration(µg/ml)	% recovery
6	0.14	6	100
10	0.16	11	110
16	0.185	17.25	107.813
20	0.205	22.25	111.25
26	0.228	28	107.692
30	0.245	32.25	107.5
34	0.262	36.5	107.353
Mean (n=7)			107.373
SD			3.573

Figure: 19 Standard curve of mean absorbance at 400 nm against concentration of saccharine (µg/ml)



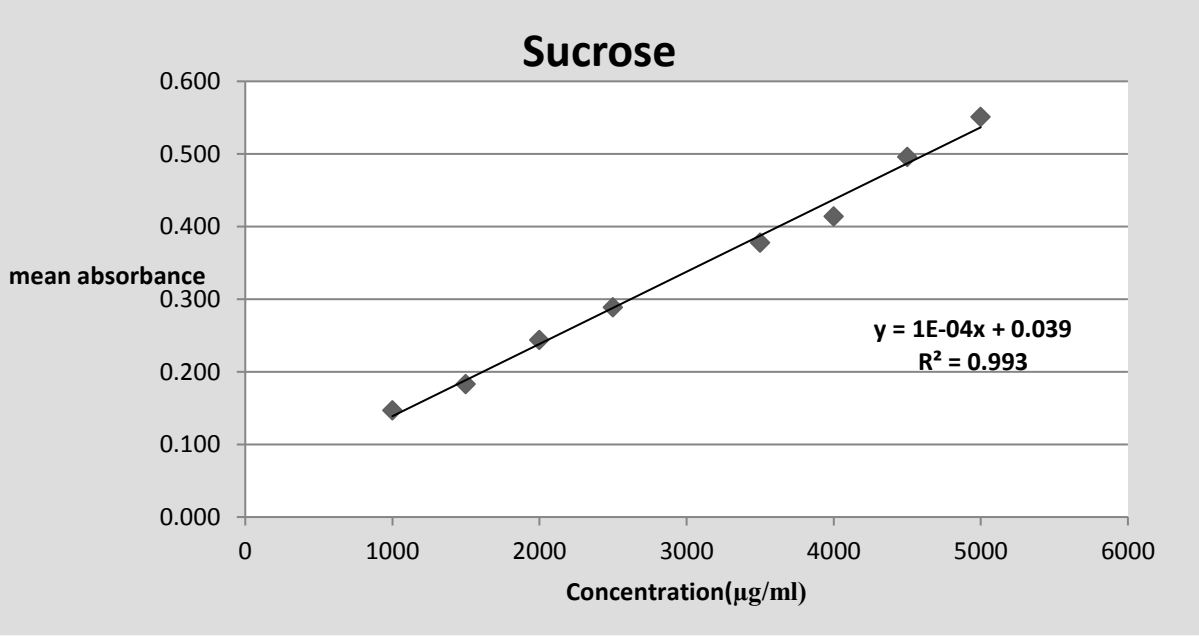
3.1.3 Sucrose

The result obtained in Table: 7 shows that the absorbance reading at 580 nm increased as the amount of sucrose in the solutions increased. Five thousand microgram of sucrose solution had the highest mean absorbance value which was 0.551 whereas 1000 µg solutions had the lowest mean absorbance value which was 0.147.

TABLE: 6 Sucrose standard curve

concentration (µg/ml)	mean absorbance	Observed concentration(µg/ml)	% recovery
1000	0.147	1080	108
1500	0.183	1440	96
2000	0.244	2050	102.5
2500	0.289	2500	100
3500	0.378	3390	96.857
4000	0.414	3750	93.75
4500	0.496	4570	101.556
5000	0.551	5120	102.4
Mean (n=8)			100.133
SD			4.523

Figure: 20 Standard curve of mean absorbance at 580 nm against concentration of sucrose (µg/ml)



