# DETERMINATION OF BENZOIC ACID IN PACKED FRUIT JUICE AND SOFT DRINK SAMPLES BY UV-VIS SPECTROPHOTOMETRY AND TITRATION METHODS

**BY:**

**ABEBA YOHANNES**

**FABRUARI, 2021**

**JIMMA, ETHIOPIA**

# DETERMINATION OF BENZOIC ACID IN PACKED FRUIT JUICE AND SOFT DRINK SAMPLES BY UV-VIS SPECTROPHOTOMETRY AND TITRATION METHODS

**BY:**

**ABEBA YOHANNES**

**Advisor: EPHREM TILAHUN (ASSI.PROF.)**

**Co-Advisor: ABEBE DIRO (MSc.)**

**MSC THEESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES COLLEGE OF NATURAL SCIENCE JIMMA UNIVERSITY INPARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE INCHEMISTRY (ANALYTICAL).**

**DECLARATION**

This research report is my original work and has not been presented for the award of degree in any other University.

Signature _____

Date: _____

Abeba Yohannes

**JIMMA UNIVERSITY**

**SCHOOL OF GRADUATE STUDIES**

**COLLEGE OF NATURAL SCIENCE**

**DEPARTMENT OF CHEMISTRY**

## MSc THESIS APPROVAL SHEET

We, the undersigned, member of the Board of Examiners of the final open defense by **Abeba Yohannes have** read and evaluated his/her thesis entitled **"Determination of Benzoic Acid in Packed Fruit Juice and Soft Drink Samples by UV-Vis Spectrophotometry and Titration Methods"** and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree Master of Science in Chemistry **(Analytical)**

| | | |
|---|---|---|
| **Mr. Ephrem Tilahun** | _____ | _____ |
| Name of Major Advisor (1) | Signature | Date |
| **Mr. Abebe Diro** | _____ | _____ |
| Name of Major Advisor (2) | Signature | Date |
| **Dr. Fekadu Melak** | _____ | _____ |
| Name of the Internal Examiner | Signature | Date |
| **Dr. Tesfa Badhasa** | _____ | _____ |
| Name of the External Examiner | Signature | Date |

## Table of Contents

**Acknowledgment**

First, I want to thank God, the Almighty, for his grace and blessings throughout my entire life. To my advisor Mr. Ephrem Tilahun (Ass.Prof.) as well as my co-advisor Mr. Abebe Diro (MS.c) I express my sincere and deep thanks for the invaluable and constructive guidance and for the comments with important motives from selecting the topic to the end of this Research. I also want to thank Demelash Jado (M.Sc.) for his moral support and for helping me by running the UV-Vis Spectrophotometer. I would also like to thank the staff of the chemistry department for their full support and cooperation in the development of this research thesis in all my difficulties. And for the kindness, concern, encouragement and cooperation of all my classmates and my best friends. My sincere thanks go to my family who have inspired me with their ongoing follow-up and wonderful guidance to reach this level.

**List of abbreviations**

| | |
|---|---|
| FAO | Food and Agriculture Organization |
| WHO | World Health Organization |
| FDA | Food and Agriculture Organization |
| FEMA | Federal Emergency Management Agency |
| JECFA | Joint Expert Committee on Food Additives |
| ADI | Acceptable Daily Intake |
| GC | Gas chromatography |
| HPLC | High performance liquid chromatography |
| UV-Vis | Ultraviolet visible |
| ICH | International Conference on Harmonization |
| EFSA | European Food Safety Authority |
| LD | Lethad Dose |
| GRAS | Generally Recognized as Safe |
| RSD | Relative standard deviation |
| $R^2$ | correlation coefficient |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| ANOVA | Analysis of Variances |

**List of tables**

**List of figures**

**Abstract**

*Benzoic acid is one of the most commonly used food preservatives. The use of these* preservatives *in food products, however, should be monitor in order to assure their safe dose for not exceed the limit stated by regulatory body. Therefore, it is important to assess the amount of benzoic acid in soft drinks by cost effective and simple method for frequent monitoring. The objective of this work was to extract and investigate the concentrations of benzoic acid present in fruit juices and soft drinks by UV-Vis spectrophotometric and titrimetric method. A total of 39 samples were collected from Jimma market. To find the best extraction condition different parameters such as extraction solvent, volume and stability were assessed and optimized. Benzoic acid residues obtained from the sample by optimized extraction condition were dissolved in 10 mL of methanol and detected at 225 nm. The level of Benzoic acid in fruit juice and Soft drink samples by using UV-Vis spectrometry were in the range of 81.5 ± 0.1 – 385.0 ± 0.1 and 284.0 ± 0.07– 400.9 ± 0.4 respectively. While the level of Benzoic acid in fruit juice and Soft drink by using titration were in the range of 139.0 ± 0.1 – 182.5 ± 1.0 and 215.0 ± 0.4 – 221.0 ±0.2 respectively. The linearity and* $r^2$ *for UV-Vis method was 1-10 ppm and* $r^2$ *0.996 respectively. The limit of detection (LOD) and the limit of quantification (LOQ) of benzoic acid was 0.16 ppm and 0.47 ppm by the UV-Vis method, while the method of titration was 0.58 ppm and 1.92 ppm respectively. The two-method demonstrated good accuracy with % recovery ranges 94.89-104 % for the UV-Vis method and 80-103% for the titration method. The percent relative standard deviation of UV-Vis method ranged from 0.18-1.35, for intra-day, and from 1.1-5.8 for inter-day variation. Benzoic acid levels in all of the samples tested within the maximum acceptable ranges for fruit juice and soft drinks (<1000 ppm), as indicated by the joint FAO/WHO Food Standards Program Codex Committee on Food Additives and Contaminants. But, for the imported samples the Benzoic acid were higher, which indicates that there should be monitoring of the level of preservatives for safe conception before distribution. At P<0.05 both methods show significance differences.*

***Key Words****: Benzoic Acid, Fruit Juice, Soft drink, UV-VIS spectrometry, Titration*

# 1. Introduction

## 1.1 Background

Preservatives are substances that prevent the development of spoilage and chemical degradation of food components by micro-organisms [1]. Chemical preservatives are commonly used in processed foods to prevent the growth of bacteria, yeasts or other gross micro-organisms that might spoil our food [2]. Benzoic acid is also used as a preservative for drinks in the form of its salts, sodium benzoate and potassium. [3, and 4]. Benzoates are converted under acidic conditions to un-dissociated benzoic acid [5]. Due to their increased solubility, benzoates are often preferred to benzoic acid [6, 7].

In margarine, in salads, in marinades, in cider, in soft drinks, in pickles, in fruit salads, in wafers, in bakeries, in jams, jellies, juices, biscuits, cakes and muffins, tomato paste and soy sauce, sodium benzoate is mainly used as a preservative [8 -11]. There are also studies available on the use of this product in wine, beer and olives [12-14]. Sodium benzoate is bacteriostatic and fungistatic under acidic conditions, but regular intake can have adverse skin effects, such as rash, non-immunological urticarial touch, and metabolic acid. In various countries, specific laws prohibit the use of food additives.

According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the safety of the use of an additive can be expressed in terms of its allowable regular intake (ADI), which is the quantity of substances that can be consumed without any health hazards on a daily basis, even for a lifetime. JECFA [15-16] has developed Group ADIs of 0–5 mg/kg body weight for benzoate salts. Adverse effects, such as metabolic acidosis, convulsions, hyperactivity and hyperpnoea, have been identified in the use of benzoic acid as an antimicrobial agent in experimental animals and humans with very high doses of benzoic acid [17]. Some studies have also documented the occurrence of allergic reactions in humans to benzoates such as urticaria, non-immunological contact urticaria and asthma [18-19]. In soft and fruit drinks containing both benzoates and ascorbic acid, it is possible to produce carcinogenic benzene at a very low level (ppb level). The reaction [20] is further enhanced by heat and light exposure.

Consumption of juices is now a very popular habit in Ethiopia, like other countries in the world. Juices and soft drinks are particularly popular with children. So, the composition of non-alcoholic

beverages should be governed by legislation. The goal of ensuring the quality of foodstuffs, the sanitary status and economic value of many products is determined on the basis of chemical measurements.

Numerous methods, including gas chromatography (GC) [21, 22], high-performance liquid chromatography (HPLC) [23], spectrophotometric UV-Vis [20-24] and titration [25], are available for the determination of benzoic acid and food materials. In order to ensure juice protection and purity, the detection of preservatives in juices is compulsory worldwide. It is therefore also important to find easy, inexpensive and effective methods for constant preservative monitoring. Therefore, for the determination of benzoic acid from fruit juice and soft drink samples, validated UV-Visible spectrophotometry and titration methods were used.

### 1.2 Statement of the problem

Refined packaged fruit juices and soft drinks are consumed by people, regardless of age, religion, gender and culture. Most bottled juices and soft drinks are imported from other countries, and there are companies that pack juices in Ethiopia. There are different forms of additives in the various types of packaged juices and soft drinks available on the market, of which preservatives play a larger role. In order to improve the shelf life and preserve the consistency and protection of packaged juices, these preservatives, such as benzoic acid, are added. Benzoic acid in the form of its salts is frequently applied to beverages as a preservative; sodium benzoate, potassium benzoate, calcium benzoate, benzene carboxylic acid and phenyl carboxylic acid [3, 4]. Excess benzoic acid intake causes many health problems due to its toxicity, including gastrointestinal, respiratory and even neurological adverse reactions [15].

