Quantitative determination of artificial sweeteners and sucrose in energy drinks and mango juice available in Dhaka city by UV-Spectrophotometric method

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE BACHELORS OF SCIENCE IN BIOTECHNOLOGY

Submitted by-

Kabirul Islam Kanon

ID: 12236002

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Biotechnology Program

Department of Mathematics and Natural Sciences

BRAC University

Bangladesh

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled "Quantitative determination of artificial sweeteners and sucrose in energy drinks and mango juice available in Dhaka city by UV- Spectrophotometric method" submitted by the undersigned has been carried out under the supervision of Dr. M. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

Candidate:

_____

Kabirul Islam Kanon

Certified:

_____

Dr. M. Mahboob Hossain

Supervisor

Professor

Department of Mathematics and Natural Sciences

BRAC University, Dhaka

Dedicated

TO

My Parents

Acknowledgement

I acknowledge my esteem to Professor **A F M Yusuf Haider**, Chairperson of MNS Department of BRAC University for allowing me and encouraging me to complete my undergraduate thesis. I am much beholden to Professor **A. A. Ziauddin Ahmad**, former Chairperson of MNS department of BRAC University

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Abbreviations

µg	**microgram**
µg/ml	**microgram per milliliter**
µl	**microliter**
ASB	**Artificially Sweetened Beverage**
CTAB	**Cetyl trimethyl ammonium bromide**
DNSA	**3,5-Dinitrosalicyclic acid**
et al	**And Others**
FDA	**Food and Drug Administration**
g/mol	**gram per mole**
GRAS	**Generally Recognized as Safe**
IUPAC	**International Union of Pure and Applied Chemistry**
kHz	**kilo Hertz**
M	**Molar**
mg	**milligram**
mg/ml	**milligram per milliliter**
ml	**milliliter**
mM	**milli Molar**
NHL	**Non-Hodgkin Lymphoma**
nm	**nanometer**
rpm	**Rotation per minute**
SSB	**Sugar Sweetened Beverage**
v/v	**volume by volume**
w/v	**weight by volume**

Abstract

Energy dinks and mango juice are popular beverages. Apart from the natural ingredients and some additives present in these drinks, sugar is an important component of both. It has been established that other than providing sweetness, sugars are potent to bring about health consequences to its consumers. Sweeteners, both artificial (aspartame, cyclamate and saccharin) and natural (sucrose) were, our center of interest. The aim of this study was to determine the presence and if present then the levels of these sweeteners in energy drinks and mango juice. Simple spectrophotometric methods were used to determine the concentration of the mentioned sugars. For this purpose, a total of 42 samples of 7 different brands were collected from different locations of the Dhaka city, Bangladesh. The methods were found to be linear over the concentration range of 10-26 µg/ml (r^2= 0.9989), 137-320 µg/ml (r^2= 0.9891), 2.5-24 µg/ml (r^2= 0.9915) and 2354-2784 µg/ml (r^2= 0.9985) for aspartame, cyclamate, saccharin and sucrose, respectively. Mango juice contained relatively lower amount of saccharin compared to energy drinks. In case of aspartame, one brand of energy drinks had the least amount. Moreover, both energy drinks and mango juice had a similar content of cyclamate but one brand of mango juice had a relatively low content of cyclamate.

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Chapter 1

INTRODUCTION

1. Introduction:

1.1 General overview

Energy drinks and mango juice are two different branches of cold beverage. Undoubtedly, these two branch lies on opposite horizon based on their constituents and taste. At two points, both of them are considered to be same, as both tastes sweet due to the presence of sugars in them and both are consumed as a substitute of water. Although, energy drinks are believed to provide energy and enhance performance; but mango juice are consumed in order to get the taste of lovely mango regardless of it, being a seasonal fruit. May be mango juice are playing the role of providing the taste of mango throughout the year to the mango lovers. Serious health outcomes may arise to consumers of these two-beverage due to their composition. Moving apart from the lime light of health consequences from caffeine in energy drinks and mango juice being composed of synthetic colours and flavours, there comes the topic of sugar, a silent killer. Sugars, both natural and artificial sweeteners are much less talked about topics in our part of the world. Earlier this year, an article has been published in a popular daily bulletin stating about the adverse effects of consuming energy drinks (The Independent, 24 March, 2017). More importantly, both the group target the young citizens and children through their advertisements. However, among the consumers of energy drinks and mango juice, children and young adults are far away ahead than consumers of other age group.

1.2 A brief of energy drinks

Energy drinks is a different class of beverage which are said to provide instant energy. This class is the new extension to beverage family. Energy drinks are usually composed of caffeine, taurine, l-carnitine, glucuronolactone, vitamins and other herbal supplements like ginseng and guarana among others. Caffeine content of energy drinks may increase due to the presence of additives like guarana, yerba mate, cocoa and kola (Ishak *et al.*,2012). Ibrahim and Iftikhar (2014) states that energy drinks contains sugar at a higher amount. Manufacturer of these drinks affirms that energy drinks are potent to provide increased energy, attention and improve sports performance and concentration time; nonetheless, health professionals are concerned about the inauspicious effects of its consumption (McGraw, 2013). The energy drinks industry has since grown exponentially, becoming a multibillion dollar market. Consumption of energy drinks in the UK has increased by 155%, from 235 to 600 million

liters from 2006 to 2014 (Visram *et al.*, 2016). Target group of energy drinks marketing is commonly youth oriented and young adults of age between 18-34 years. In 2011, the European Food Safety Authority (EFSA) commissioned a study to gather consumption data for energy drinks in 16 countries of the European Union. They found that young people aged 10–18 years had the highest reported consumption prevalence (68%), compared with adults over 18 years (30%) and children under 10 years (18%). Energy drinks is first marketed by a Japanese pharmaceutical company, Taisho, with the brand name of Lipovitan D. Initially the target group was employees working at night shift in 1961. From there, the idea was picked by an Austrian businessman, Dietrich Mateschitz, who later started the company Red Bull GmbH along with his two Thai business partners (Hossain *et al.,*2014).

1.3 Mango from trees to PET bottles

Mango (Mangifera *indica L.*) belongs to the family of Anacardiaceae (Cashew family). Mango is a sweet delicious fleshy and juicy fruit, which is indigenous to South Asia. Mango is known as "the king of fruits" due to its popularity as seasonal fruit in tropical regions (Usman *et al.,* 2001). Due to its unavailability during other seasons, there is a vast demand of its unique taste and flavour to general people. Although mango was grown in India about 5000 years ago but mango seeds has traveled from Asia to the Middle East, East Africa and South America with humans starting from 300 or 400 AD. (http://mango.org). To meet the peoples demand for the crave of mango, manufacturer have come up with its fruit extract as juice. There are several brands of mango juice available in Bangladesh, main target group of these juice are young people, although they have a popularity in common people irrespective of their age.

1.4 Yesterday, today and tomorrow of sugar

Sugar is the name collectively given to substances which tastes sweet. Sweetness is a substantial sensation to human being. Sugars are used in order to make foods and drinks taste sweet. These substances are white crystalline in appearance, which is composed of carbon, hydrogen and oxygen in fixed proportion. Sucrose is a carbohydrate known as table sugar and abundantly consumed as a sweetening agent. Previously, sucrose has been utilized in great quantities as it was the only type available then. It plays a key role in taste of food and drinks,

as well as meeting the demand of carbohydrate in the body. Honey has been source of sweetness from ancient time (Bright, 1999). Although, there are various sources of natural sucrose, which includes raw honey, dates, coconut sugar, maple syrup, blackstrap molasses, brown rice syrup, real fruit jam etc. In spite of being sweet in taste and calorie giving, sugar has a direct co-relation with obesity, tooth-decay diabetes and heart disease.

In order to avoid the straight harmful consequences of sugar, world has shifted to the usage of artificial sweetening agent. At present, sweetening agent has been classified into two categories based on their contribution of energy: caloric sugar and non-caloric sugar. Sucrose falls under the category of caloric sugar. Artificial sweeteners come underneath the class of non-caloric sugar which is also known as low-calorie sweeteners, intense sweeteners. Moreover, artificial sweeteners are derivatives of different chemical compound that are potent to forge sensation of sweet taste (Swithers, 2013). Non-caloric sweeteners have a higher intensity of sweetness compared to caloric sweeteners (Gardner *et al.,* 2012). Since, the discovery of artificial sugar, their demand is perpetual and their usage has turned versatile by capturing the market within a short span of time due to their promotional tagging of being "Non-caloric". To be precise, this write-up focuses on three of the approved non-caloric sugar (aspartame, cyclamate and saccharin) and most abundantly used caloric sugar (sucrose).

In the last century, several artificial sweeteners have been discovered, where some of them are accidentally produced and some of them are intentionally synthesized in order to meet the relentless demand of non-caloric sugar as well as to compete with existing ones (Lindley, 1999). This phase of discovery can be categorized into „two generations". Saccharin, cyclamate and aspartame belongs to first generation, on the other hand acesulfame-K, sucralose, alitame, neotame and stevia comes under the „new or second" generation (Bright, 1999).