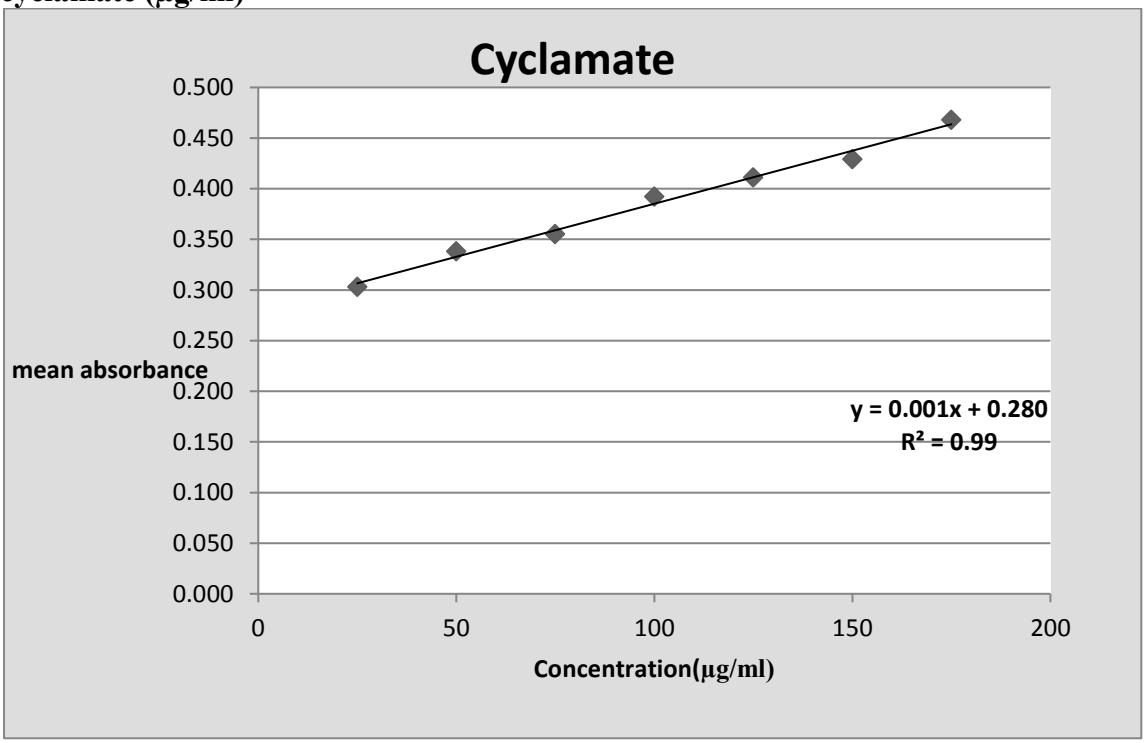
3.1.4 Cyclamate

The result obtained in Table: 8 shows that the absorbance reading at 560nm increased as the amount of cyclamate in the solutions increased. Twenty five micrograms of cyclamate solution had the lowest mean absorbance value which was 0.303 whereas 200µg solutions had the highest mean absorbance value which was 0.52.

TABLE: 7 **Cyclamate standard curve**

concentration (µg/ml)	mean absorbance	Observed concentration(µg/ml)	% recovery
25	0.303	23	92
50	0.338	58	116
75	0.355	75	100
100	0.392	112	112
125	0.411	131	104.8
150	0.429	149	99.333
175	0.468	188	107.429
200	0.52	240	120
Mean (n=8)			106.445
SD			9.338

Figure: 21 Standard curve of mean absorbance at 560 nm against concentration of cyclamate (µg/ml)



3.2. Practical determination of the studied NAS in soft drink samples

In all, eight original brands of soft drinks were analyzed and each of these brands had three batches. Under each batch, two samples were analyzed, replicating the results twice which bring the number of samples analyzed under each brand to 12.The brands and their batch numbers are stated in Table: 2.

The amount of saccharin, aspartame, sucrose, and cyclamate in soft drinks samples was determined by substituting their respective absorbance values into the computed calibration graph. The mean concentration of artificial sugars (±SD) in four samples under each batch was calculated. The maximum and minimum value in the total 12 samples analyzed per batch is also recorded.