Since preservative amounts in most processed packaged fruit juices are not listed under the product label, it is difficult to know if the preservative intake is still at a safe level. In addition, packaged fruit juices and soft drinks in Ethiopia are widely consumed by infants, sick and injured individuals without any prescribed dosage. The consumer might not be aware of the amount of preservatives found in packaged fruit juices and compared to the maximum allowable amounts for regulatory bodies and scientific inputs. To ensure the quality and consistency of juices, it is mandatory to analyze the preservatives in juices worldwide. The purpose of this analysis is, therefore, to

determine the concentration of benzoic acid in the sample of packed fruit juice and soft drinks using the double-beam UV-Vis spectrophotometer and the titrimetric method and to address the following research questions:

1. Do the value of benzoic acid in the analyzed samples exceed from recommended limits?
2. The value of benzoic acid obtained by the two-method different or not?

### 1.3 Objective of the study

#### 1.3.1 General objective

To extract and investigate the concentrations of benzoic acid present in fruit juices and soft drinks sample by UV-Vis spectrophotometric and titrimetric method

#### 1.3.2 Specific objectives

- To optimize extraction condition that affect extraction efficiency of the target analyte.
- To validate the method under optimized condition for the determination of benzoic acid
- To determine the extracted benzoic acid concentration using UV -Vis spectrophotometry method and titration methods.
- To compare the benzoic acid concentration obtained from the two methods and the minimum recommended standard level.

### 1.4 Significant of the study

Benzoic acid is added to improve the shelf life and preserve the consistency and protection of packaged juices. Because of their toxicity, the excess benzoic acid intake causes many health problems, including gastrointestinal, respiratory and even neurological adverse reactions.

This analysis therefore has the following significance: -

- To give information on the presence of benzoic acid and its level the samples
- Give warning for consumers if the amount exceeds the recommended limit
- To demonstrate the efficiency of titration method as substitute of UV–Vis spectrophotometry if the instrument not available.
- Document the obtained result as a base line information related to the study.

## 2.Literature review

### 2.1 Food Additives

Throughout its journey from factories or industrial kitchens, during transportation to warehouses and shops, and finally to customers, additives are required to ensure that processed food remains healthy and in good condition. Food additives are the backbone of the modern food industry and play an important role in improving the colour, smell and taste of food, changing its nutritional composition, improving the conditions of production and extending its shelf life [26].

In fact, the Food Protection Committee of the US National Research Council described food additives as a substance or a mixture of substances other than basic foods that are present in a food as a result of an element of manufacturing, processing, storage, or packaging[27].According to the US Food and Drug Administration (FDA), a food additive is any substance whose intended use results in, or is reasonably expected to result in, directly or indirectly being, or otherwise affecting, the characteristics of any food[28]. Food additives are also classified as chemical substances intentionally added to food products, in known amounts, directly or indirectly, for the purpose of aiding food processing, preservation of foodstuffs or improving the taste, texture or appearance of foodstuffs [29]. It is possible to directly or indirectly use food additives. Direct additives are those that are purposely applied for a particular purpose to foods, while indirect additives are those to which foods are exposed during manufacturing, packaging or storage [30]. Sweeteners, coloring agents, preservatives, emulsifiers, stabilizers, thickeners, taste enhancers and miscellaneous [31] are the numerous food additive categories.

#### 2.1.1 E-numbering

The E-System, developed by the European Economic Community, is a list of food additives that is periodically updated, including additives that are considered healthy, allowing foods to move within the common market from country to country [32]. Each additive is assigned a unique number, known as 'E-numbers', which is used worldwide for all approved additives to govern these additives and notify consumers [33]. As food additives pass safety tests, chemical compounds and other species are consistently added to the list of safe-to-use food additives. The official UK food

standards agency was able to obtain an updated list of food additives and their E numbers. Table 1 [34,35] provides the general list of the E numbers of food additives.

**Table** 1: E numbers of food additives

| E– numbers | Food additives | Example |
|---|---|---|
| E100-E199 | Colors | Carotene(E160) |
| E200-E299 | Preservatives | Benzoic acid (E210) |
| E300-E399 | Antioxidants and acidity regulators | Ascorbic acid (E300) |
| E400-E499 | Thickeners, stabilizers and emulsifiers | Pectin(E440) |
| E500-E599 | Anticaking agents | Sodium carbonate(E501) |
| E600-E699 | Flavor enhancers | Monosodium Glutamate (E621) |
| E700-E799 | Antibiotics | Calcium propionate |
| E900-E999 | Glazing agents and sweeteners | Aspartame(E951) |
| E1000-E1599 | Additional chemicals | Lysozyme(E1105) |

### 2.2 Food preservation

Preservatives have been widely used in fruit, cosmetics, and pharmaceutical products as additives. These preparations are protected by the addition of preservatives which avoid alteration and degradation of the formulation of the product [36].

Preservatives are used to prolong the shelf-life of some goods over the prolonged period to ensure their protection. Most significantly, they prolong bacterial degradation, which can contribute to toxin development and food poisoning [37]. Food preservatives are substances that, once added to a given food, can prevent or delay changes induced by the action of microorganisms, enzymes and/or physical agents [38]. Its high use by the food industry is due to the increasing demand for foods that are chemically stable, safe and durable [39].

Long-term preservatives can be natural (salt and sugar) or chemical, and this is the most powerful form of preservative [40]. Chemical preservatives interact with micro-organisms' cell membranes, their enzymes or genetic mechanisms [41]. Preservation typically includes preventing bacteria, fungi, and other microorganisms from developing, as well as retarding the oxidation of rancidity-

causing fats. It also involves processes used to inhibit natural aging and discoloration, such as the enzymatic browning reaction in apples after they are cut [42], which may occur during food preparation. In the food and beverage industries, many types of chemical preservatives are currently being used, such as benzoates, sorbets, vitamins, fruit extracts, sodium salts, etc., as shown in Figure.1 [43].

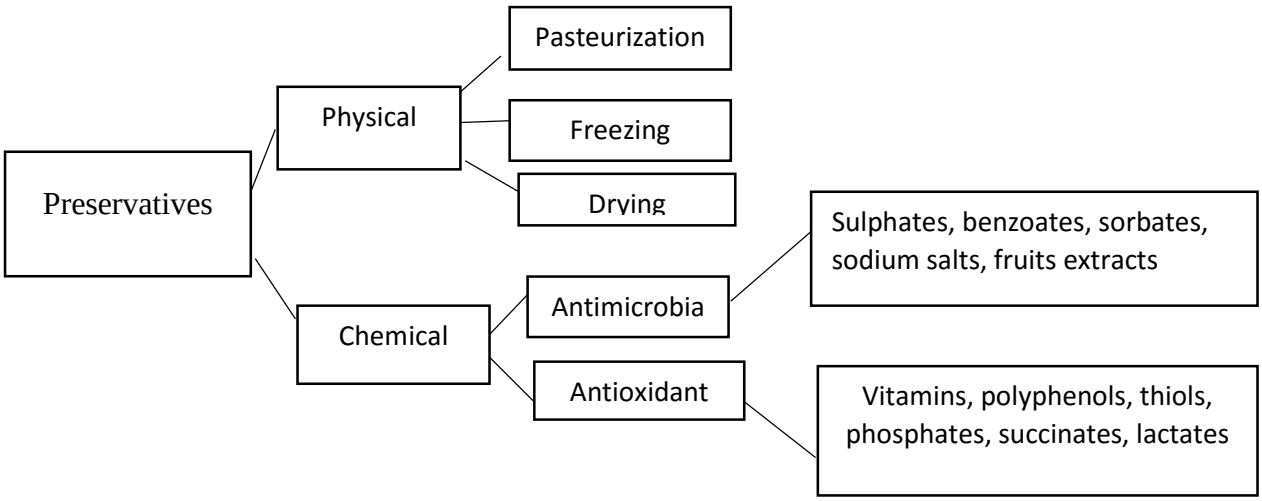



**Figure 1**: Diagrammatic representation of usages of preservatives

### 2.2.1 Benzoic acid

One of the most used preservatives is benzoic acid. Due to its solubility, this preservative is more commonly used in its salt form than in the acid form [44]. Sodium benzoate is a sodium salt with a molecular weight of 144.1 g.mol-1, defined by the chemical formula $C_7H_5O_2Na$, an odorless compound that is soluble in water and ethanol. It is usually used in many products in the cosmetic, pharmaceutical and food industries as a preservative [45]. It is used in the pharmaceutical industry for the treatment of various diseases, such as urea-cycle disorders, liver diseases and multiple sclerosis [46]. In the food industry, sodium benzoate is used in food and beverages because, in addition to providing easy application, it is effective in inhibiting the growth of fungi and bacteria during storage [47]. The preservation of margarines, sauces, marmalades, jelly, liqueurs, beers, fruit juices and soft drinks [48] is suggested. In spite of its presence in many foods, population studies suggest that the primary dietary sources of this preservative are soda and juice in cartons

[49]. Due to its strong stability and excellent solubility in water [50], sodium benzoate has been used for many years as a preservative.

The FDA considers it to be "Generally Regarded as Safe" and may be present in foods at concentrations above 0.1% [17]. For FAO and WHO, the IDA is 5 mg.kg-1 body weight for sodium benzoate foods [45, 48]. The maximum limit for sodium benzoate as a preservative is 1000mg/L [3], according to Joint FAO/WHO Food Standards Program Codex Committee on Food Additives and Contaminants. The Lethal Dose (LD50) for a preservative is 2000 mg.kg-1[51], according to the European Food Safety Authority (ESFA). The FDA has never set a limit on sodium benzoate and considers it as very safe, although, in the light of publications that show inconsistencies, it has been criticized as negligent in its assessments [52]. Although the IDA is almost impossible to be exceeded by the average customer, the ADI may be exceeded by the large regular consumers of soft drinks and juices [38].