Sweeteners	Key market areas
Acesulfame-K	North America, Europe and Asia
Alitame	Oceania, South/ Central America
Aspartame	North America, Europe and Asia
Cyclamate	Europe and Asia
Neohesperidine DC	Europe and Japan
Neotame	Under FDA review
Saccharin	Asia, Europe and USA
Stevioside	Asia
Sucralose	North America
Thaumatin	Europe and Asia

Table 1: List of artificial sugars and their market areas (Lindley, 1999)

1.4.1 The pioneer of a new era: Saccharin (E954)

Saccharin is the first artificial sweetener accidentally discovered in 1879 by Constantine Fahlberg at John Hopkins University while working on coal tar derivatives in order to identify new preservatives (Kauffman & Priebe, 1978). It is 300 times sweeter than sucrose, but has a bitter or metallic aftertaste (Yang, 2010). IUPAC name of saccharin is 1,1-dioxo-1,2-benzothiazol-3-one (benzoic sulfinide) and a chemical formula of $C_7H_5NO_3S$. It is easy to synthesize, cheap and stable when heated up, considering it to be suitable for bakery usage. Saccharin is 2 fused rings: one phenyl ring and another, 5 membered ring with a carboxyl group, a nitrogen in the ring (making the ring heterocyclic), and a sulfone group next to the nitrogen. By 1979, 44 million Americans used saccharin on daily basis (Hicks, 2010), this has been the accumulated result of two World Wars and preference of people to be slim-fit by controlling energy intake. Saccharin is mostly used in soft drinks, dessert mix, yogurt and as a table-top sweetener.

Sodium and calcium salts of saccharin were first added in the FDA Generally Regarded as Safe (GRAS) list in 1959 and ammonium saccharin was added in 1961. In 1972, it was removed from GRAS list based on a study report from Wisconsin Alumni Research Foundation (WARF) (Tisdel *et al.,* 1974) on the doubt of its safety of consumption. Food and Drug Administration (FDA) recommended a ban on the use of saccharin in 1977, based on the findings of a group of researchers, who concluded it to be a weak carcinogen of bladder cancer in rats (Arnold *et al*., 1980). Although FDA could not ban the use of saccharin due to the Saccharin Study and Labeling Act (SSLA), though it mentioned about a mandatory labeling of its immanency as "USE OF THIS PRODUCT MAY BE HAZARDOUS TO YOUR HEALTH. THIS PRODUCT CONTAINS SACCHARIN WHICH HAS BEEN DETERMINED TO CAUSE CANCER IN LABORATORY ANIMALS" (Schiffman and Gatlin, 1993).

1.4.1.1 Synthesis of Saccharin

Toluene is the basic raw material for the synthesis of saccharin. Cl_2SO_2OH is added to toluene, forming 2 products: one with the SO_2Cl group attached at the *ortho* site of the phenyl group, and one with the SO_2Cl group at the *para* site of the phenyl group. The *para* product is discharged and the other product is used in the next reaction, where ammonium carbonate is added to the reaction mixture. Ammonium chloride is formed, as ammonium ion replaces the chlorine in the molecule and forms the new product. The reaction is purified and separated. Potassium permanganate

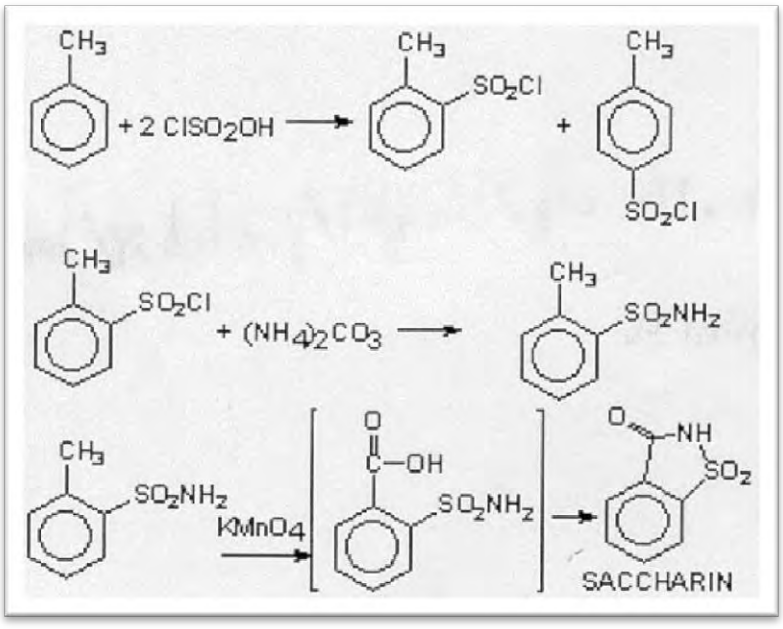

Figure 01: Sequence of chemical reaction in synthesis of saccharin. (Courtesy: Hodgin, G. (n.d.). The History, Synthesis, Metabolism and Uses of Artificial Sweeteners. Retrieved November 12, 2017, from http://monsanto.unveiled.info/products/aspartme.htm)

of higher concentration and the purified newly formed product are allowed to react at temperature of 150 ^{0}C. The methyl group (-CH_3) in the molecule is oxidized to form a carboxyl group, COOH. Alongside, the formation of carboxyl group, the molecule self-cyclizes forming saccharin by removing one molecule of water, when this happens, the molecule self-cyclizes, releasing water and producing saccharin. After this, a base is added to exchange the hydrogen on the nitrogen atom with a metal ion, like sodium or calcium.

1.4.2 Another invention by accident: Aspartame (E951)

The discovery of aspartame is also inadvertent, in 1965, James M. Schlatter, a chemist at G.D. Searle and Company was working on the production of an anti-ulcer drug. In order to detect efficacy of new anti-ulcer drug, he was synthesizing a tetra-peptide which is naturally produced in the stomach. In the process of the synthesis, he produced a dipeptide as an intermediate, aspartyl-phenylalanine methyl ester, which is known as aspartame. Schlatter unintentionally tasted a sweet taste on his finger. As a consequence, he and his lab partner Harman Lowrie, tasted the dipeptide in 10 ml of black coffee, he thought as the compound is amino acid derivative so it should safe for human consumption (Walters, 2001).

Aspartame (ASP), 1-methyl N-L-a-aspartyl-l-phenylalanine is a dipeptide composed of aspartic acid, phenylalanine and methanol (Figure 02) (Ashok *et al.,* 2013a). There are two forms of aspartame, an alpha and a beta form, whereas only the alpha form is sweet (Gimba *et al.,* 2014). It is a white crystalline powder and has a sweetness of 200 times compared to sucrose (Mazurek & Szostak, 2011) and has a molecular formula of $C_{14}H_{18}N_2O_5$. Aspartame has a dulcet sweetness without a bitter aftertaste (Oyama *et al*. 1984). At present, more than 200 million people worldwide consumes aspartame as a sweetener and used nearly in 6000 food products, it is used as a non-nutritive sweetener, which includes dry beverage mixes, chewable multi-vitamins, breakfast cereals, chewing gum, puddings and fillings, carbonated beverages, refrigerated and nonrefrigerated ready to drink beverages, yoghurt products, and pharmaceuticals (Okasha, 2016). The FDA approved application of aspartame in dry products such as breakfast cereals, chewing gum, instant tea and coffee, dairy products and as a table-top sweetener. However, in a short time FDA ordered a stay on its use, which was lifted in 1981 according to the 1974 ruling. In 1984, it was allowed to use in beverages although in 1983 additional usage of aspartame was approved by the United States (Schiffman and Gatlin, 1993).

Aspartame is more stable in solid form and should be stored in an airtight container. Certain changes in moisture, temperature and pH can cause hydrolysis of the compound and would gradually lose its sweetness. As a consequence, aspartame is suitable for usage in frozen desserts, chocolate, UHT drinks or beverages while it is unsuitable for baking.

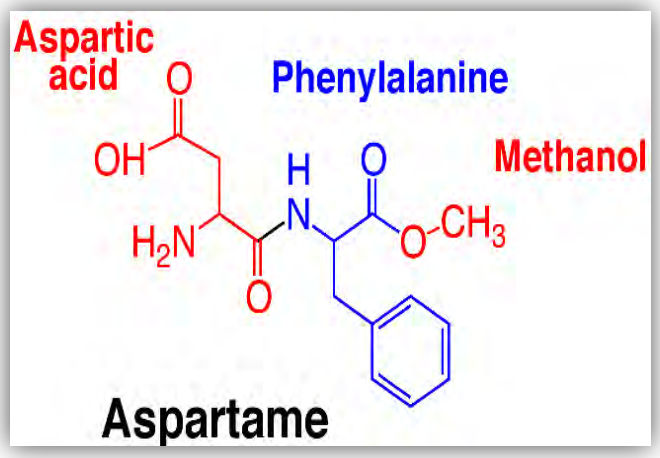

Figure 2: Structure of Aspartame (courtesy: (n.d.). Retrieved November 14, 2017, from http://www.sweetenerbook.com/aspartame_2.html)

Unlike other artificial sweeteners, it readily hydrolyzes to its constituent amino acids, phenylalanine (50%), aspartic acid (40%) and methanol (10%) in the gastrointestinal tract (Singh *et al*., 2013). Aspartic acid is a non- essential amino acid, which means body can itself synthesize it. Aspartic acids play in role in synthesis of DNA and urea and as a neurotransmitter in the brain (Walters, 2001). Surplus aspartic acids are broken down to fumarate, which then releases energy through the tricarboxylic acid cycle. Whereas phenylalanine is an essential amino acid, so it should be supplemented through diet. Phenylalanine acts as a predecessor of tyrosine and several neurotransmitter biogenesis. Abundance phenylalanine is catalyzed to fumarate and acetoacetate, both of which release energy. However, people with phenylketonuria lacks the enzyme that catalyzes phenylalanine to tyrosine, as a consequence excess phenylalanine is converted to phenylketones. Usually phenylketones are discharged through urine, if this condition remains untreated; it may lead to intellectual malfunction and other serious health problems (Genetics home reference, 2017). Therefore, FDA has made it obligatory to label products containing aspartame (phenylalanine), in order to alert the phenylketonurics about the presence of aspartame (phenylalanine) as an inactive ingredient (US-FDA). In humans, methanol is metabolized into formaldehyde (HCHO) in the liver by alcohol dehydrogenase (Cederbaum, 2012). Formaldehyde is oxidized to formic acid (HCOOH) by formaldehyde dehydrogenase.