3.2.1 Determination of Aspartame content in samples

The amount of aspartame in soft drinks samples was determined by substituting their respective absorbance values into the computed calibration graph which was y=0.002x+0.023

Table: 8 Statistical analysis for levels of aspartame in soft drink sample

Sample	Mean Concentration ± SD (µg/ml)			Min. (µg/ml)	Max. (µg/ml)
	Batch-1	Batch-2	Batch-3		
Coke, Co	45.5±6.285	45.5±5.686	44.375±5.023	37	54
Pepsi, Pep	84±2.828	87.5±3.582	100.125±2.869	82	103.5
Mojo, Mo	85.25±1.472	113.75±3.304	106.75±3.663	89	116
7-up, SP	11.875±0.75	11.25±0.866	11.625±1.495	10	12.5
Mirinda, Mir	82.125±1.315	97.5±3	109.875±1.75	81	112
Clemon, Cl	13±0.913	11.625±1.25	12.875±0.947	10	14
Mountain Dew, MD	10.5±1.080	10.875±1.315	13.875±0.629	09	14.5
Lemu, L	62.125±2.496	71.375±2.496	74.25±1.433	60	76

All analyses were repeated four times from each batch. The aspartame concentrations are shown in Table: 11. Aspartame was found in all the samples analyzed. The results obtained shows that mean aspartame concentration ranged from 10.5 – 113.75 µg/ml. The highest concentration of aspartame was found in Mojo while the least concentration was found in Mountain Dew. There is significant variation in the range of aspartame used in the soft drinks analyzed. For most drinks analyzed, it was observed that the content of each one of the sugars present in the sample varies from bottle to bottle, with the exception of Coke, 7-up, and Clemon which showed very little variation per bottle.

3.2.2 Determination of saccharine content in samples

The amount of saccharine in soft drinks samples was determined by substituting their respective absorbance values into the computed calibration graph which was $\mathbf{y = 0.004x + 0.116}$

Table: 9 Statistical analysis for levels of saccharine in soft drink sample

Sample	Mean Concentration ± SD (µg/ml)			Min. (µg/ml)	Max. (µg/ml)
	Batch-1	Batch-2	Batch-3		
Coke, Co	01±0.645	1.438±0.718	2.438±1.329	0.25	3.5
Pepsi, Pep	2.063±0.944	8.189±1.546	7.438±1.008	01	9.5
Mojo, Mo	15.75±0.989	17.813±1.560	12.125±0.854	11.25	19.25
7-up, SP	4.75±0.707	2.313±0.826	6.563±0.688	1.5	7.25
Mirinda, Mir	0.875±0.595	1.375±0.595	1.063±0.747	0.25	2
Clemon, Cl	6.688±0.966	9.125±0.968	12.125±0.968	5.75	13
Mountain Dew, MD	1.563±0.554	0.625±0.332	0.813±0.554	0.25	2.25
Lemu, L	3.875±0.520	2.5±0.791	3.5±1.555	1.25	4.75

The results obtained in Table 12 showed that the mean saccharine concentration in Mojo was highest than the rest of the brands and lowest in Mountain Dew.

3.2.3 Determination of cyclamate content in samples

The amount of cyclamate in soft drinks samples was determined by substituting their respective absorbance values into the computed calibration graph which was $\boldsymbol{y = 0.001x + 0.269}$

Table: 10 Statistical analysis for levels of cyclamate in soft drink sample

Sample	Mean Concentration ± SD (µg/ml)			Min. (µg/ml)	Max. (µg/ml)
	Batch-1	Batch-2	Batch-3		
Coke, Co	37±8.124	70.5±13.892	31±6.683	22	87
Pepsi, Pep	71.75±18.998	55±12.038	66±4.397	46	89
Mojo, Mo	67±23.622	110.5±27.294	66.5±4.041	41	141
7-up, SP	96.25±9.465	100.5±12.396	102.5±14.060	83	115
Mirinda, Mir	148.25±14.104	151.5±12.041	168±10.551	131	179
Clemon, Cl	51±14.674	76.75±15.435	73.5±13.204	32	92
Mountain Dew, MD	131.5±12.179	142.75±15.966	147.5±29.693	120	189
Lemu, L	164±19.647	166.5±19.070	160.5±16.836	123	183