Sodium benzoate (a metabolite of benzyl alcohol) is quickly absorbed by the gastrointestinal tract as soon as it is ingested, then conjugated with glycine to form pyruvate in the liver [53]. This transformation happens in the mitochondria by means of two steps [54]. The preservative is converted into benzoyl-CoA by means of an adenosine triphosphate (ATP)-dependent acid, reaction 1, upon entering the cell. CoA is subsequently converted by means of glycine Nacyltransferase to pyruvate, reaction 2. SB results in the consumption of ATP, glycine and coenzyme A in the mitochondria. The ingestion of this preservative results in a rise in benzoate serum and also in pyruvate serum [55]. Within the first 6 h, the resulting hippuric acid is quickly excreted in the urine and the remaining dose is fully removed within 2 to 3 days [53,54].

### 2.2.2 Physical and chemical properties of benzoic acid

Benzoic acid ($C_7H_6O_2$ or $C_6H_5COOH$); benzene carboxylic acid, phenyl carboxylic acid [E 210 (EU Food Labelling Regulation)], molecular weight 122.13) is a white solid that begins to sublimate at 100 °C, with a melting point of 122 °C and a boiling point of 249 °C. Its water solubility is poor (2.9 g/L at 20 °C) and its water solution is weakly acidic (dissociation constant at 25 °C = $6.335 \times 10^{-5}$; pKa 4.19) [56]. It is soluble in ethanol and very slightly soluble in benzene and acetone. It has a partition coefficient (log Kow) of 1.9 for octanol/water. Its vapor pressure

ranges from 0.11 to 0.53 Pa at 20 °C. The determined constant of Henry's law at 20° C was given as 0.0046-0.0222 [57].

Sodium benzoate ($C_7H_5O_2Na$); sodium salt benzoic acid [E 211 (EU Food Labelling Regulation)]; molecular weight 144.11) has a melting point of 300 °C. It is very water soluble (550-630 g/L at 20 °C) and is hygroscopic at over 50 percent relative humidity. It was soluble in Ethanol, methanol and ethylene glycol [56].

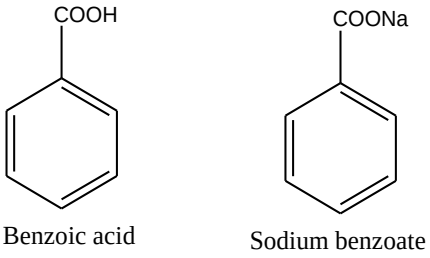



**Figure 2** :Chemical structures of benzoic acid and Sodium benzoate

### 2.2.3 Uses of benzoic acid

Benzoic acid and its derivatives are commonly used in food, cosmetics, hygiene products, and oral, parenteral and medicinal drugs as antibacterial and antifungal preservatives [58]. Their minimum microbicide concentrations for various bacterial or fungal species vary from 20 to 1200 mg/L at pH 6.0 and their minimum inhibitory concentrations range from 50 to 1000 mg/L. [59] Benzoic acid and its derivatives are also used as flavoring agents in meat, cosmetics and hygiene products [60].

Benzoic acid and its salts are commonly used in the manufacture of plasticizing agents. They are also used in the treatment of some urea cycle disorders and other genetic enzymes as corrosion inhibitors in coolants, active ingredients in pesticide products, biocidal products in veterinary hygiene, bactericidal agents, hydroxyl-radical scavengers with antioxidant activity, diuretics and therapeutic agents, promoting the alternative route of nitrogen excretion in the body [59,61]. Due to their large range of antimicrobial activity, solubility in water and high stability, they are used as preservatives in foods, cosmetics, pharmaceuticals, hygiene and personal care products [62].

### 2.2.4 Toxicology and adverse effects

Benzoic acid and a great variety of related compounds are generally recognized as safe (GRAS) substances and their use as additives and/or flavouring agents in foods, cosmetics, pharmaceutical and hygiene products, is permitted by FAO/WHO and FDA/USDA. In 2006, the European Food Safety Authority (EFSA) recommended that the approval of propylparaben (E216) and its sodium salt (E217) be removed after more than 85 years of prolonged use, although the appropriate daily intake (ADI) of 10 mg/kg body weight/day remains for the remainder of the parabens and that its use in cosmetics be allowed with a maximum concentration of 0.4%.and a total maximum level of 0.8% [63].

From a human health point of view, benzoic acid and its salts are regarded by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as a single category group with benzyl alcohol, benzyl acetate and benzyl aldehyde, as they are rapidly metabolized and excreted in urine within 24 hours. [64]. These compounds are absorbed from the gastrointestinal tract and, within 1-2 hours, reach their peak plasma concentration, and may also be partially absorbed (22-89%) through dermal and inhalation routes [59]. Benzyl alcohol is degraded by alcohol dehydrogenase and cytochrome P450 mediated oxidation into benzyl aldehyde in the liver and kidneys, and benzyl aldehyde is further oxidized into benzoic acid by aldehyde dehydrogenase [65]. Alkyl benzoate esters in various tissues and locations such as the respiratory tract, skin, blood and gastrointestinal tract are degraded to benzoic acid and the related alcohols by enzymatic activity [66].

Benzoic acid can also induce digestive mucous membrane irritation, and doses higher than ADI (i.e., 1000 mg/kg body weight/day for 5 consecutive days) can cause nausea, headache, burning of the esophagus, and decrease the coefficient of digestive use by inhibiting oxidizing enzymes such as pepsin, trypsin, polypeptidases, and D-amino acid [59,67]. Other adverse effects related to benzoic acid include diarrhea, muscle fatigue, metabolic acidosis, convulsions, tremors, hypoactivity and emaciation, which may be correlated with improper use of glycine to detoxify benzoic acid, resulting in a decrease in the amount of glycine and interfering with the metabolic processes in which it is involved, resulting in a reduction in creatinine, glutamine, urea and uric acid production [68,59 ].Benzoic acid and its salts were also related to childhood hyperactivity or hyperkinetic syndrome[69,71].

Benzoic acid and benzoates are used not only as preservatives in foods and cosmetics, but also in medicines and other pharmaceuticals, and their ability to displace bilirubin from albumin, which can lead to hyperbilirubinemia, encephalopathy and kernicterus, especially in neonates, is a major safety concern in this context [72]. In addition, sodium benzoate is used at doses of 180-650 mg/kg body weight/day for up to several years for the treatment of urea cycle disorders, which may cause adverse effects such as hyperactivity, impulsiveness and inattentive behavior, especially in children.

**2.3 Benzoic acid occurrences and surveys**

A survey of the determination of preservatives in fruit juice products has been carried out in Bangladesh [73]. A total of 50 different samples of commercial juices have been obtained in Dhaka, Bangladesh from the local markets. 28 were domestic products, while 22 were products imported. Benzoic acid levels in samples ranged between 99,1-441ppm.

The survey concluded that the utilization of benzoates is significantly lower than the maximum authorized levels. A survey on the detection of benzoic and sorbic acids was carried out in Brazil [18]. The analysis included 56 samples including soft drinks, fruit juice, margarine, cheese and yogurt. Benzoic acid levels in the samples were from not detected to 804 mg/L. The survey concluded that the utilization of benzoates is significantly lower than the maximum authorized levels. A survey on Benzoic Acid (IV), Sulfur (IV) Oxide and Sorbic Acid in Carbonated Food products was conducted in Lagos [74] in Nigeria. 33 samples including Commonly consumed carbonated drinks (14), fruit juices (14), sport drinks (2) and dairy drinks (3) purchased in different Lagos markets. Benzoic acid levels in the samples ranged between 168 and 799 ppm. In the study, the use of benzoates was considerably lower than the permitted maximum levels.

In India, research is being conducted in Study and Quantification of Preservative (E211) In Carbonated Soft Drink Samples [20]. Commercial soft drink samples, Sprite and Marinda from three different batches were collected from different areas of Chennai city. The average amount of benzoic acid in sprites ranged from 168 to 175 ppm and in Marinda samples from 396 to 398 ppm.

In Nigeria, research is being conducted in Analysis and Health Risk Assessment of Sodium Benzoate and Potassium Sorbate in Selected Fruit Juice and Soft Drink Brands [75]. A total of 20 samples were bought in Nigeria in different markets. Benzoic acid levels range from 25.8-245.10 ppm.

### 2.4 Analytical Methods

Analytical methods for the determination of benzoic acid include gas chromatographic (GC) methods, which are more sensitive and precise but require long sample preparation and derivatization prior to determination, include analytical methods for the determination of benzoic acid[22,23], high-performance liquid chromatography (HPLC), which has a high specificity but consumption during the study of large quantities of organic solvents and extensive sample preparation step[24], and double beam UV-Vis spectrophotometer and titrimetric method, is a simple, inexpensive and reliable method for determining benzoic acid in processed fruit juices[25,26].

#### 2.4.1 Titrimetric analysis

Titration is a common method used for quantitatively determining an identified analyte's unknown concentration. As the calculation of volume is important in titration, it is also called volumetric analysis. Based on the types of reactions they use, there are several types of titrations. Acid-base titration and redox titration are the most common types [76-80]. A pH indicator is normally applied to the analyte solution for acid-base titration to indicate the endpoint of titration [81]. The analyte concentration based on the stoichiometry reaction can be determined from the titrant volume reported at the endpoint. The pH can be used as a function of the added titrant volume during the acid-base titration. The point of influence on the curve, the point at which the stoichiometric volume of acid and base is equal in solution, is referred to as the point of equivalence, and the point at which the color shift is detected is the endpoint of titration [81].

### 2.4.2 Spectrophotometric method

Spectroscopy is the science that deals the study of interaction between electromagnetic radiation and matter [82]. It is a powerful instrument for researching and analyzing the wide sample range of atomic and molecular structures. The electromagnetic spectrum area of 100 to 400 nm is covered by optical spectroscopy.