1.4.3 Descendants of the new era: Cyclamate (E952)

Sodium cyclamate or sodium cyclohexylsulfamate was synthesized by Ludwig Frederick Audrieth and Michael Sveda in 1937 at University of Illinois. The sweet taste of cyclohexylamine was accidentally witnessed by Sveda during their work (Bopp *et al.,* 1986). Three different compounds like cyclamic acid, calcium cyclamate and sodium cyclamate are commonly known as cyclamate (Mortensen, 2006). The salts of cyclamate are stable in heat, cold and have good shelf-life. The stability and solubility of cyclamate in water makes it preferable for usage in foods and beverages. Cyclamate is 30 times sweeter than sugar (Sardesai & Waldshan, 1991). Although it has least sweetness level among the artificial sweeteners, but in combination with other intense sweeteners it provides a synergistic effect by cloaking the aftertaste of a single intense sweetener. In 1957 Abbot Laboratories formulated a blend of cyclamate and saccharin in the ratio 10:1, as saccharin is approximately 10 times sweeter than cyclamate. The blend was marketed as Sucaryl®. Taste of the blend was really good as the aftertaste of saccharin has gone due to cyclamate‟s synergistic effect. This formulation leads the path of producing Zero- and Low-calorie foods and beverages without major changes in taste.

Moreover, cyclamate was first marketed in 1950 by Abbot Laboratories, both salts of cyclamate (sodium and calcium) were classified as GRAS (Generally Regarded as Safe) by US FDA, after the enactment of the Food Additive Amendment in 1958 (Schiffman & Gatlin, 1993). Kojima and Ichibagase (1966), reported that cyclamate can be metabolized by gut bacteria to cyclohexylamine. A specific genus of gut flora known as enterococci has the potential to breakdown cyclamate, which are not present in all human; its presence was observed in one of three people, accounted by Drasar, Renwick and Williams in 1972. As a consequence, FDA has removed

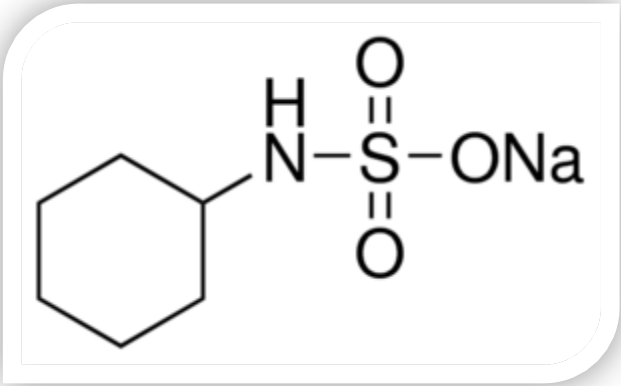

Figure 3: Structure of cyclamate
(Courtesy: Sodium Cyclamate 47827. (n.d.). Retrieved November 15, 2017, from https://www.sigmaaldrich.com/catalog/product/supelco/47827?lang=en®ion=BD)

cyclamate from GRAS list. Price *et al*. (1970) stated that cyclamate at higher concentration with saccharin caused bladder cancer in rats and therefore, its usage as food additive in USA is banned by FDA. Although, the halt was relieved internationally, currently 50 countries permitted the sale of cyclamate but not USA (Cyclamate- regulatory controversy, n.d.).

1.5 Consequences of consuming artificial sweeteners or non-caloric sweeteners:

- **Risk of cancers**

  Relation between consumption of artificial sweeteners and risk of lymphoma and leukemia were discussed by Schernhammer *et al.* (2012). The research has been performed for both mice and humans, the results for rats are directly corelated for humans. They have observed a positive association between diet beverages and total aspartame intake and risks of Non-Hodgkin lymphoma (NHL) and multiple myeloma elevates significantly for subjects who consumed at least one serving per day compared to non-consumers in multivariable models. On contrary, no evidence of association to NHL and multiple myeloma is observed for women.

- **Glucose intolerance**

  Artificial sweeteners are said to be „metabolically inert" and are expected to have no physiological effect on the consumers (Suez *et al.,* 2015). A recent paper published in Nature states that artificial sweeteners can cause glucose intolerance to the consumers by altering the gut micro-flora (Suez *et al.,* 2014). The schematic experimental design is illustrated in Figure 4.

- **Weight gain**

  The San Antonio Heart Study observed changes in weight between men and women over a period of 7-8 years. According to Fowler *et al.*, (2008), possibility of putting in weight and obesity has a positive correlation with the consumers of artificially sweetened beverages (ASB) compared to non-consumers of ASB, regardless of being normal weight or over weight at baseline. On a cross-sectional study conducted for 2 years on adolescents showed ASB consumption was associated with increase in body mass index (BMI) and increased body fat in male and female. On contrary, a

longitudinal study showed that sugar sweetened beverage (SSB) consumption resulted in BMI increase only in males, whereas, there was no such evidence associated with females for SSB consumption (Laska *et al*., 2011). Although, none of the study mentioned ASB consumption was associated with reduced risk of weight gain or increased body fat percentage.

- **Metabolic syndrome:**

  There has been reports of metabolic syndrome resulted from consumption of ASB (Artificially Sweetened Beverages), in a variety of cohort study (Swithers, 2013). A study of CARDIA (Coronary Artery Risk Development in Young Adults), showed positive guild of metabolic syndrome with ASB, it also stated that the risk increases approximately by 17% (Duffey, *et al.*,2012). In addition, two other study conducted by Framingham offspring and MESA concluded a similar health outcome due to consumption of greater than one serving of ASB per day. Sample sizes of the studies were 6039 and 5011 men & women respectively and were conducted for 4 years and 2-5 years for the latter (Dhingra, *et al*., 2007) (Nettleton *et al.*, 2009). The magnitudes of risk associated with the consumption of SSB (Sugar Sweetened Beverages) were similar to as consumption of ASB, reported from these researches.

- **Type 2 diabetes**

  Manufacturers usually endorses the non-caloric product (ASB) as diabetic product to target people with T2D. Despite that, a study of European E3N showed; risk of T2D was more than doubled for frequent consumers of ASB compared to the non-consumers and SSB consumption was also linked with increased likelihood of T2D (Bhupathiraju *et al.*, 2013). Data from the Nurses‟ Health Study (NHS) revealed that possibility of T2D would be triggered due to consumption of one ASB or SSB per day at minimum. Notably, a recent finding states that chances of getting T2D would be elevated with intake of ASB in persons who are normal weight at baseline (Romaguera *et al.*,2013).