From Table: 12, it is seen that in each brand significant amount of cyclamate is present. The total cyclamate content was found between 46.167-163.667 µg/ml with the highest mean concentration being in Lemu (163.667± 19.823 µg/ml) and lowest being in Coke (46.667 ± 21.288 µg/ml). Most variation in mean saccharine concentration per batch is seen among Coke and Mojo. The SD value for Mojo was high which indicates the low precision of the method for that brand. This could be due to the use of blend of several NAS in the soft drink sample analyzed.

3.2.4 Determination of sucrose content in samples

The amount of cyclamate in soft drinks samples was determined by substituting their respective absorbance values into the computed calibration graph which was $\mathbf{y = 1E - 04x + 0.039}$

Table: 11 Statistical analysis for levels of sucrose (µg/ml) in soft drink sample

Sample	Mean Concentration ± SD (µg/ml)			Min.	Max.
	Batch-1	Batch-2	Batch-3		
Coke, Co	1892.5 ± 22.174	1960 ± 28.284	2192.5 ± 17.078	1870	2210
Pepsi, Pep	1795 ± 19.149	1905 ± 17.321	2030 ± 25.820	1780	2060
Mojo, Mo	2027.5 ± 25	1937.5 ± 20.616	1852.5 ± 28.723	1820	2060
7-up, SP	1860 ± 18.256	1940 ± 11.5547	1937.5 ±17.078	1840	1960
Mirinda, Mir	1780 ± 21.602	1860 ± 16.330	1917.5 ± 12.583	1750	1930
Clemon, Cl	1825 ± 23.805	2020 ± 21.602	2017.5 ±20.078	1810	2040
Mountain Dew, MD	1840 ± 14.142	1930 ± 16.230	1965 ±12.910	1830	1980
Lemu, L	1775 ± 19.149	1840 ± 18.257	1895 ± 23.805	1750	1920

Sucrose which is a non-reducing sugar was found in considerably high concentration in all the samples analyzed. The results obtained shows that mean sucrose concentration ranged from 1836 – 2015 µg/ml. The highest concentration of sucrose was found in Coke while the lowest concentration was found in Lemu. The sucrose concentration was in close range to each other in the soft drinks analyzed.

3.3. Summary

Table: 14 summarize the mean concentration (± SD) of aspartame, saccharin, sucrose and cyclamate found in the eight brands of the soft drink samples analyzed. RSD or Relative Standard Deviation was also calculated for each brand and each NAS. It is a statistical measurement that describes the spread of data with respect to the mean and the result is expressed as a percentage. The %RSD function is used extensively by chemists as the interpretation is based on a percent result and not some abstract value and is used to assess the variation of sets of data.Besides, the RSD (%) can be used to make comparisons across dissimilar results (Torbeck 2010).

Table: 12 The levels of aspartame, saccharin, sucrose and cyclamate in soft drinks

	Sweeteners							
	ASPARTAME		**SACCHARINE**		**CYCLAMATE**		**SUCROSE**	
SAMPLE	X±SD	%RSD	X±SD	%RSD	X±SD	%RSD	X±SD	%RSD
COKE	45.175± 5.175	11.468	1.625± 1.063	65.431	46.167± 21.856	46.106	2015± 135.814	6.74
PEPSI	90.542± 7.762	8.57	5.896± 3.048	51.694	64.25± 13.994	21.781	1910± 102.069	5.344
MOJO	103.667± 10.521	10.149	15.229± 2.879	18.907	81.333± 28.703	35.291	1939.167± 77.982	4.02
CLEMON	12.5± 1.148	9.185	9.313± 2.482	26.652	67.083± 17.273	26.426	1954± 97.277	4.978
MIRINDA	96.5± 12.013	12.44	1.164± 0.628	56.672	155.917± 14.343	9.199	1852.5± 60.921	3.289
MOUNTAIN DEW	11.75± 1.840	15.66	1± 0.496	49.6	140.583± 19.988	14.218	1911.667± 56.542	2.958
7-UP	11.583± 0.733	6.332	4.54± 1.939	42.692	115± 11.297	11.327	1912.5± 41.369	2.163
LEMU	69.25± 1.148	8.315	3.292± 2.482	34.247	163.667± 19.823	12.112	1836.667± 54.495	2.967