Ultraviolet-Visible spectrophotometer: UV-Visible spectrophotometer involves measurement of the number of ultraviolet radiations absorbed by any substance in a solution. Instrument that measures the ratio or function of the intensity ratio of two light beams in the U.V-visible region [83]. Beer's law states that with the number of absorbing molecules, the power of a beam of parallel monochromatic radiation declines exponentially. The law of Lambert states that the intensity of a beam passing through a medium of homogeneous thickness of parallel monochromatic radiation decreases exponentially. The Beer-Lambert rule yields a mixture of these two laws [84].
Beer-Lambert law: When beam of light is passed through a transparent cell containing a solution of an absorbing substance, reduction of the intensity of light can occur. Mathematical expression of Beer- Lambert law is as follows [83].

$$A = a\ b\ c$$

Where, A=absorbance or optical density

a= extinction coefficient

b= length of radiation path through sample (cm)

c= concentration of solute in solution.

"a" and "b" are constant. So "a" is directly proportional to the concentration "c"

### 2.4.3 Liquid-liquid extraction

Solvent extraction is a method used widely in both industrial and laboratory applications. A number of techniques are used, such as liquid liquid extraction (LLE), liquid solid extraction (LSE), supercritical fluid extraction (SFE), and other special techniques. LLE is an extraction method applied using a liquid extracting medium to liquids, liquid samples, or samples in solution. Liquid liquid extraction (LLE) is one of the separation techniques in which one or more solvents

in the feed are moved to another liquid that is immiscible. It is one of the frequent methods of separation used for isolating one or two components from the mixture. The principle of extraction is based, on the equilibrium distribution of the substance in the two immiscible phases, one of which is the solvent extraction phase [85,86]. The solvent must be pure, but with the propensity to react to the variable of interest selectively. In the phase, the mass transfer phenomenon is regulated. This is the way a liquid solvent removes substances from solids or liquids. The difference in solubility of the solute in two separate immiscible liquids is the main principle of the method. The extraction efficiency of the analyte was affected by different factors in liquid liquid extraction. These are viscosity, tension of the surface, pressure of vapor, density.
There are two general types of liquid-liquid extractions [87]: -

**Extraction with organic solvents**: -An organic solvent extraction in which an organic solvent with a high affinity for the desired compound is used to extract the compound from another solution.

**Acid-base extraction: -** An acid-base extraction, in which an organic acid or base is extracted from an organic solvent by using an aqueous solution of an inorganic base or acid, respectively. A neutralization occurs which converts the compound into an ionic, water-soluble salt, causing it to transfer from the organic phase to the aqueous phase.

### 2.5 Method validation

Validation of an analytical method is used to prove that the method built is appropriate for its intended purpose. The method was validated in terms of Linearity (Calibration curve construction), limit of detection (LOD), limit of quantification (LOQ), intraday and inter-day precisions, and recovery (R %) [88].

## 3. Materials and Methods

### 3.1 Study area and Period

The study area is about 346 km from Addis Ababa and is located at Jimma University, Oromia Regional State, Ethiopia. The research was performed from November 2019 to February 2021. The sample of soft drink and fruit juice was purchased from the local jimma market, which was based on the commonly consumed and most available in the local jimma city market (Appendix.1).

### 3.2 Chemicals and reagents

Analytical grade Benzoic acid ($C_7H_6O_2$, 99 %), sodium hydroxide (NaOH) and sodium chloride (NaCl) were obtained from the fine chemistry research laboratory Industries (India). The organic solvent Methanol ($CH_3OH$, 99.8%), diethyl ether ($(C_2H_5)_2O$, 99%), dichloro methane ($CH_2Cl_2$,99.5%), petroleum ether, were obtained from LOBA CHEMIE (Indian), chloroform ($CHCl_3$) was obtained from Bluuxul (India), and ethanol ($C_2H_5OH$, 99.9%) were obtained from Carlo Erba (Paris, France). Phenolphthalein, hydrochloric acid (12M HCl) obtained from Sigma Aldrich (UK), anhydrous sodium sulfate ($NaSO_4$) obtained from FINKEM (England) and ammonium hydroxide ($NH_4OH$) obtained from ROMIL (Cambridge, UK), were used during the experimental studies.

### 3.3 Instruments and Apparatus

Extraction and determination were performed using SPECORD 200PLUS UV-Vis spectrophotometry analytikjena (Germany), Quartz cuvette (1 cm in diameter), Sonicater obtained from IM LAB(Germany), Centrifuge machine (4000 r/min) obtained from CENTRIFUGE MODEL 800(China), Elma ultrasonic water bath obtained from (Germany) and filter paper (Whitman filter paper C80 No1001090 (90 cm) obtained from Whatman International (England). Equipment's such as Water Bath (Germany), Micro-pipettes, analytical balance (Kern, ABJ-220NM, Germany), and refrigerator. Apparatus such as, Separation funnel, Iron Stand, Burette, centrifuge (10 mL) tubes, beakers, volumetric flasks, measuring cylinders, reagent bottles, spatulas and paper labels were used in this study.

### 3.4 Sample collection and design

Ten different packed fruit juices with different flavors such as 3D mango Prigate, Rani Carton and Bottled, Frootine, Junire, 7star, tomato, orange, guava and three soft drinks as sprite, Fanta and Marinda were purchased from various supermarkets and local markets in Jimma City. In three separate shops, one package of each sample was sold and combined to create one representative sample. The list of fruit juice and soft drinks collected is included in the table.

**Table 2**: List of fruit juice and soft drink samples collected

| Sample code | Type of Juice | Local | Imported | Quantity |
|---|---|---|---|---|
| M1 | 3DMango | 1 | | 3 |
| M2 | Prigate | 1 | | 3 |
| M3 | Rani Bottled | | 1 | 3 |
| M4 | Rani Carton | | 1 | 3 |
| M5 | Frootine | | 1 | 3 |
| M6 | Junire | | 1 | 3 |
| M7 | 7star | | 1 | 3 |
| T1 | Tomato | | 1 | 3 |
| O1 | Orange | | 1 | 3 |
| G1 | Guava | | 1 | 3 |
| S1 | Sprite | 1 | | 3 |
| S2 | Marinda | 1 | | 3 |
| S3 | Fanta pineapple | 1 | | 3 |
| Total | | 5 | 8 | 39 |

### 3.5 Sampling Preparation

20 g of each different brand of fruit juice and the degassed soft drink samples were taken into a beaker in the presence of saturated sodium chloride solution and the solutions made alkaline to litmus paper with 10 % sodium hydroxide solution. The solutions mixed well with using glass road

and make up to 60 mL with saturated sodium chloride solution and allowed to stand for 30 min with frequent shaking. Finally, the solutions were filtered with Whitman filter paper and the filtrates were used for further extraction step.

### 3.5.1 Optimization strategy for extraction of benzoic acid

Liquid-Liquid Extraction was used for benzoic acid extraction from samples. One consideration at the time of the experiment was carried out to determine the optimum extraction state of benzoic acid from samples, which means finding the best condition of one variable by fixing the other constant. 60 mL of each type of sample was extracted from four types of extraction solvents, namely Diethyl ether, Chloroform, Dichloro methane and Petroleum ether, at different volumes ranging from 15 to 105 mL, and the stability of the solvent used to dissolve the residue of extracted benzoic acid was tested for 10 days prior to UV-Vis analysis (Diethyl ether, Ethanol and Methanol).

#### 3.5.1.1 Selection of scanning wavelength

Previous methods of spectrophotometry recorded different scanning wavelengths. UV/Visible benzoic acid spectra in the range of 200-400 nm were obtained using double beam spectrophotometry. For further analysis, the wavelength corresponding to the spectrum with maximum response was chosen.

#### 3.5.1.2 Selection of extraction solvent

Selecting a suitable extraction solvent is a crucial parameter for the LLE process. To attain the target of near-complete extraction of the solutes of interest, it is very important to select a solvent system. Optimizations of the extraction solvent were carried out by keeping the amount of extraction and solvent steady at 60 mL and methanol respectively. In order to extract 60 mL of pre-treated sample solutions, four extraction solvents of diethyl ether, chloroform, dichloro methane and petroleum ether were used. The best solvent for extraction that provided the highest absorption of benzoic acid was chosen.

#### 3.5.1.3 Selection of volume of extraction solvent.

The selection of a suitable amount of extraction solvent increases the extraction efficiency of the targeted analyte. By keeping the extraction solvent (Diethyl ether) and methanol unchanged, the extraction solvent volume was measured. For the extraction of 60 mL of pre-treated sample solutions, selected extraction solvents with different volumes of 15-105 mL were used. The best amount of extraction solvent was chosen to provide the maximum absorption of benzoic acid.

#### 3.5.1.4 Solvent stability

The option of a stable solvent is an important necessity for the analyte not to undergo any chemical alteration and to remain stable in the solvent in question. Stability at room temperature was monitored for 10 days by measuring absorbance changes twice a day at the maximum wavelength. Three solvents (diethyl ether, ethanol, and methanol) were examined to analyze the effect of solvent stability and the most stable solvent was selected.

### 3.5.2 Extraction procedure

#### 3.5.2.1 Extraction procedures for determination of Benzoic acid from fruit juices and soft drinks

In the 100 mL beaker, 60 mL of filtered sample was taken, then the solution was definitely acidic to litmus with hydrochloric acid and 4 mL of hydrochloric acid excess was applied to acidify the solution. Prepared solutions were transferred to a 250 mL separation funnel and three times extracted with 60 mL of diethyl ether. The mixture was well shaken at each extraction to ensure maximum extraction (break emulsions by standing, stirring or centrifuging) and vented several times in the fume hood. The aqueous process has been drained and thrown away. The combined ether extracts were washed with 30 mL hydrochloric acid portions and discarded hydrochloric acid washed. The ether solution was extracted three times with 30 mL portions of 0.1% ammonium hydroxide and the ether layer was discarded. The combined extract of ammonium hydroxide was neutralized with hydrochloric acid and an additional 1 mL of excess was tested. The acidified solution was extracted three times using 60 mL of ether. To avoid mineral acid, the combined ether extract was transferred into the separatory funnel and washed with distilled water, then the water

phase was drained out. Over anhydrous sodium sulfate, the ether layer was dried and the solvent was distilled away. The last traces of the solvent were removed overnight under an air current at room temperature, after that residue of benzoic acid was collected with dissolving it in 10 mL of methanol [89].