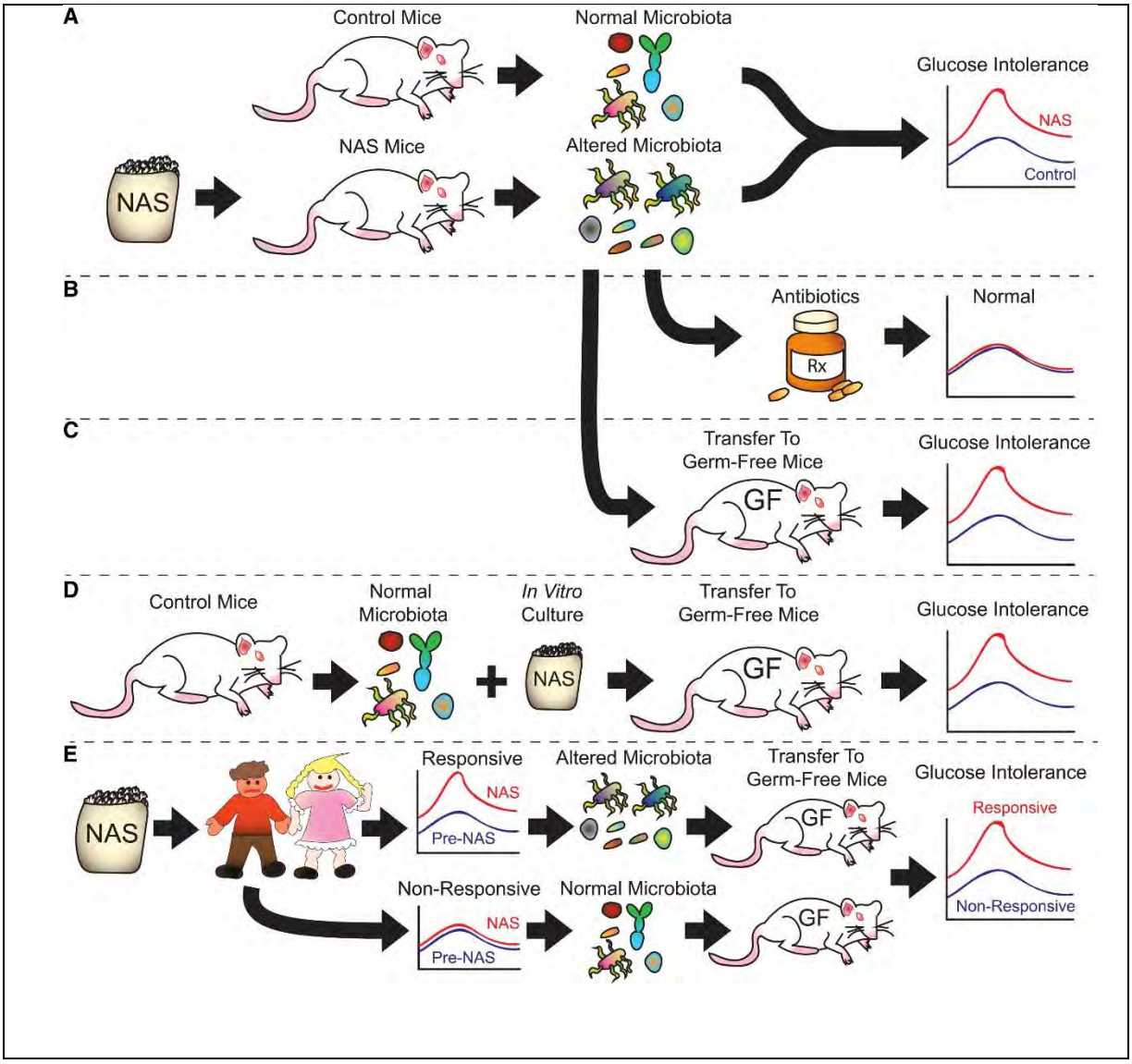

Figure 4: Non-caloric artificial sweeteners (NAS) inducing glucose intolerance in human and mice (NAS induction). NAS induction (A): Mice fed NAS developed altered intestinal microbial glucose intolerance. Antibiotic suppression (B): treating these mice with antibiotics countered this effect, indicating microbial involvement. Microbial transfer (C) from NAS-fed mice to germ-free (GF) mice fed normal chow induced glucose intolerance, compared to GF mice receiving control microbiota. NAS directly affects microbiota (D): Microbiota from control mice were grown in the presence of NAS in vitro and transferred to GF mice, inducing glucose intolerance compared to microbiota cultured without NAS. Personalized human response depends on microbiota (E): The responsiveness of adult human patients to NAS-induced glucose intolerance depended on prior microbial composition. When transferred to GF mice, microbiota from NAS-responsive patients induced glucose intolerance, while microbiota from NAS-non-responsive patients did not.

1.6 Research Objectives

Energy drinks and mango juice are frequently consumed by adolescents. These drinks are sweet in nature, so are expected to contain sugars like sucrose and artificial sweeteners like aspartame, cyclamate and saccharin. Artificial sweeteners have adverse effects on the consumers. As a consequence, the necessity to observe the amount of caloric and non-caloric sweeteners present on these drinks arises. The research focuses on the quantitative determination of caloric and non-caloric sugars present in Energy drinks and Mango juice available in Dhaka, Bangladesh.

Chapter 2

Materials & Methods

2.1 Sample collection:

In total 42 samples were collected, in which two samples for each batch and three batches of seven brands. Out of the seven brands, there were four brands of energy drinks (three of them were national and one was international) and three brands of mango juice (all of them were national).

Table 2: Brand names of all samples along with their batch number and manufacturing dates

Brands (Manufacturer)	**Batch Numbers, Manufacturing date (Mfg)**		
	01	02	03
Speed (Akij Food & Beverage Ltd.)	Batch No.: 856 S 417 (0857) Mfg:10.09.2017	Batch No.: 736 S 417 (0237) Mfg: 12.08.2017	Batch No.:823 (2345) Mfg: 06.08.2017
Power (PRAN Foods Ltd.)	Batch No.: 02 (L-3 05:28) Mfg: 11.01.2017	Batch No.: 58 (L-3 00:05) Mfg:12.06.2017	Batch No.: 136 (L-2 01:12) Mfg: 19.08.2017
Royal Tiger (Globe Soft Drinks Ltd)	Batch No.: 321605 (T 04:13) Mfg: 17.09.2017	Batch No.: 317905 (T-04) Mfg:18.08.2017	Batch No.: 322005 (T 19:55) Mfg:20.09.2017
Red Bull (Red Bull Gmbh)	Batch No.: 1514499 (16:49) Mfg: no date given	Batch No.:1518788 Mfg:23.11.2016 (09:30D 3)	Batch No.: 15164598 Mfg:
Pran Frooto (PRAN Foods Ltd.)	Batch No.: 09 “এ” Mfg: 23.08.2017 (P-2)	Batch No.:07 “এ” Mfg: 23.07.2017 (P-1)	Batch No.: 01 “এ” Mfg: 07.08.2017 (P-1)
Frutika (Akij Food & Beverage Ltd.)	Batch No.: 194 F.M. 17 Mfg:30.07.2017 (2252)	Batch No.: 163 F.M.17 Mfg:06.07.2017 (0210)	Batch No.: 215 F.M. 17 Mfg: 21.08.2017 (0634)
Mangolee (AST Beverage Ltd.)	Batch No.:12303 (T:12:19) Mfg: 19.04.2017	Batch No.:41403 (T:15:29) Mfg: 09.08.2017	Batch No.: 32504 Mfg:

2.2 Sample preparation

All the energy drink samples were decarbonated using ultrasonic bath at 40 kHz frequency for 10 minutes in test tube.

Fruit juice is usually composed of pulps or solid fruit parts, which are likely to interfere the result of spectrophotometer. As a consequence, fruit juice will be treated with several chemicals to obtain the desired result (artificial sweetener). With the demand of expected result, mango juice sample should be treated differently. Artificial sweeteners or sugars were dissolved in the liquid portion of juice or fruit drinks. Initially, 5 ml of sample was dissolved in distilled water to make a total volume of 50 ml (10 x dilutions). The mixture was shaken gently using vortex machine to homogenize the solution. At first the solution was filtered with cotton and then by Whiteman type 1 filter paper. Pale yellow or white filtrate were obtained and stored for further analysis.

2.3. Saccharin

A simple extractive spectrophotometric method was used for the determination of saccharin from energy drinks and mango juice. This method has been proposed by a group of scientists (Mathew *et al.,* 2006). The method was based on the bromination of saccharin to form N-bromo- derivative, which then releases iodine by reacting with potassium iodide. Upon addition of the surfactant cetyl trimethyl ammonium bromide (CTAB), magnitude of the yellow colour increases. Absorbance is taken at 400 nm followed by extraction of the reaction mixture with isoamyl alcohol.

2.3.1 Equipment

- Fume hood (LabTech, Canada)
- Digital Balance (Shimadzu Corporation, Japan)
- Separating funnel (SIMAX, Czech Republic)
- Water bath (Biobase, USA)
- Ultrasonic bath (HumanLab Instrument Co., Korea)
- Spectrophotometer (Model: EMC-61PC-UV) (EMCLAB, Switzerland)

2.3.2 Reagents

- Saccharin powder (for standard)
- Bromine water (Merk Specialities Pvt. Ltd, India)
- Potassium iodide (Merk Specialities Pvt. Ltd, India)
- Formic acid (Merk Specialities Pvt. Ltd, India)
- Cetyl trimethyl ammonium bromide (CTAB) (Sigma-Aldrich, Germany)
- Sulphuric acid (Merk, Germany)
- Hydrochloric acid (Merk, Germany)
- Sodium hydrogen carbonate (Merk, Germany)
- Isoamyl alcohol (Sigma-Aldrich, Germany)
- Diethyl ether (Merk Specialities Pvt. Ltd, India)

2.3.3 Preparations

1. Saccharin standard solution: Fifty milliliters of 1 mg/ml stock solution was prepared by dissolving 50 mg of saccharin powder in 50 ml of water. Serial dilution was performed in order to prepare standard solution of concentration ranging from 2µg – 34 µg.
2. Formic acid: Equal volume of formic acid and water was added to prepare 50% v/v. For example, to prepare 50 ml of 50% v/v formic acid, 25 ml of formic acid was added with 25 ml water.
3. Potassium iodide: To prepare 1% w/v of potassium iodide 1g was dissolved in 100 ml water.
4. Cetyl trimethyl ammonium bromide (CTAB): Molecular mass of CTAB($C_{19}H_{42}BrN$) is 364.45 g/mol. A stock of 0.1 M was prepared by dissolving 1.822 g of CTAB in 50 ml water. The stock solution was diluted accordingly to prepare a solution of 1 mM.
5. Sulphuric acid: Concentrated sulphuric acid (98%) was diluted to 10% using the formula, $C_1V_1=C_2V_2$.
6. Hydrochloric acid: Concentrated hydrochloric acid (37%) was converted to 5% using the same formula.
7. Sodium hydrogen carbonate: Molar mass is 84 g/mol. Two grams of $NaHCO_3$ was dissolved in 100 ml water to prepare 2% solution of $NaHCO_3$.

2.3.4 Procedure

2.3.4.1 Standard curve establishment

The extraction and determination procedure for standard and sample were based on the method described by Mathew *et al.* (2006).

According to Mathew and his group, to an aliquot of 5 ml of standard solution containing 2 µg – 34 µg of saccharin, 0.5 ml of bromine water was added and shaken gently for 2 minutes. Dropwise formic acid was added to dissolve the excess bromine, then 1 ml of potassium iodide was added. The yellow solution obtained was shaken for few seconds and 1 ml of cetyl trimethyl ammonium bromide was added and shaken well. Then the volume of the solution was made to 50 ml by adding water and transferred to a 100 ml separating funnel. Reaction mixture was washed twice, using 3 ml of isoamyl alcohol and the lower layer was extracted (yellow layer) (Figure 4). Absorbance was taken at 400 nm and the values were recorded. A blank was also prepared using 5 ml of water. A standard curve was plotted using the standard saccharin concentration and absorbance obtained.