X= Mean Concentration (µg/ml); SD= Standard Deviation; RSD= Relative Standard Deviation

From the table, it is seen that the mean aspartame concentration was the highest in Mojo (103.667± 10.521 µg/ml) and lowest in Mountain Dew and 7-up (11.75± 1.840 µg/ml and 11.583± 0.733 µg/ml, respectively). The level of saccharine was moderately very low in all the samples analyzed. The highest being is in Mojo (15.229± 2.879 µg/ml) and lowest in Mountain Dew (1± 0.496 µg/ml). The RSD value is highest in almost all of the soft drinks in the analysis

of saccharine. Hence, this indicates that the smaller the amount of saccharin content in a sample, the lower is the precision of its absorbance reading at 400 nm. The sucrose content was significantly high in all of the soft drinks, highest in Coke and lowest in Lemu. The SD and % RSD was very low in the analysis of the sucrose and hence it can be stated that its absorbance at 580nm. Samples of Coke showed the lowest mean concentration of cyclamate (46.167± 21.856 µg/ml);) while Lemu contained the highest (163.667± 19.823 µg/ml).

The result concluded that all of the samples of carbonated soft drinks had non-nutritive sweeteners in them. However, the calculated SD and RSD were very high for saccharine. High values of SD and RSD indicated that this analytical method for saccharine produced low precision and reproducibility. The RSD of saccharine content analysis in the samples showed as high as 65.4% while the RSD of sucrose content was as low as 2.1%. Thus, the data for sucrose concentration was most precise followed by aspartame, then cyclamate and finally saccharine.

Chapter: FOUR

Discussion

Soft drinks have been part of our global lifestyle since the nineteenth century. They are non-alcoholic water-based flavored drinks that are optionally sweetened, acidulated and carbonated. Frequently; soft drinks contain a number of additives that can affect the beverage's taste and characteristics (Khatri and Shalini, 2008). It is added by the manufacturer and since the discovery of artificial sweeteners; they are being used in place of sugar or corn syrups.

Globally, the consumption of soft drinks has increased since the 1980s. The five fastest-growing soft drink markets between 1996 and 2001 were from Asia, East Europe, and the Middle East. The five fastest developing markets during 2001 and 2006 were all from Asia. Amongst them, Pakistan had the highest percentage growth rate, while India made sizeable volume gains, as affluence spreads to more of its vast population (Johnson et al., 2006). Simultaneously, Bangladesh has become an important soft drinks business region for many international companies due to its increasingly high population and the public, especially the young generation, trying to pick-up "Western" habits. In addition to it, Muslim countries like ours where alcohol consumption is prohibited soft drinks remain high in demand (Feng, 2015).

In Bangladesh, the soft drink industry is virtually unregulated, which make it profitable to some class of local companies to establish their own products. With this comes the uncontrolled use of NAS, usage of which reduces the production cost substantially. Limited research has been conducted on the determination of aspartame, saccharine, cyclamate and sucrose levels in soft drinks in Bangladesh. We did not find any work based on the simultaneous and quantitative analysis of four non-nutritive sweeteners in soft drinks in Bangladesh. The results obtained in this study will be compared with the analysis done in different countries, around the globe.