### 3.6 Standard and working solution preparation

By dissolving an accurately weighted quantity of benzoic acid in methanol, stock solutions containing 1000 mg/L of standard benzoic acid were prepared and different standard solutions (1-10 mg/l) were also prepared by diluting the required volume of each standard concentration from the intermediate solution, and then stored in the refrigerator at $4^{o}C$.

#### 3.6.1 Preparation of 0.05N NaOH Solution

0.5 g of NaOH was taken into a 250 mL volumetric flask and distilled water was applied and shaken until dissolved and filled with distilled water to the mark.

#### 3.6.2 Preparation of 0.1%$NH_3OH$ solution

In a 100mL volumetric flask, 1ml of $NH_3OH$ was taken and distilled water was filled to the mark and the solution homogenized.

#### 3.6.3 Preparation of 0.5 % of $FeCl_3$ solution

In a 100 mL volumetric flask, 0.5 g $FeCl_3$ was taken and distilled water was added and shaken until dissolved, then the flask was filled up with distilled water to label.

#### 3.6.4 Preparation of 10% Sodium hydroxide solution

0.5 g $FeCl_3$ was taken into a 100 mL volumetric flask and distilled water was added and shaken until dissolved, then the flask was filled with distilled water to label.

### 3.7 Qualitative test for extracted benzoic acid (Ferric chloride test)

The presence of benzoic acid was tested by dissolving the residue of benzoic acid in 10 mL of hot water and 3 drops of 0.5% ferric chloride solution. The presence of benzoic acid is suggested by the detection of salmon color precipitation of ferric benzoate.

### 3.8 Quantitative analysis of benzoic acid by spectrophotometric and titration methods

#### 3.8.1 Spectrophotometric method

The benzoic acid was extracted using diethyl ether from the prepared sample and the residue was dissolved in a methanol solvent. The Double Beam UV-visible spectrophotometer measured the absorbance at 225 nm against the blank sample. The amount was determined using the calibration curve of standard benzoic acid and express ppm concentrations were determined.

#### 3.8.2 Titration method

The extracted benzoic acid was dissolved in 10 mL of methanol and the solution was titrated with 0.05 N sodium hydroxide, adding phenolphthalein as an indicator. The titration end point was indicated by a light pink color and the amount of sodium hydroxide consumed was recorded.
The following equation was used to calculate the contents of benzoic acid in samples [25,89]:

$$Benzoic\ acid(ppm) = \frac{122\ xTitre\ xDilution\ x\ 1000\ x\ ml\ of\ 0.05N\ sodium\ hydroxide}{weight\ of\ sample\ x\ aliquot\ taken(60\ ml\ of\ filtrate)}$$

### 3.9 Method validation

For the validation of the spectrophotometric method for the determination of benzoic acid in the samples, the following performance parameters were verified: linearity, limits of detection and quantification, accuracy (recovery) and accuracy (recovery) (intraday and inter-day precisions).

**Linearity**

Linearity of response for standard was evaluated by triplicate assaying using six concentration levels ranging from 1-10 mg/L benzoic acid, covering all expected values and measuring the regression coefficient ($R^2$) from the concentration versus absorption plot. Similarly, the benzoic acid standard ranging from 30 to 150 mg/L evaluated the linearity of the titration process.

**Limit Detection (LOD)**

Limit of detection is the minimum concentration that can be detected by the analytical method with a given certainty. It is also the smallest concentration or amount of an analyte that can be reliably shown to be present or measured under defined condition [90].

**For UV-Vis method:** The LOD was calculated by three times standard deviation of the blank divided by the slope.

$$LOD=3.3\times SD/b,$$

**For Titration method:** LOD = 3 x standard deviation of the 5-blank measurement in mg/L

**Limit of quantification (LOQ)**

The limit of quantification (LOQ) of individual analytical procedure as the lowest amount of analyte in a sample which can be quantitative determined with suitable precision and accuracy [90]. **For UV-Vis method:** LOQ were calculated ten times standard deviation of blank divided by slope.

$$LOQ=10\times SD/b$$

**For Titration method:** LOQ = 10 x standard deviation of the 5-blank measurement in mg/L

**Recovery test**

As there is no any certified reference material used to compare the results with, the efficiency of the method used was assessed by spiking experiments. In order to validate the method accuracy, the recovery tests were performed by the analysis of the samples spiked with three different concentrations of standard benzoic acid. Then, the absorbance of solution before spiked and after spiked was measured by UV-Vis spectrometer. The percent of benzoic acid recovered from the solution were calculated by using formula [91]:

%Recovery = (C spiked sample – C unspiked sample x100)/ (C added (Spiked))

**Precision**

The precision of the developed method was expressed in terms of the relative standard deviation (RSD, %) [92]. Intraday variations were performed by analysis of three different concentrations (1,4&8mg/L) of the benzoic acid three times on the same day. The Inter-day precision were performed by analysis of three different concentrations (1,4&8 mg/L) of the benzoic acid for five days and % RSD was calculated.

**Statistical analysis**

For the comparison of the benzoic acid content of the food sample, statistical tools such as one-way ANOVA and person correlation were used. Single factor Analysis of variance (ANOVA) was used to test the significance difference between the two methods at alpha = 0.05[93]. Variation in the levels of benzoic acid between the samples and the methods were checked to determine it was due to random error.

## 4.Result and Discussion

### 4.1 Optimization of Spectrophotometry condition

### 4.1.1 Selection of wavelength

The wavelength selection was done with the help of UV spectra of Standard Benzoic acid in the range of 200 – 400 nm. The maximum absorbance was observed at wavelength of 225 nm (figure.3). Therefore, 225 nm was used for the determination of benzoic acid in the sample. [20,75]

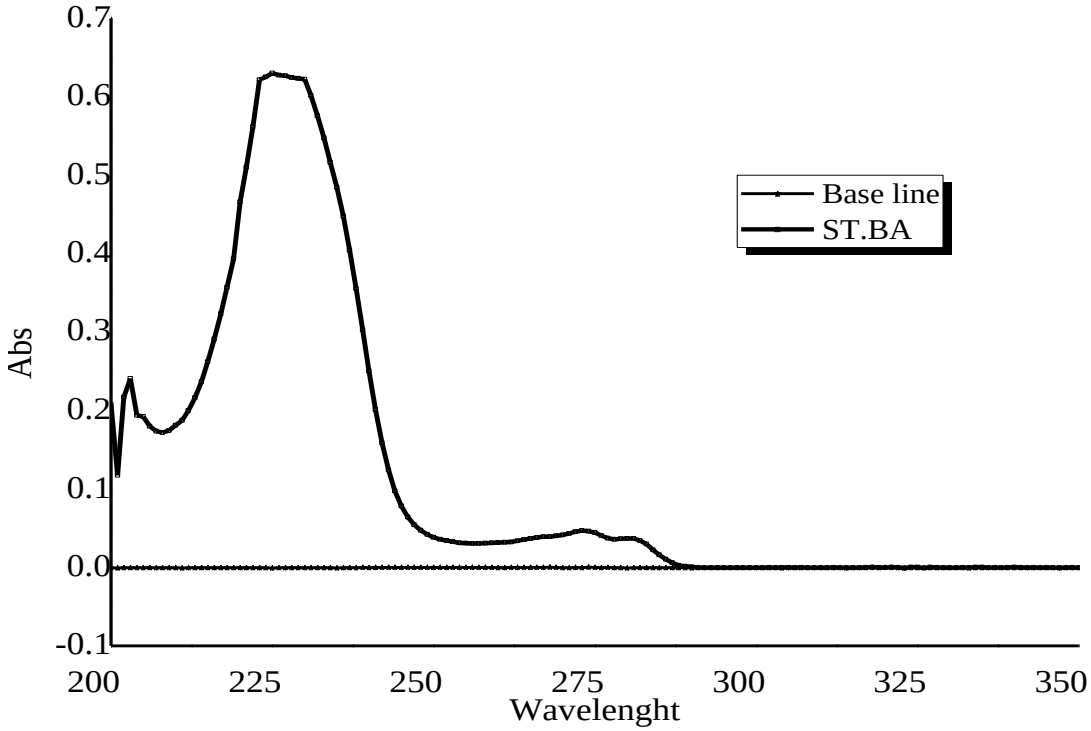



**Figure 3**: UV spectrum of Benzoic acid

### 4.1.2 Selection of extraction solvent

When choosing LLE as a sample preparation technique, a number of factors are important. Among these considerations, it is very important to choose a solvent method to achieve the objective of near-complete extraction of solutes of interest. When choosing a solvent, consideration should also be given to the boiling point, the degree of miscibility of the two phases, the relative basic densities, viscosity and propensity to form emulsions. When making a choice, consideration must also be given to the safety, toxicity, and flammability of the organic solvent. [94] Accordingly, four forms

of ESs were prepared and tested as an extraction solvent (DEE, CHL, DCM and PE). The results acquired (Figure. 4) showed that diethyl ether was selected as solvent extraction.