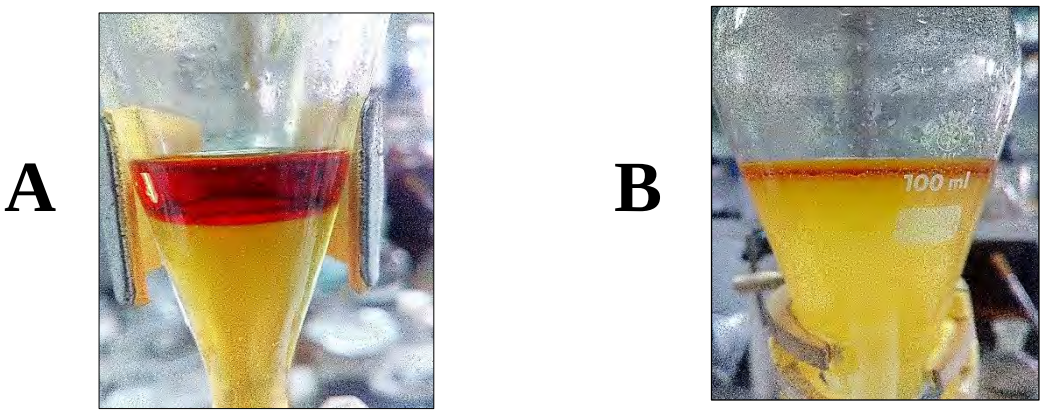

Figure 5: Extraction with isoamyl alcohol. A: First time extraction. B: Second time extraction.

2.3.4.2 Working with sample

Extraction of saccharin from sample:

Ten milliliters of decarbonated energy drinks or juice extract was transferred to a separating funnel. One milliliter of 10% sulphuric acid was added and shaken gently. The mixture was extracted twice with 6 ml of diethyl ether and the lower layer was discarded. The upper ether layer was extracted twice with 2% sodium hydrogen carbonate and the ether layer was

discarded. The aqueous layer was acidified with 2 ml 5% hydrochloric acid and extracted twice with 5 ml of diethyl ether into a test tube. Then the test tube was heated in a water bath at 80℃, so that the ethereal extract evaporates. The residue on the test tube was dissolved in water and the volume was made up to 5 ml.

Using this dissolved extract, the procedure mentioned in 2.3.4.1 was repeated and the absorbance reading was recorded in a table. From the plotted standard curve, the concentration or amount of saccharin present in the sample can be obtained.

2.4 Aspartame

A simple spectrophotometric method has been developed by Lau and his group (1988) for the determination of aspartame in soft drinks based on the reaction of aspartame with ninhydrin. Extraction with propylene carbonate has improved the selectivity of the method. Optimum conditions for the extraction of aspartame and for the reaction of aspartame with propylene carbonate were maintained. The absorbance measurement was taken at 585 nm. The standard calibration curve plotted was linear in the range 5 µg/ml to 50 µg/ml. Assuming the standard curve as a reference, amount of aspartame present on different samples of energy drinks and fruit drinks were obtained.

2.4.1 Equipment

- Ultrasonic bath (HumanLab Instrument Co., Korea)
- Centrifuge machine (Model: DSC-200T) (DigiSystem Laboratory Instrument Inc., Taiwan)
- Water bath (Biobase, USA)
- Spectrophotometer (Model: EMC-61PC-UV) (EMCLAB, Switzerland)
- Digital balance (Shimadzu Corporation, Japan)
- Micropipette
- Centrifuge tube (15 ml)
- Vortex machine (SCILOGEX, USA)

2.4.2 Reagents

- Anhydrous sodium sulphate (Scharlab, Spain)
- Acetate buffer
- Propylene carbonate (EMD Millipore, USA)
- Absolute ethanol (EMD Millipore, USA)
- Ninhydrin

2.4.3 Preparation:

1. Acetate buffer: The buffer was prepared by mixing 50 ml of 0.2 M acetic acid solution with 50 ml of 0.2 M sodium acetate solution.

2. Ninhydrin: To produce 4% ninhydrin solution, 4 g of ninhydrin was dissolved in 100 ml absolute ethanol.

3. Aspartame standard solution: Ten milligrams of aspartame powder were dissolved in 10 ml propylene carbonate to prepare a stock of 1 mg/ml. Accurate serial dilution were performed to prepare standard solution of concentration ranging 5 µg/ml to 50 µg/ml.

2.4.4 Procedure:

The extraction and determination procedure for analysis of samples were based on the method described by Lau *et al.* (1988) and Celik *et al.* (2014).

2.4.4.1 Working with aspartame standard solution:

To an aliquot of 0.75 ml of standard aspartame solution 0.5 ml of acetate buffer of pH 3.5 was added in a centrifuge tube. 3 ml of propylene carbonate and 2 ml of absolute ethanol was added to the centrifuge tube. The mixture was placed in an ultrasonic bath for 5 minutes at a frequency of 40 kHz. Then centrifuged at 5000 rpm for 5 minutes for extraction of aspartame with the assistance of propylene carbonate. 3.5 ml of lower phase was collected in screw cap test tube and was dried with anhydrous sodium sulphate. After 20 minutes of settling, 2 ml of the dried solution was carefully taken into another screw cap test tube followed by addition of 2 ml 4% ninhydrin solution. Reaction mixture was

boiled in a hot water bath at 80℃ for 20 minutes. The solution was then cooled followed by dilution with ethanol to volume of 5 ml. Absorbance reading was taken at 585 nm and recorded in a table, using the absorbance value and the corresponding concentration a standard curve was plotted. For the blank, 0.75 ml of water was used.

2.4.4.2 Working with sample:

Same procedure was followed with decarbonated energy drinks and treated fruit drinks to obtain the concentration of aspartame present in them.

2.5 Cyclamate

A new method has been developed using ultrasound-assisted emulsification micro-extraction (USAE-ME) for the determination of cyclamate, the process is coupled with UV-Vis spectrophotometry. Basically, the method is supported by protonation of cyclamate ions in acidic medium. The cyclamic acid formed is extracted as fine droplets in presence of chloroform as an extraction solvent which contains rhodamine B (RhB) reagent. A highly coloured ion-pair complex of [cyclamate][$RhBH^{+}$] was formed when the extracted cyclamic acid further reacts with rhodamine B. In turn, the ion-pair complex formed can be used to spectrophotometrically detect the amount of cyclamate present. The proposed method can be efficiently used to determine the concentration of cyclamate in beverages and sweetener tablets. It is said the method is simple, rapid, inexpensive, accurate and remarkably free from interference effects.

2.5.1 Equipment

- Micropipette
- Ultrasonic bath (HumanLab Instrument Co., Korea)
- Centrifuge tube (15 ml)
- Centrifuge machine (Model: DSC-200T) (DigiSystem Laboratory Instrument Inc., Taiwan)
- Spectrophotometer (Model: EMC-61PC-UV) (EMCLAB, Switzerland)

2.5.2 Reagents

- Cyclamate standard solution
- Rhodamine B (RhB) (Sigma-Aldrich, Germany)
- Sulphuric acid (Merk, Germany)
- Chloroform (RCI Labscan Limited, Thailand)

2.5.3 Preparations

1. Cyclamate standard solution: Ten milligrams of cyclamate were dissolved in 10 ml of water to prepare a solution of concentration 1 mg/ml. The 1 mg/ml solution was in turn diluted 10 times to obtain a solution of concentration of 100 µg/ml. Thus, using appropriate serial dilution, standard solution of 25 µg/ml to 200 µg/ml were prepared.
2. Sulphuric acid: To obtain 100 ml of 0.1 M sulphuric acid, 8 ml of concentrated (18.4 M) acid was dissolved in 92 ml of water. Calculation was done using the formula, $C_1V_1=C_2V_2$.
3. Rhodamine B: 10 mg of rhodamine B powder was dissolved in 104.2 ml of chloroform to prepare RhB solution of concentration 2×10^{-4} M.

2.5.4 Procedure:

The extraction and determination procedure for standard and sample were based on the method described by Hashemi *et al.* (2015).

2.5.4.1 Working with standard cyclamate solution:

To an aliquot of 5 ml cyclamate standard solution, 5 ml of 0.1 M sulphuric acid was added in centrifuge tube. The tubes were immersed in ultrasonic bath for 1 minute. Liquid in centrifuge tube and ultrasonic bath should be in same level for efficient mixing. Two hundred microliter of RhB solution was injected into the solution using a micropipette. Emulsification and extraction was performed at 40 kHz of ultrasonic frequency for 20 seconds at 25±1℃. As a result, oil-in-water emulsions of chloroform in water was formed. After that, the emulsion was centrifuged at 3500 rpm for 5

minutes, which resulted in the formation of a coloured organic layer at the bottom of the tube. In case of blank, 5 ml of water was used instead of standard cyclamate solution. Then, 100µl of settled organic layer was dissolved in 2.9 ml of chloroform (30x dilution) in order to take the spectrophotometric reading. Absorbance reading were recorded in a table, standard curve was plotted against concentration and absorbance reading to determine the concentration of cyclamate present in various sample.

2.5.4.2 Working with sample:

Decarbonated samples of energy drinks should be 5x diluted and diluted solution should be treated in the same way as described in 2.5.4.1.