Over 48 various soft drink samples were analyzed using the method described. All products were bought from local stores. Most of the samples contained artificial sweeteners. Every sample was analyzed twice. Samples studied are positive for all the four sweeteners, out of which three are

authorized (aspartame, saccharine and sucrose) and one authorized by EFSA and EU (cyclamate) but is disapproved by FDA. Aspartame, Cyclamate and sucrose were found to be the most commonly and heavily used sweeteners. Saccharin is used in very small amount when compared to the rest.

Beverages are the most interesting group of foods in terms of sweeteners composition. Almost all of the tested drinks contained three or four components of sweetener blends. There is a wide variation in the content of individual artificial sugar in the range of soft drinks analyzed. The average aspartame, saccharine, sucrose and cyclamate contents were found to be 11-104, 1-9, 1800-2000 and 45-165 µg/ml respectively. One of the main reasons for the differences in the content of individual sugar and the inconsistency of the result might have been for the use of multiple artificial sweeteners, at the same time, in soft drinks. For example, saccharin is often used along with cyclamate, while aspartame is used alongside acesulfame-K (another artificial sweetener) in a range of products, including soft drinks. The sweeteners work in synergy, thereby, allowing the blending of low concentrations of acesulfame-K and saccharin, both of which can leave a bitter aftertaste when used in high concentration but can be eliminated when used with aspartame and cyclamate respectively (Behrens et al., 2017). A study published on September 14th in the journal *Cell Chemical Biology* have suggested this in their paper about the synergistic behavior of blending artificial sweeteners.

The concentration of aspartame in each of the sampled soft drink is given in Table 14. The results obtained shows that aspartame concentration ranged from 11.58– 103.67 µg/ml. The values obtained in soft drinks in Turkey were noticeably lower, around 155-240 mg/ml (Elif et al., 2014). Grembecka et al. (2013) determined the mean aspartame concentration soft drinks in the range 4.4-6.5 mg/100 ml (almost equivalent to 44-65 µg/ml), in Poland. Alghamdi et al (2005) found the mean concentration of aspartame to be in the range 79-550 µg/ml in 29 samples of different beverages analyzed, in Riyadh. In the same kind of drinks in Portuguese, 48 different samples were analyzed of which 25 being soft drinks, had a mean concentration of aspartame of 89 mg/ml (Lino et al., 2008). A study on the mean aspartame concentration in Polish soft drinks was found to be 162 mg/l (Rój et al., 2007). The aspartame level in 41 soft drinks from plant extracts, 11 fruit juices and 26 artificially and flavored drinks analyzed in Croatia was found to be between 153.69-876.42, 80.29-435.05 and 198.22-709.36 mg/l respectively (Serdar and

Knežević, 2011). The results from soft drink samples collected from Romanian market ranged between 41.94-881.98 mg/l (Oroian et al.,2013)The values differ notably from country to country. When compared to this study, the values are very high in samples analyzed in Saudi and Croatia.

Saccharin concentration in all the soft drinks evaluated in this study is represented in Table: 14. The result indicates that the amount of saccharine in the eight brands analyzed range between 1-15.229 µg/ml. The results suggested that the amount of saccharin content in soft drinks varied significantly from brand to brand. Similar studies on the analysis of saccharin concentration in soft drinks from various countries are looked at. A study on the mean concentration of saccharine in Cola-flavored soft drinks in Malaysia was found to be 6.945 µg/ml (SL and SS, 2017). Another research analyzed 30 samples of carbonated drinks from the Romanian market and found that the concentration of saccharin to be 9.72 µg/ml (Oroian et al., 2013). The concentration of saccharin, ranged between 11.6-173.7 mg/ml, was found to be one of the most commonly used artificial sweeteners in the majority of 31 sweetener containing samples analyzed (17 samples contained saccharine), in Poland in the year 2011 (Zygler et al., 2011). Similarly, another study in 2013 in Poland found the mean concentration of saccharine to be as low as 6.74 mg/100 ml (equivalent to 0.0674 mg/ml) (Grembecka et al., 2013). On the other hand, a study conducted in Taiwan found no saccharine in soft drink samples collected from the market (Chang and Yeh, 2013). A research conducted in the analysis of saccharin levels in Pakistan reported the range to be between 4.62-13.4 µg/ml in different Cola brands (Javed et al., 2001). The mean saccharine concentration in the samples collected from local Chinese market was found to be 0.0443 mg/ml (Chen et al., 1997). The analytical data from the present survey and that carried out in different countries is very alarming in the sense that the amount of saccharine found in soft drink samples is not uniform.