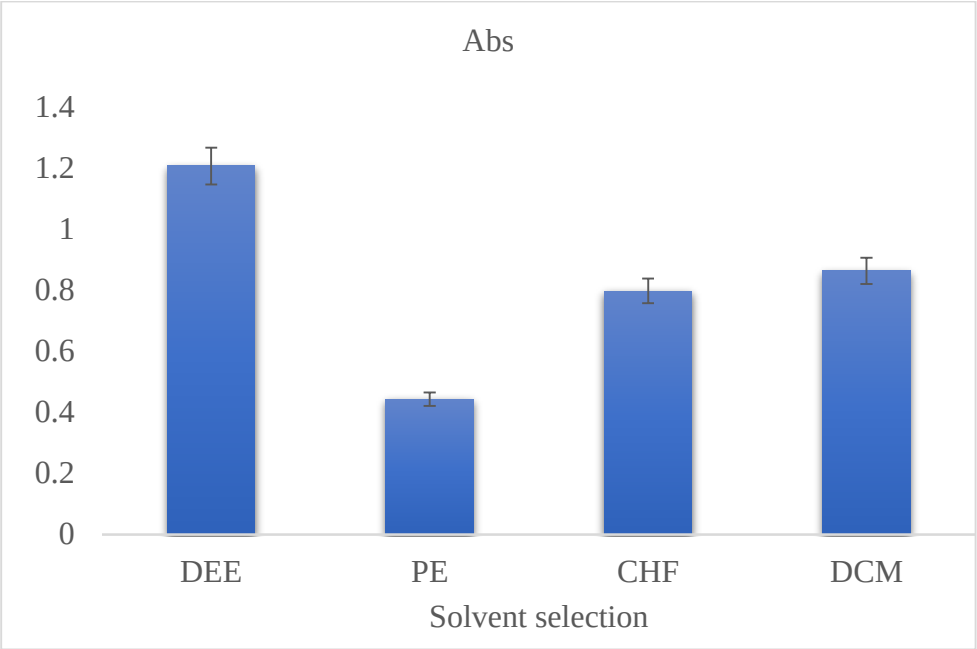



**Figure 4**: Effect of the type's extraction solvent. Extraction conditions: sample (20 g), volume of the extraction solvents (60 mL), extraction time (30min), wavelength(225nm), solvent methanol(10mL) and centrifugal time (25 min at 4000 rpm).

### 4.1.3 Volumes of extraction solvent

The amount of solvent extraction may play an important role in the efficient extraction of analytes. The volumes of extraction solvent in the 15-105mL range were examined to analyze the impact of extraction solvent thickness. Results obtained showed (Figure.5) that the recovery of benzoic acid at 60 mL was quantitative. However, the collection of extraction solvent was difficult in amounts of less than 60 mL and analyte loss occurred. On the other hand, dilution was observed for volumes greater than 60 mL [95]. For the remainder of the job, 60mL of extraction solvent was then used as an extraction solvent.

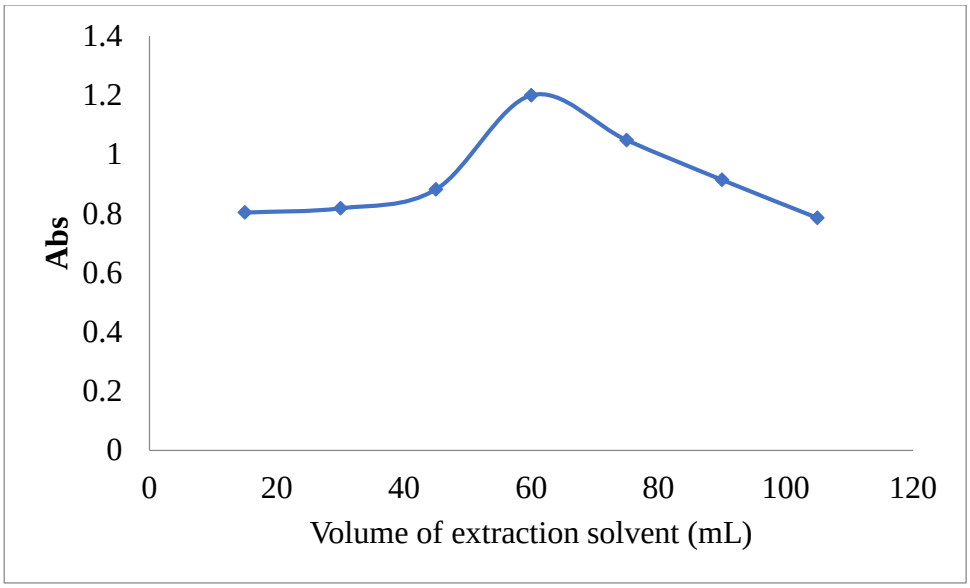



**Figure 5**: Effect of the Volumes of extraction solvent. Extraction conditions: sample (20 g), type of the extraction solvents Diethyl ether (DEE), extraction time (30min), solvent methanol(10mL), wavelength(225nm) and centrifugal time (25 min at 4000 rpm).

### 4.14 Solvent stability

The solvent stability is a measure of the extent to which the studied benzoic acid is stable in a solvent being used for the assay over a particular period of time under specified conditions. It is an essential requirement that the analyst should not undergo any chemical change and should remain stable in the particular solvent. [96] To examine the effect of solvent stability different solvent **(**DEE, Ethanol and, Methanol) were tested. Obtained results revealed (Figure.4) that Benzoic acid was readily soluble in methanol and showed good stability. Hence methanol was selected as solvent**.**

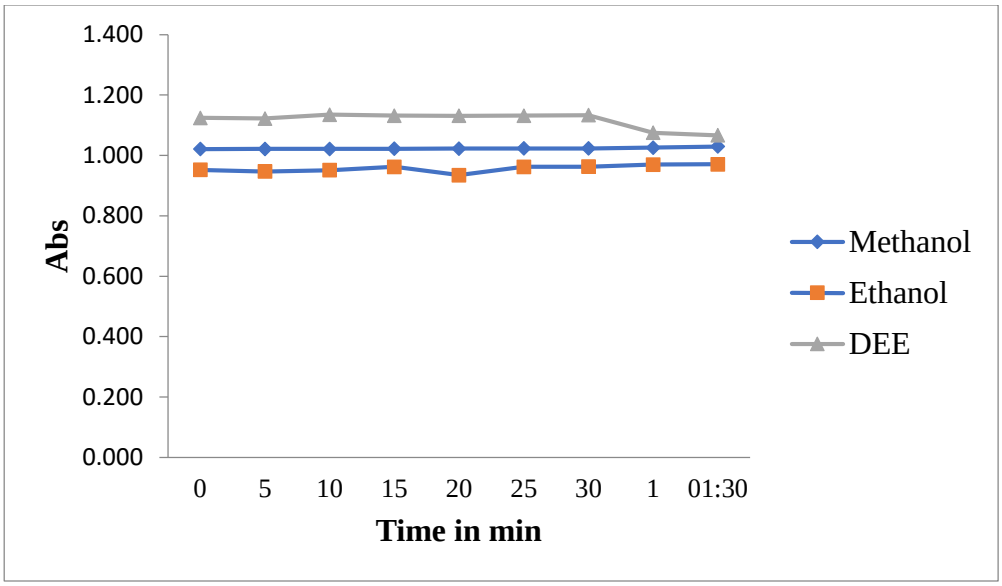



**Figure 6:** Effect of Solvent stability. Extraction conditions: sample (20 g), type and volume of the extraction solvents (DEE and 60mL) extraction time (30min), wavelength(225nm) and centrifugal time (25 min at 4000 rpm.

### 4.2 Validation methods

#### 4.2.1 Linearity

Linearity of response for standards was tested by assaying in triplicate using six levels of concentrations, ranging from 1 – 10 mg/L benzoic acid, which covers all expected values and the regression coefficient ($R^2$) was calculated from plot of concentration versus absorbance. The UV-Vis method showed good linearity with an acceptable correlation between absorbance and concentration with the correlation coefficient $R^2$=0.9955 and the regression equation (y= 0.1807x+0.1867) (Figure. 7)

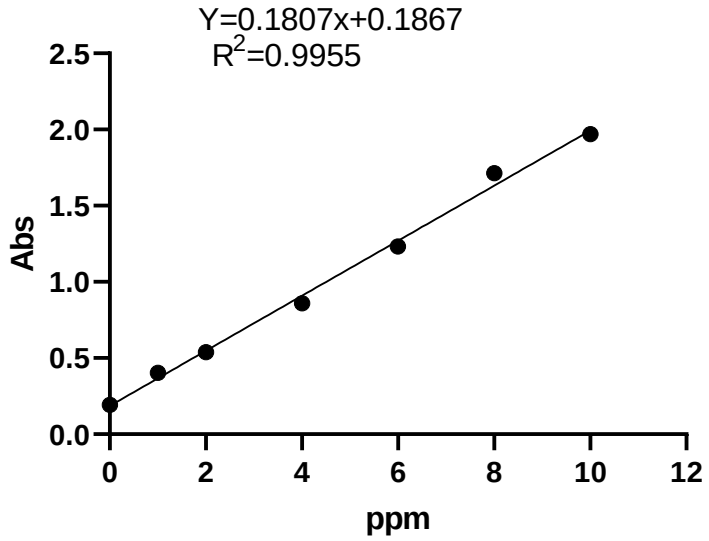



**Figure 7**: Calibration curve for benzoic acid, expressed on a linear scale

### 4.2.2 Detection and quantification limits

LOD and LOQ were determined from the residual standard deviation and the slope of the linear calibration equation obtained. The limits of detection (LOD) and quantification (LOQ) of the UV-Vis method were calculated using 3xSD/slope and 10×SD/slope respectively, while for titration method LOD and LOQ were calculated using 3 and 10 times of SD of blank measurement. Accordingly, LOD and LOQ of the proposed method were presented in Table 3.

**Table 3**: LOD and LOQ of the two methods in ppm

| Method | LOD | LOQ |
|---|---|---|
| UV-Vis Spectrophotometry | 0.156 | 0.467 |
| Titration | 0.58 | 1.92 |

### 4.2.3 Precision

The precision of the developed method was expressed in terms of the relative standard deviation (RSD, %). Intraday variations were performed by analysis of three different concentrations

(1,4&8mg/L) of the benzoic acid three times on the same day. The Inter-day precision were performed by analysis of three different concentrations (1,4&8 mg/L) of the benzoic acid for five days and % RSD was calculated. The low RSD values indicate that there were no significant variations in the analysis of benzoic acid at the given concentration levels. Table.3 show the Intraday and inter-day precision of the method.