2.6 Sucrose

The method for the determination of sucrose in beverage has been developed by Gimba *et al.* (2014). This method is based on the acid digestion of sucrose followed by addition 3, 5-dinitrosalicylic acid (DNSA), which provided the colorimetric change which enabled us to detect the presence of sucrose.

2.6.1 Equipment

- Water bath (Biobase, USA)
- Spectrophotometer (Model: EMC-61PC-UV) (EMCLAB, Switzerland)
- Micropipette
- Vortex machine (SCILOGEX, USA)

2.6.2 Reagents

- Standard sugar solution
- Hydrochloric acid (Merk, Germany)
- Sodium hydroxide (Merk Specialities Pvt. Ltd, India)
- 3, 5-dinitrosalicylic acid (DNSA) (Sigma-Aldrich, Germany)

2.6.3 Preparation

1. Standard sugar solution: 100 mg of sucrose was dissolved in 100 ml of water to prepare a standard solution of 1 mg/ml. Serial dilution was performed to produce standard solution of concentration ranging 100 µg/ml to 1000 µg/ml.
2. Hydrochloric acid: Hundred and eighteen milliliters of concentrated hydrochloric acid (10.14 M) was added with 82 ml of water to prepare an acid solution of 6 M of 200 ml.
3. Sodium hydroxide: Ten grams of sodium hydroxide pellet was dissolved in 100 ml of water to produce a solution 2.5 M strength.
4. 3, 5-dinitrosalicyclic acid (DNSA): Chemical formula: $C_7H_4N_2O_7$. Molecular mass is 228.12 g/mol. To prepare 0.05 M of DNSA, 1.1406 g of DNSA powder was dissolved in 100 ml of water. Mild heating and shaking is required to dissolve the solute properly.

2.6.4 Procedure

2.6.4.1 Working with sucrose standard solution

Two milliliter of sucrose standard solution was pipetted into a test tube and 2 ml of water was added to it. 2 ml of 6 M hydrochloric acid was added to the test tube. Then, placed in a boiling water bath for 10 minutes at 80℃. The test tubes were removed and 8 ml of 2.5 M sodium hydroxide was added followed by 2 ml of 0.05 M 3,5-dinitrosalicyclic acid (DNSA). After addition of DNSA, it was immediately vortexed followed by inversion for proper mixing of the reacting components. Again, the tubes were placed in water bath for 5 minutes and equal time duration was maintained for all. The mixture was quickly transferred to ice water bath for 10 minutes after removing from boiling water bath. For blank, 2 ml of water was added instead of standard sucrose solution. The solutions were diluted 10 times before taking the spectrophotometric reading. Absorbance reading was taken at 580 nm against blank and the dates were recorded in a table to plot a standard curve of concentration against absorbance value.

2.6.4.2 Working with sample

The samples were treated in the same way as explained in 2.6.4.1 section, to determine the concentration of sucrose present in each sample.

All the reagents used in the following experiments are of Analysis or Synthesis grade, name of the manufacturer is given on the parenthesis besides the name of the chemicals on the reagent section. Double distilled water has been used throughout the experiments. All the apparatuses were washed with detergent followed by rinsing with 0.1 M nitric acid and acetone. followed by heating in a thermal heater for 20 minutes at 100℃.

2.7 Data Analysis

All the data were analyzed using Microsoft Office Excel version 2010.

Chapter 3

Results

The research work here focuses on the determination of the amount of sugar and three artificial sweeteners, aspartame, saccharin and cyclamate present in three local and one international energy drinks along with three local mango juice. The concentration was obtained from the standard curve plotted for each of the sugar element. The results found were completely experimental and might vary from batch to batch for each of the brand.

Standard curves were plotted by using the coefficient of multiple determinations for multiple regression or R-squared statistics in Microsoft Excel, with the aim to obtain best fit line. Averages of the three absorbance were used for plotting the standard curve for each known concentration of standard. The standard curve was plotted, keeping the known concentration of standard solutions on the x-axis and the obtained corresponding absorbance on the y-axis. In addition, unknown concentrations were obtained using the using the formula given by Excel. Batch mean of each brand for specific sugar is shown in separate tables along with the plotted standard curve for the respective sugar.

3.1. Sucrose

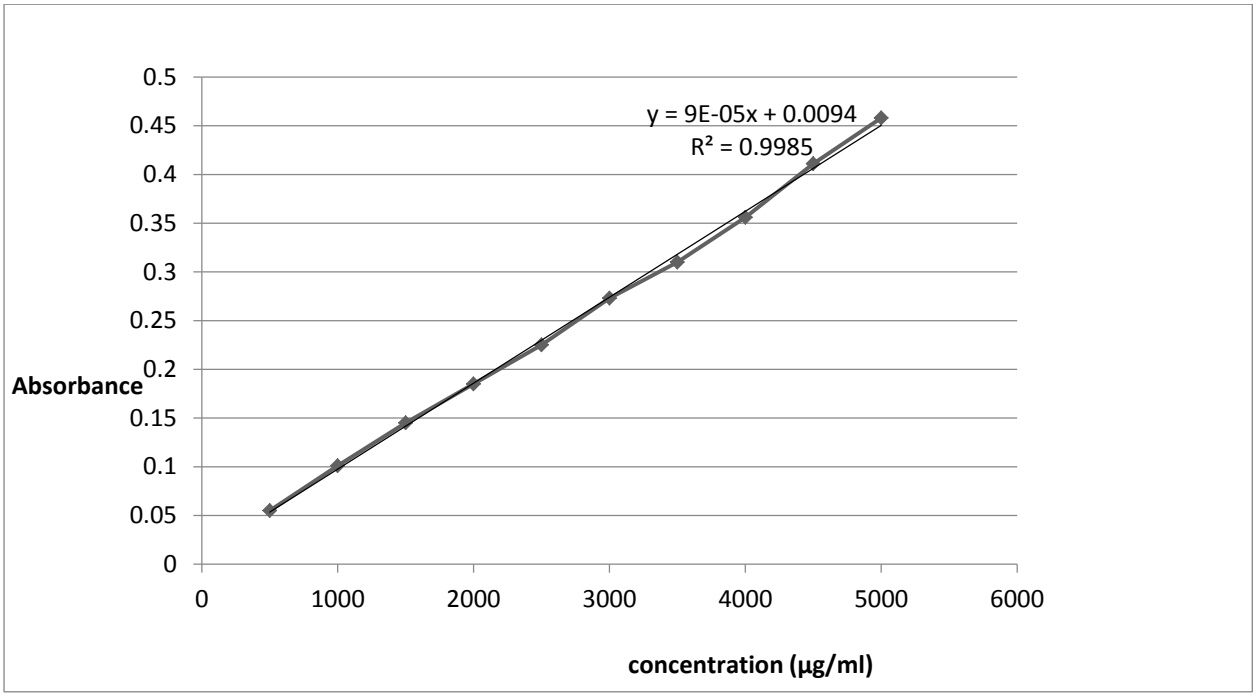

Figure 06: Standard curve for Sucrose

Table 3: Mean concentrations of sucrose present in three different batches of each brand

	Speed (µg/ml)	**Power** (µg/ml)	**Tiger** (µg/ml)	**Red Bull** (µg/ml)	**Frooto** (µg/ml)	**Frutika** (µg/ml)	**Mangolee** (µg/ml)
Batch 1	2537.22	2470.56	2353.89	2784.44	2567.78	2678.89	2484.44
Batch 2	2526.11	2417.78	2409.44	2706.67	2592.78	2509.44	2403.89
Batch 3	2467.78	2473.33	2445.56	2762.22	2487.22	2662.22	2415.00

The standard curve of sucrose had an equation, y = 0.00009x + 0.0094. The value of absorbance was inserted into the equation to obtain the unknown concentration. The r^2 (r-squared) value of the graph was 0.9985 and the mean recovery percentage was 98.51± 2.31%.

Mean sucrose content in Red Bull was highest. Variations in mean content for batches were more in Frutika. Lastly, the range of mean sucrose content was 2353.8 µg/ml to 2784.4 µg/ml.