The concentration of cyclamate in the present study was found in the range between 46.17-163.67 µg/ml, tabulated in table: 14. Limited researches related to the cyclamate analysis in the soft drinks have been seen in Bangladesh, as well as in different countries. A Chinese study found the mean cyclamate concentration to be 1.48 mg/ml in local soft drinks samples (Chen et al., 1997). However, the reported mean concentration of cyclamate in a research conducted in Croatia was 80.13-627.38 mg/ml (Serdar and Knežević, 2011) and in Hungary, it was 113.14-

280.67 mg/ml (Croitoru et al., 2011). A study carried out in Poland on 31 samples of soft drinks collected from their local stores found only 10 samples with the presence of cyclamate in them. They reported cyclamate within a range between 95.5-237.7 mg/l (Zygler et al., 2011). The experimental values for the concentration of cyclamate levels in the samples were within the range established by the literature values, except for the values in Croatia.

The results of soft drink samples analysis for sucrose content was within the range 1852.5-2015 µg/ml, as tabulated in table:14. With sucrose being a disaccharide made of one molecule of fructose and one molecule of glucose, they undergo hydrolytic reaction during storage because of the acidic pH of soft drinks (Vidal- Valverde et al., 1981). The amount of sucrose varies from bottle to bottle of same drink for this reason. In one study, samples from locally collected soft drink from the markets of Spain, divided into three categories: Cola, Lemon and Orange, had the range of sucrose content between 0.122-7.97 g/100 ml, 4.51-10.70 g/100 ml and 4.54-12.84 g/100 ml respectively (Vidal- Valverde et al., 1981). Another study carried out in Spain to determine the sucrose content in soft drink samples found the range to lie between 2.26-8.25 g/100 ml, 0.3-10.49 g/100 ml and 3.98-9.17 g/100 ml for Cola, Lemon and Orange, respectively (Vidal–Valverde et al., 1981). Surprisingly, a study published in the Obesity Journal of USA found no sucrose in the samples of soft drinks analyzed. One of the reasons for this might have been due to the differences in the content of individual sugars like glucose, fructose, and sucrose being used in the manufacturing process.

Artificial sweeteners were found in all analyzed products. The safety concerns for the consumption of these four sugars have been considered by a range of regulatory organizations and the interested scientists. The main international body that addresses the safety of ADI level of artificial sweeteners is the Joint Expert Committee on Food Additives (JECFA) of the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO). The US Food and Drug Administration or FDA is also another prominent regulatory organization. The FDA established amount, ADI, for aspartame and saccharine, along with some other popular sweeteners are given in table: 1. The exception lies with cyclamate. FDA has banned cyclamate while it is approved by JECFA and is being used in the European Union. Different countries around the globe have different regulatory laws on the acceptable amount of

consumption but the amount matches closely with the ones stated by these two major organizations.

From the results obtained in table: 14, the following conclusions can be drawn:

1. **Concentration of aspartame:** highest in Mojo followed by Mirinda, Pepsi, Lemu, Coke, Clemon, Mountain Dew, and lowest being in 7-Up.
2. **Concentration of Saccharine:** Mojo had the highest mean concentration of saccharin followed by Clemon, Pepsi, 7 Up, Lemu, Coke, Mirinda and lastly, Mountain Dew.
3. **Concentration of Cyclamate:** Lemu had the highest mean concentration of cyclamate and then Mirinda, Mountain Dew, 7-Up, Mojo, Clemon, Pepsi and least in Coke.
4. **Concentration of sucrose:** the highest concentration was in Coke followed by Clemon, Mojo, 7-Up, Mountain Dew, Pepsi, Mirinda and Lemu.