**Table 4**: Intraday and Inter-day precision

| **Precision** | **%RSD** | **%RSD** | **%RSD** |
|---|---|---|---|
| | Level I | Level II | Level III |
| Intraday precision | 1.36 | 0.83 | 0.19 |
| Inter-day precision | 5.29 | 5.88 | 1.14 |

### 4.2.4 Accuracy

Recovery is the fraction of the analyte determined after addition of a known amount of the analyte to a sample. The recovery test was carried out by spiking three different concentration of standard solution of benzoic acid in selected three processed packed fruit juices and soft drink samples. Then, the absorbance of solution before spiked and after spiked was measured by UV-Vis spectrometer and the volume of sodium hydroxide consumed before spiked and after spiked was recorded for titration method. The %R of benzoic acid for UV-Vis and titration were in the range 94.9 – 104.0% and 80 – 103% respectively. These values are in the literature range 80-120%. This indicates the optimized methods are accurate/valid for determination of benzoic acid, shown in Table 5. The recovery (%R) of the sample was calculated by:

$$\%R = \frac{\text{Cspiked conc} - \text{Cunspiked conc}}{\text{conc of standard added}} X100$$

**Table 5:** Results of recoveries test for optimized procedure of three food samples (n=3)

| Method | Sample | Mean Recovery(%R) |
|---|---|---|
| UV-Vis | 3D | 94.88 ± 10.96 |
| | 7star | 96.09 ± 2.96 |
| | sprite | 104.00 ± 4.92 |
| Titration | 3D | 80.00 ± 2.73 |
| | 7star | 98.74 ± 6.53 |
| | Sprite | 103.00 ± 12.40 |

### 4.3 Qualitative test for extracted benzoic acid

**Ferric Chloride Test**

Benzoic acid reacts with neutral FeCl3 to give a Salomon colored (light brown) precipitate of ferric benzoate (Figure.8). The result of this analysis of fruit juice and soft drink confirms all samples contained benzoic acid.

$$3(C_6H_5COOH) + FeCl3 \rightarrow 3(C_6H_5COO)_3Fe + 3HCl$$

**Figure 8:** Light brown Precipitate of ferric benzoate

## 4.4 Quantitative analysis of Benzoic Acid by Spectrophotometric and Titration Methods

### Spectrophotometric method

UV-Visible spectrophotometer involves measurement of the number of ultraviolet radiations absorbed by any substance in a solution. Instrument which measures the ratio or function of the ratio of the intensity of two beams of light in the UV-Visible area [75].

### 4.4.1 Determination of benzoic acid from soft drink and fruit juice samples by UV-Vis spectrophotometry

The aim of this study was to investigate and quantify commonly used preservatives in marketing fruit juices and soft drinks. The spectrophotometry method was used to carry out the analysis at 225 nm in the UV region. From the corrected absorbance and the calibration graph obtained using standard benzoic acid solution, the amount of benzoic acid was determined.

For this purpose, 10 different brand of fruit juice and 3 soft drink samples in triplicate were collected, extracted using optimized condition and finally the level of benzoic acid were analysed at 225 nm by UV-Vis spectrophotometry, the results are presented in (Table.6). All the fruit juice products contained benzoic acid. The benzoic acid concentration in fruit juice and Soft drink samples by using UV-Vis spectrometry were in the range of 81.5 ± 0.1 – 385.0 ± 0.1 and 284.0 ± 0.07– 400.9 ± 0.4 respectively. The obtained results showed that all fruit juice and soft drink samples contain benzoic acid below the permissible limit, 1000 mg/L [3]. Among the fruit juices sample M5 contains the highest amount of benzoic acid while M1 was the lowest. Out of the analyzed samples six of them have labeled but the other they don’t have, which could create obstacle for regulatory purpose. In addition, almost all imported samples M3-G1 are higher BA concentration compared to the local one.

**Table 6**: Concentrations of benzoic acid in fruit juice and soft drink samples by UV-Vis Spectrophotometry

| Fruit juices samples | Concentration benzoic acid (ppm) in 20µL | Concentration of benzoic acid(ppm) in the sample |
|---|---|---|
| M1 | 1.63 ± 0.18 | 81.50 ± 0.09 |
| M2 | 3.75 ± 0.21 | 187.40 ± 0.10 |
| M3 | 3.25 ± 0.20 | 163.80 ± 0.09 |
| M4 | 2.63 ± 0.19 | 131.60 ± 0.07 |
| M5 | 7.70 ± 0.29 | 385.00 ± 0.07 |
| M6 | 2.74 ± 0.19 | 137.00 ± 0.04 |
| M7 | 2.78 ± 0.19 | 139.00 ± 0.07 |
| T1 | 4.62 ± 0.19 | 128.00 ± 0.07 |
| O1 | 4.95 ± 0.22 | 231.00 ± 0.09 |
| G1 | 2.56 ± 0.23 | 247.50 ± 0.09 |

| Soft drink samples | Concentration benzoic acid (ppm) in 20µL | Concentration of benzoic acid(ppm) in the sample |
|---|---|---|
| S1 | 5.67 ± 0.24 | 284.00 ± 0.07 |
| S2 | 8.01 ± 0.29 | 400.90 ± 0.41 |
| S3 | 6.28 ± 0.25 | 314.50 ± 0.16 |

### 4.4.2 Quantification of benzoic acid from soft drinks and fruit juices by Titration method

The analysis of benzoic acid content in the samples was based on titrimetric method. The benzoic acid concentration in the sample were determined.

The benzoic acid contents calculated as follows:

$$Benzoic\ acid(ppm) = \frac{122\ xTitre\ xDilution\ x\ 1000\ x\ ml\ of\ 0.05N\ sodium\ hydroxide}{weight\ of\ sample\ x\ aliquot\ taken(60\ ml\ of\ filtrate)}$$

The result of benzoic acid concentration in fruit juice and soft drink samples by the titration methods is presented in (Table.7). All the fruit juice products contained benzoic acid. The benzoic acid concentrations in fruit juice and soft drink samples ranged between 139.00 ± 0.25–182.50 ± 1.00 mg/L and 208.00 ± 0.21–221.00 ± 0.20 mg/L respectively. A limit of 1000 mg/L have been set for benzoic acid, [3]. The result on Titration method showed that all samples also within permissible limits

**Table 7**: Concentrations of benzoic acid in fruit juice and soft drink samples by titration method

| **Fruit juice samples** | **Concentration benzoic acid (ppm) in 20µL** | **Concentration of benzoic acid(ppm) in the sample** |
|---|---|---|
| M1 | 3.26 ± 0.50 | 163.00 ± 0.50 |
| M2 | 3.65 ± 0.47 | 182.20 ± 0.47 |
| M3 | 3.52 ± 0.57 | 176.20 ± 0.57 |
| M4 | 3.13 ± 0.43 | 156.50 ± 0.43 |
| M5 | 3.00 ± 0.10 | 150.00 ± 0.10 |
| M6 | 3.65 ± 1.00 | 182.50 ± 1.00 |
| M7 | 2.87 ± 0.25 | 139.00 ± 0.25 |
| T1 | 3.39 ± 0.49 | 169.50 ± 0.49 |
| O1 | 3.39 ± 0.12 | 169.60 ± 0.12 |
| G1 | 3.52 ± 0.11 | 176.00 ± 0.11 |

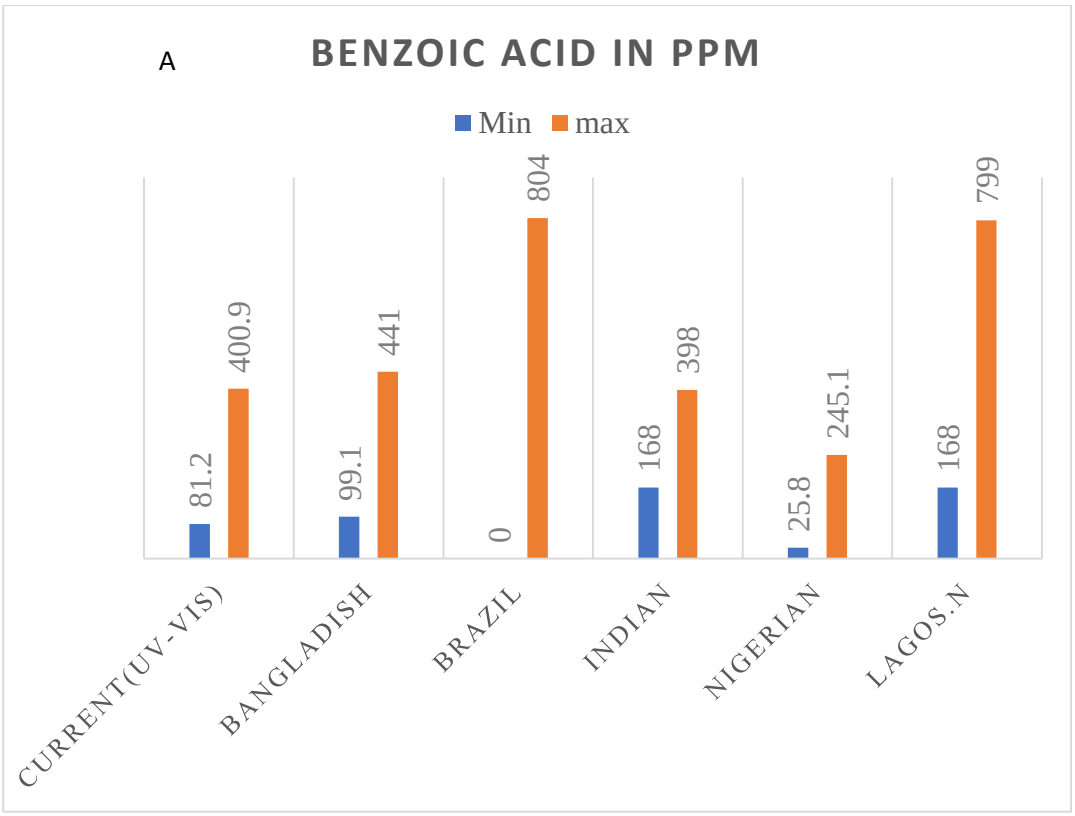
| **Soft drink samples** | **Concentration benzoic acid (ppm) in 20µL** | **Concentration of benzoic acid(ppm) in the sample** |
|---|---|---|
| S1 | 4.18 ± 0.21 | 208.00 ± 0.21 |
| S2 | 4.44 ± 0.20 | 221.00 ± 0.20 |
| S3 | 4.30 ± 0.40 | 215.00 ± 0.40 |

### 4.5 Statistical analysis of the two method

One way a nova was used to express the significance difference of the two method, UV-Vis spectrophotometry and titration. from a nova data (Apendex.2) the two method were significantly different at $P<0.05$, but at sample M7 the two method are not significantly different at $P<0.05$. The correlation within the sample and between the sample show the correlation of the sample (Appendix.3).