3.2 Saccharin

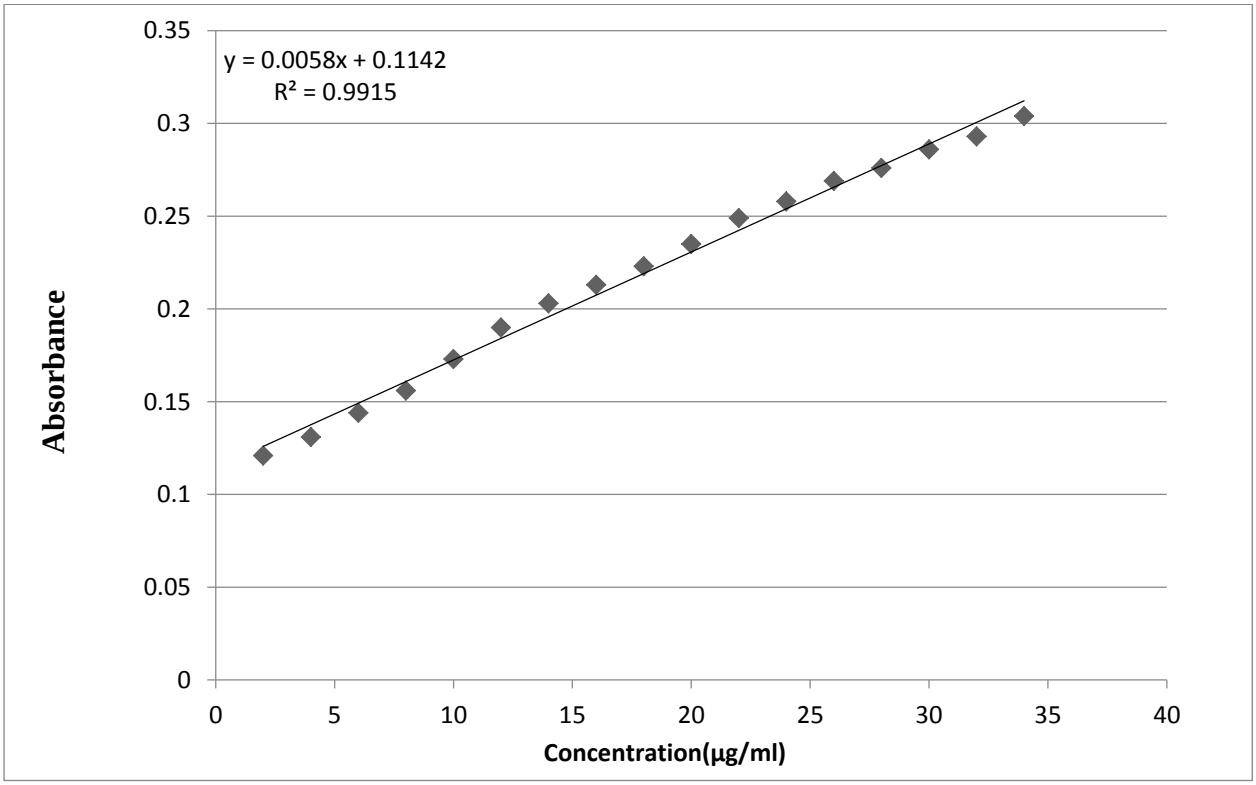

Figure 07: Standard curve of Saccharin

An approximate best fit line was obtained for the standard solution of saccharin, the curve had an equation of y = 0.0058x + 0.1142 with r-squared value of 0.9915. The extraction method of saccharin had the lowest mean recovery of 96.25 ± 12 % for standard. However, Frooto and Frutika were found to have least amount of saccharin. Although, the highest concentration of saccharin was obtained in Power. In addition, Red Bull had a more consistent aspartame content among its batches. Rest of the brand had a variation among their batches. The mean saccharin content had a range of 2.46 µg/ml to 23.81 µg/ml.

Table 4: Mean concentrations of Saccharin observed in three different batches of each brand

	Speed (µg/ml)	**Power** (µg/ml)	**Tiger** (µg/ml)	**Red Bull** (µg/ml)	**Frooto** (µg/ml)	**Frutika** (µg/ml)	**Mangolee** (µg/ml)
Batch 1	20.08	23.47	10.93	17.71	2.46	2.62	13.31
Batch 2	17.54	19.07	12.63	19.24	5.00	5.51	8.73
Batch 3	16.52	23.81	14.15	18.56	8.05	7.37	8.05

3.3 Aspartame

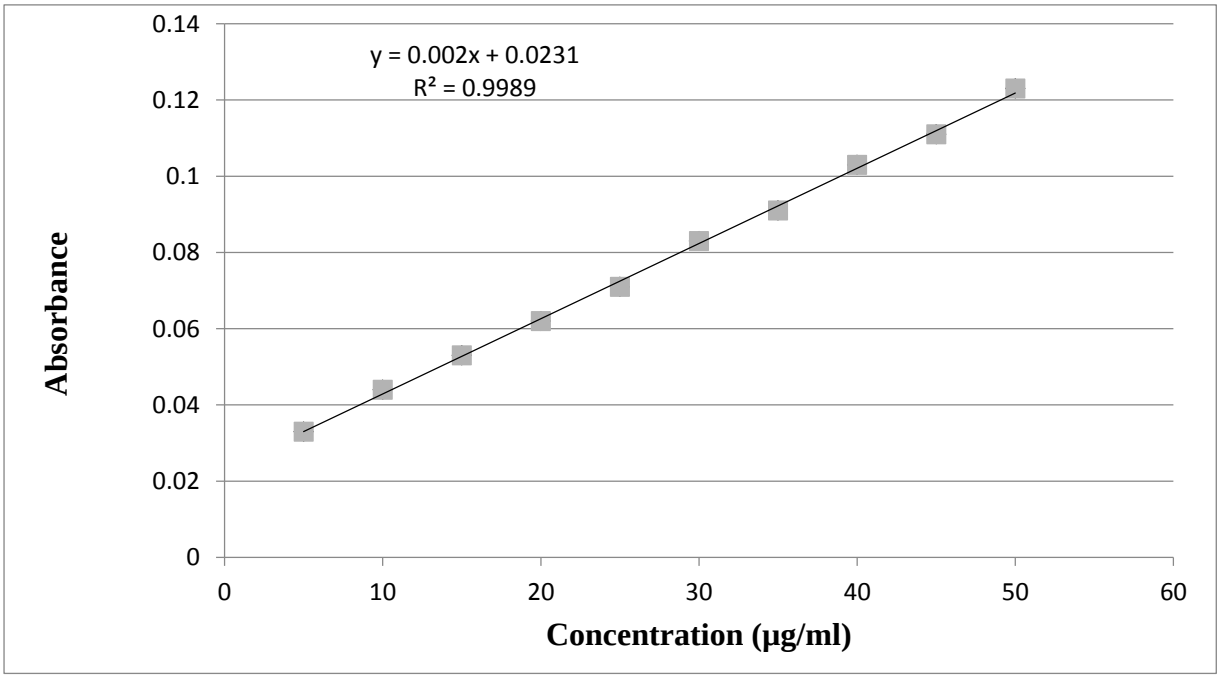

Figure 08: Standard curve for Aspartame

Table 5: Average concentrations of Aspartame in each batches of different brands

	Speed (µg/ml)	**Power** (µg/ml)	**Tiger** (µg/ml)	**Red Bull** (µg/ml)	**Frooto** (µg/ml)	**Frutika** (µg/ml)	**Mangolee** (µg/ml)
Batch 1	11.83	10.20	22.33	21.33	17.45	22.95	15.08
Batch 2	15.20	11.70	18.58	20.20	12.55	25.95	17.70
Batch 3	11.95	10.83	15.08	20.58	22.95	25.20	15.83

Unknown concentration was calculated using the equation, y = 0.002x + 0.0231. R-squared value of the curve was 0.9989. The method applied had a mean recovery percentage for standard solutions was 99.0 ± 2.4%. Moreover, all the unknown concentrations were calculated using Excel to avoid human error. Variation in aspartame content on different batches had been observed; however, Red Bull had more consistency in mean aspartame level among batches of different brands. Furthermore, Frutika had the highest mean for aspartame, as well as it had the highest amount of aspartame among the brands that were tested. On the other hand, power energy drinks had least content of aspartame. Range of mean aspartame composition in sample was 10.2 µg/ml to 25.95 µg/ml.

3.4 Cyclamate

Table 6: Mean concentration of Cyclamate in three different batches of each brand

	Speed (µg/ml)	**Power** (µg/ml)	**Tiger** (µg/ml)	**Red Bull** (µg/ml)	**Frooto** (µg/ml)	**Frutika** (µg/ml)	**Mangolee** (µg/ml)
Batch 1	320.10	310.00	314.23	280.00	283.08	136.73	290.96
Batch 2	318.08	294.23	310.96	289.23	254.62	140.96	298.27
Batch 3	311.34	287.69	295.77	304.04	235.96	152.65	299.23

The equation for the standard curve of cyclamate showed a gradient of 0.0013 and an intercept of 0.269. Mean recovery percentage was 97.4 ± 5.8 % for the standard along with the r-squared value of 0.9891. Mean cyclamate content for the batches of Mangolee was much closer to each other. Speed and Frutika lies on two different poles on the basis of mean cyclamate content, the latter had least cyclamate content. The extent of mean cyclamate content among the batches of different brand ranged from 136.73 µg/ml to 320.10 µg/ml.