In general, there is a wide variation in the range of the content of individual sugars analyzed present in the samples. Coke seems to contain less amount of artificial sweetener and highest amount of sucrose. On the other hand, Mojo contained the maximum amount of aspartame and saccharine, when compared to the rest of the analyzed products. Mountain Dew had the moderate level of blend of the artificial sweetener in it.

It is vital to distinguish the limitations of this study. First and foremost reason is the scarcity of data in the analysis of NAS in soft drinks in Bangladesh. No comparison with similar study can be done for the soft drinks which are produced locally like Clemon, Mojo, Lemu etc. The variation in the content of different sugars for the brands might have been due to differences in the manufacturing technologies. The discrepancy in the analysis of artificial sugar content within the same brand may have been due to different production batches. The comparison of data on the levels in this study with other countries is also difficult. One of the major explanation for this will be the difference in surrounding environment, type of soft drink analyzed, even the brand of soft drink being investigated. The origin of production of the soft drink is another major limitation of this study.

Other limitations that should be distinguished in this study include the analytical method that has been implicated in the study. The determination of the quantitative analysis of the artificial sweetener in soft drinks will yield different results, depending on whether HPLC is being used or ion Chromatography or Spectrophotometry or dispersive Raman spectroscopy among others. The difference maybe significantly diverse for this reason. In addition to, for the samples containing lower sugar content, especially in case of saccharine, the method requires further optimization which could be achieved through enhancing the sensitivity of the detection. Also, variability in the study of the same analysis may exist, considering that only one laboratory was used for investigation. In addition, the soft drink samples were purchased from stores in Dhaka only.

However, in order to minimize the error, the analysis of samples was done twice for each sample, under one batch containing two sample bottles. The standard solutions were repeated thrice for each concentration and an average value was calculated to establish the standard curve. Further research can be carried out in the same laboratory with a fresh batch of samples of the same brands or different brands to curtail some of the potential errors. The work can be taken ahead and carried out in other laboratories to explore possible disparity by laboratory or by analysis methodology used.

This work of mine is significant in view of it being the opening study of soft drinks in Bangladesh. Works cited in the paper are mainly gathered from research done in European countries, very few being from neighboring countries. The present study will be valuable taking into account that very little information exists for these artificial sweeteners in the South Asian region.

Chapter: FIVE

Conclusion

In recent times, low-calorie sweeteners are extensively exploited in foods and as well as beverages, whether it is a soft drink, energy drink, fruit drink or supplementary drink. Investigations of the health implications associated with these compounds have raised questions as to whether or not they're secure for consumption. As a result, their awareness in meals and drinks should be regulated via legislation if one wants to prevent excessive intake.

Consumption of soft drinks is popular among all age group. A thorough survey is much needed to analyze the content of sweeteners in the soft drinks available in the stores of Dhaka. In this study, artificial sweeteners were found to be present in high concentration in all analyzed products. Mean concentration of aspartame, saccharine, cyclamate and sucrose were found to lie in the range between 11-104, 1-9, 45-165 and 1800-2000 μg/ml, respectively. Several studies over the years found viable links between soft drink intake and medical problems, the results of which, however, remain contested.

The findings of this research work will provide a thumbnail sketch for future analysis to examine the artificial sugar content, not only in soft drinks but also in other food materials which include baked good, yogurts etc. Although the current work does not account for potential changes over time which might occur due to different methodologies adopted by manufacturers, the different compound being used, sampling error or contamination of samples, among others but it will make key contribution to further pursue the analysis of these widely used sweeteners.

This method is very practical because it permits one to achieve a better sensitivity without the need for expensive methods such as HPLC or electrophoresis. The methodology requires little analysis time, is rapid and costs low. The practical significance of the method is validated in terms of sensitivity, linearity, reproducibility, repeatability, recovery and robustness. The method applied was completely validated and suitable for quality control laboratories, where economy and time are essential.

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