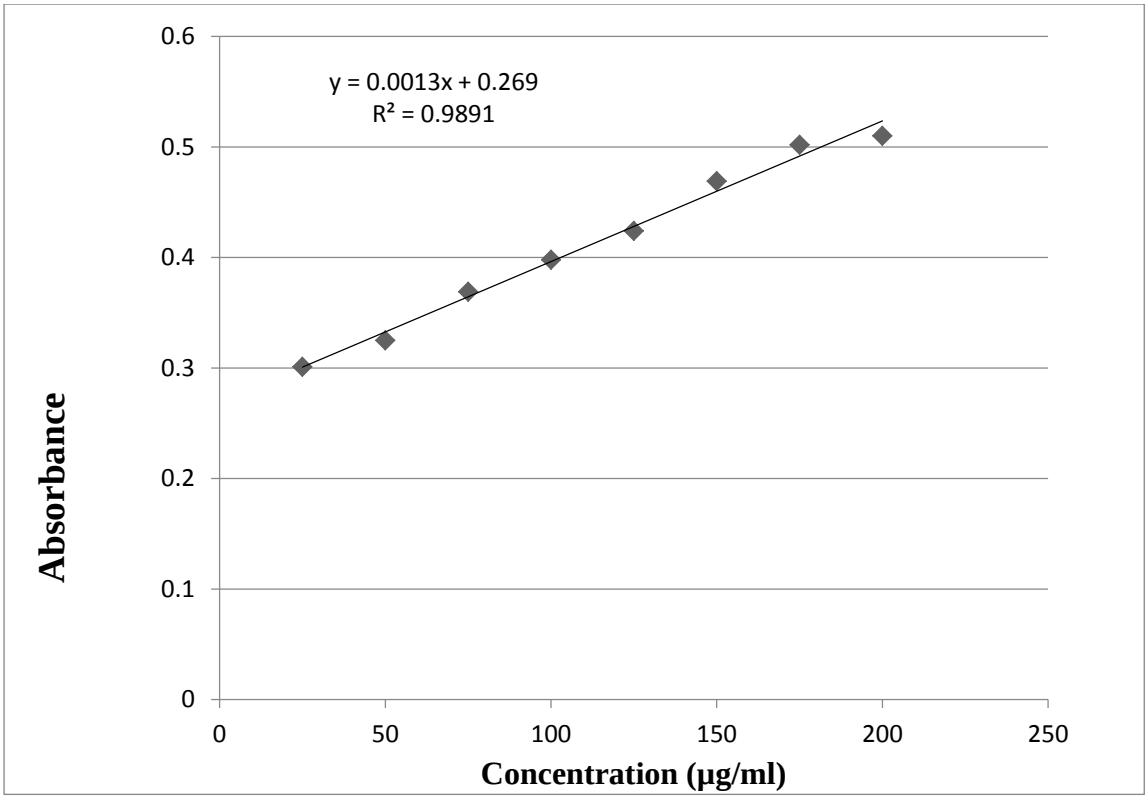

Figure 09: Standard curve for Cyclamate

Table 7: Approximate content of each sugar in a 250-ml bottle of the samples available in the market.

	Sucrose	Saccharin	Aspartame	Cyclamate
Speed	627.59 mg	4.51 mg	3.25 mg	79.13 mg
Power	613.47 mg	5.53 mg	2.73 mg	74.33 mg
Tiger	600.74 mg	3.14 mg	4.67 mg	76.75 mg
Red Bull	687.77 mg	4.62 mg	5.17 mg	72.77 mg
Frooto	637.31 mg	1.29 mg	4.41 mg	64.74 mg
Frutika	654.21 mg	1.29 mg	6.18 mg	35.86 mg
Mangolee	585.86 mg	2.51 mg	4.05 mg	74.04 mg

Summing up the above results, mean value of each of the sugars present in different sample was calculated, for a better observation. Using the obtained mean values, total content of each sugar in a 250 ml was calculated. With these values, a table was prepared with the intention to bring the complete result of the research or analysis on a single window. However, table above showed the mean amount of each sugar in each brand. In other words, it can be said that according to present investigation, the amount of these sugar, a person consumed was illustrated.

Chapter 4

Discussion

Quantitative determination of the three specified artificial sweeteners from energy drinks and mango juice has not yet been documented in Bangladesh. In the current research, simple spectrophotometric methods were used to quantitatively determine the concentration of sucrose, saccharin, aspartame and cyclamate in energy drinks and mango juice commercially available in Dhaka. Beverages are the most frequently consumed NNS (non-nutritive sweetener) containing product compared to NNS foods or packs of artificial sweeteners among male and female children and adults in NHANES 2007-2008 (Gardner *et al.,* 2012). So, more artificial sweeteners are being consumed by means of beverage (soft drinks and energy drinks). Findings of the aforementioned research work also shows a positive correlation with Gardner *et al.* (2012) as the content of artificial sweeteners in experimental samples are high. Reid *et al.* (2016) states that global consumption of energy drinks has doubled between 2006 and 2012, which can allude to serious health hazard in young people due to high sugar content. Similar to other countries, cold beverages (energy drinks and fruit juice) are popular among the youth and adults in Bangladesh.

In the present investigation it was observed that sugar (sucrose) concentration in the sampled energy drinks and mango juice ranged from 2402.9 µg/ml to 2751.1 µg/ml. Gimba and colleagues (2014), found a sugar (sucrose) concentration ranging from 91.05 µg/ml to 1686.73 µg/ml, by sampling energy drinks following the similar method. Red Bull had the highest concentration and Tiger had the lowest sucrose concentration. In the present study the concentration of sucrose found was higher than mentioned in literature, this might be due to change of country. No limit or maximum level of consumption for sugar was mentioned by the regulatory authorities like FDA.

The result of the present research showed that concentration of saccharin found in samples ranged from 5.17 µg/ml to 22.12 µg/ml. In addition, Frooto and Frutika combinedly had the least and Power energy drinks had the highest amount of saccharin. Serdar and Knezevic (2011), reported that saccharin content ranged from 40.01 µg/ml to 55.24 µg/ml for fruit juice and 65.77 µg/ml – 76.91 µg/ml for artificial and flavoured drinks. Furthermore, latest findings showed another range of saccharin concentration in liquid foods where the maximum content was 145.20 µg/ml and minimum was 9.46 µg/ml (Ramsurn *et al*., 2015). Similar to Serder and Knezevic (2011), present investigation also revealed that lower amount

of saccharin was present in mango juice and a bit higher was obtained in energy drinks. However, the results obtained here, were much lower than that stated in both the literature.

In the present study aspartame concentration ranged from 10.91 µg/ml to 24.70 µg/ml. Ramsurn *et al.* (2015) stated the concentration of aspartame in their sample from 15.03 µg/ml to 844 µg/ml and Serder and Knezevic (2011) reported that aspartame content in fruit juice ranged from 80.29 µg/ml – 435.05 µg/ml and 198.22 µg/ml to 709.36 µg/ml for artificial and flavoured drinks. The results obtained from the current research were much lower than reported in the aforementioned papers. According to the results found in this study, Frutika had the highest and Power had the least concentration of aspartame in their respective samples.

Serder and Knezevic (2011) reported that the concentration of cyclamate ranged from 70.10 µg/ml to 583.94 µg/ml in fruit juice and 203.68 µg/ml to 621.75 µg/ml in artificial and flavoured drinks. However, the results obtained in the present study ranged from 143.45 µg/ml to 306.99 µg/ml. Maximum cyclamate was present in Tiger and the minimum presence was found in Frutika. The findings here, remained within the range stated by Serder and Knezevic (2011).

In this investigation, concentration range of aspartame and saccharin obtained was much lower when compared with mentioned papers, whereas, concentration of cyclamate lies within the range reported, but concentration of sucrose outlies the range reported on literature. As, three of these artificial sweeteners and sucrose were found in all the samples, so their respective concentration was much lower. Although, no possible reason can be put forwarded for much higher concentration of sucrose present in all the variants.

The findings of the current analysis of sugars both natural and artificial can summarized as follows, sucrose content varied in a range of 576.40 mg to 654.85 mg in a container of 250 ml, Red bull contains the highest amount and tiger has the least amount. Amount of saccharine content in mango juice are nearly half compared to the tested energy drinks, on the

other hand Frutika lies on the bottom and Power lies on the top. In addition, aspartame content is relatively low in all the samples (range is 2.77 mg to 6.02 mg, per 250 ml). Cyclamate had the highest content among artificial sweeteners, the maximum concentration was observed in Speed. However, presence of sucrose was much higher than any other sugar, as expected.

The safety of the artificial sweeteners had been considered by a range of regulatory organizations, their experts advisory groups and interested scientists. The European Commission‟s Committee on Food (SCF) set the acceptable daily intake (ADI) of aspartame, saccharin at the same level as those set by the Joint FAO/WHO Experts Committee on Food and Additives (JECFA). They had approved the ADI for aspartame, cyclamate and saccharin as 40 mg/kg body weight (bw), 11 mg/kg bw and 5mg/ kg bw respectively. Although, the ADI of aspartame stated by FDA was 50 mg/kg bw but for saccharin it was same as mentioned by JECFA. No ADI level was mentioned for cyclamate by the FDA, as it was banned in USA. So, an adult of 60 kg can consume 2400-3000 mg of aspartame per day, 300 mg of saccharin and 660 mg of cyclamate per day, without any harmful consequences to their body as stated by regulatory authority like JECFA and FDA.

Conclusion:

Interestingly, a person needs to consume several cans of energy drinks and juice containing artificial sweeteners in order to meet the amount specified in ADI chart. Not only beverages contain non-caloric sweetener, moreover their usage become versatile as mentioned in introduction. Presence of three commonly used sweeteners in all the sampled brands, also indicates that manufacturers are using mixtures of sweetener along with sucrose instead of adding one or two sweeteners. From the proportion of saccharin to cyclamate, it can be assumed that a blend of these are being used, like Sucaryl®, a common blend in other part of the world. More importantly, none of the manufacturers stated the presence of these artificial sweeteners in their product. Furthermore, there should be strict monitoring on the foods and beverages that are using non-caloric sweeteners. The regulatory bodies should be scrupulous about the usage of these sweeteners, as they show no signs of harm immediately or at present, but they harm human body anonymously and in most cases, it remains unrevealed and untraced. Moreover, none of the manufacturer of the samples, maintained the code of conduct of FDA as there was no labeling for neither aspartame for PKU and nor for saccharin, which had been made mandatory for saccharin. The following research has only focused on the presence of three mentioned sweeteners, there should be attempt to observe the total sugar content in beverages which commercially available. In addition, further determinative assessment should be performed in order to observe or detect the presence of other non-caloric sweeteners (acesulfame-K, neotame, stevioside, sucralase, alitame etc.) in food products commercially available in Bangladesh. Lastly, the government should focus on the extent of usage of these artificial sweeteners on food products and if necessary they should come to a decision of banning the application and consumption of such sweeteners after performing the assessment of adverse effect caused by the sweeteners.

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