### 4.6 Comparison of the level of benzoic acid with other reported articles

The proposed method was compared with some of the methods which have recently been reported in the benzoic acid extraction and determination literature. The preservative levels obtained in this study were compared with the values reported in other countries in similar studies (Figure.9) (A and B). Determination of preservatives in fruit juice products available in Bangladesh [73] showed that the mean concentration of benzoic acid in samples of fruit juice and soft drinks was 99.1 – 441ppm. The average value recorded here is almost similar to that of 81.5– 400.8 ppm reported in the current study. Determination of benzoic and sorbic acid in Brazilian foods [18] and benzoic acid level Sulphur (IV) Oxide and Sorbic Acid in Carbonated Drinks Sold in Lagos, Nigeria [74], mean concentrations of N.D – 804 ppm and 168–799 ppm of benzoic acid were reported, respectively, which are higher than the current analysis (81.5–400.8). A study on preservative quantification (E211) reported average benzoic acid concentrations in Indian Carbonated Soft Drink Samples [20] (168-398) of benzoic acid approach to the current (284–-400.9ppm) study. Benzoic acid concentrations ranging from 25.8–245.10 ppm was reported for study and health risk assessment of sodium benzoate and potassium sorbate in selected fruit juice and soft drink brands in Nigeria [75]; these values are much lower than what we report in this study.




.

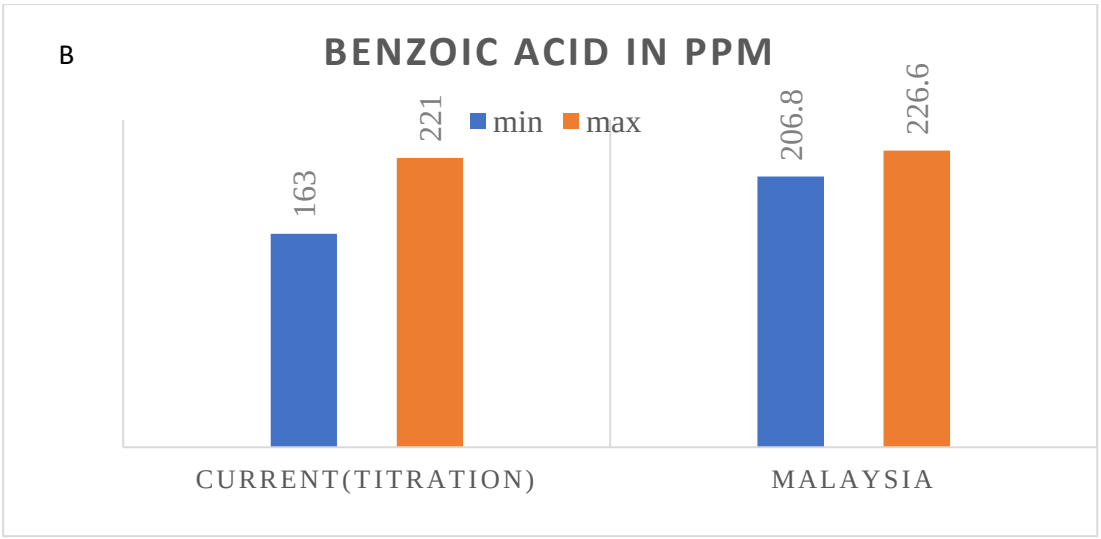



**Figure 9:** comparison of benzoic acid concentration by UV-Vis (A) and Titration (B) with other literature

## 5. Conclusion and Recommendations

### 5.1 Conclusions

Fruit juices and Soft drinks are currently a popular drink for both children and adults. However, the use of food preservatives in juices and drinks has become a serious threat to human health because they cause numerous life-threatening diseases. Our primary objective was, then, to check the Fruit juices and Soft drinks on the market to see whether they contain preservatives and their content. A UV-Vis spectrophotometric and titration method were applied in this study to assess the benzoic acid preservatives in fruit juice and soft drinking samples. The proposed UV-Vis spectrophotometry and titration method have successfully validated with a parameter such as recovery, precision (intera day and intraday), LOD, LOQ, linearity and specificity. Thirteen different fruit juice and soft drink samples have been collected for this purpose. From these six juices and three soft drinks labels to contain benzoic acid without the exact amount. Benzoic acid content was analyzed qualitatively by ferric chloride and found that all fruit juices and soft drinks were contained benzoic acid. The results obtained from the two-method showed that the benzoic acid concentration varied between different kinds of soft drink and fruit juices products. The level of benzoic acid in fruit juice and soft drink samples by using UV-Vis spectrometry were in the range of 81.5 ± 0.1 – 385.0 ± 0.1 and 284.0 ± 400.9 ± 0.4 respectively. While the level of benzoic acid in fruit juice and soft drink by using titration were in the range of 139.0 ± 0.1 – 182.5 ± 1.0 and 215.0 ± 0.4 – 221.0 ± 0.2 respectively.

The overall finding from the analysis indicates all samples analyzed by the two method were within the permissible level 1000mg/L rules set by Joint FAO/WHO Food Standards Program Codex Committee on Food Additives and Contaminants. Benzoic acid consumption is higher in soft drinks than in fruit juices according to the result obtained. This is because most manufacturers of fruit juices use pasteurization as the method in preserving their products. The findings from the ANOVA showed there are a significant difference between the result obtained by UV-Vis spectrophotometry and titration technique at $p<0.05$. The result of the two methods were comparable with the other expensive instruments so these two methods can be used as screening purpose. As per this study all samples contain preservative even if the amount is within the permissible limit however the samples are highly consumed by adolescents especially by the school going children, an excess amount of preservatives can cause many serious health problems.

One of them is behavioral change especially in children. Besides, another most serious harmful effect of preservatives is their ability to transform into carcinogen when digested. Thus, frequent use and conception of more than one or two bottle may exceed the permissible limit so too much use of preservatives should be prohibited.

## 5.2 Recommendations

Based on the findings the following recommendation are forwarded

- We recommend all marketed fruit juices should be investigated by the concerned authorities as well as independent research groups at regular interval across the country especially for the imported ones.
    - Due to cost effectiveness and easily availability of the two methods they can be used for the routine analysis of fruit juices and soft drinks.
    - Finally, the Food and Drug Standards Authority should have a routine check up on the various soft drinks, fruit juices and other consumable products on the market whether the preservatives levels are within the permissible limits.
    - Too much consumption of packed fruit juices and Soft drinks may expose for high level of preservatives and lead to health hazard we recommend to use organic unprocessed fruit, which is cheap and available in everywhere.
    - Further study by including other possible preservative, colorant will be recommended to get sufficient information about the imported and local products.

## 6. References


1. Gould, G. W.; "Preservation: past, present and future". British *Medical Bulletin*. **2000,** 56(1), 84-96.
2. Indrajit, S.; Ajay, S.; and Shrivastava, V. S.; "Determination of benzoic acid residue from fruit juice by gas chromatography with mass spectrometry detection technique". *Archives of Applied Science Research.* **2011**, 3 (2), 245-252.
3. CAC (2004) Joint FAO/WHO Food Standards Program Codex Committee on Food Additives and Contaminants: Updated as at the 36th Session of the Codex Alimentarius Commission, Rotterdam..
4. Mazdeh, F.Z.; Moradi, Z.; Moghadam.; Moradi-Khatoonabadi, Z.; Aftabdari, F.E.; Badaei, P.; and Haji Mahmoodi, M.; Determination of synthetic food colors, caffeine, sodium benzoate and potassium sorbate in sports drinks. Trop. *J. Pharm. Res*.**2016**, 15(1),183-188.
5. Dolezal, P.; Comparison of the effect of benzoic acid addition on the fermentation process quality with untreated silages, Actauniv. agric. etsilvic. *Mendel. Brun*.**2004**, 2, 15-22.
6. Ene, C.P.; and Diacu, E.; High performance Liquid Chromatography method for the determination of benzoic acid in beverages.*UPB Sci. Bull.Series B.* **2009**, 71,81-88.
7. Khosrokhavar, R.; Sadeghzadeh, N.; Amini, M.; Ghazi-Khansari, M.; Hajiaghaee, R.; Ejtemaei, M.Sh.; Simultaneous determination of preservatives (sodium benzoate and potassium sorbate) in soft drinks and herbal extracts using High Performance Liquid Chromatography (HPLC). *Journal of Medicinal Plants*. **2010**, 9(35), 80-87.
8. Turkoglu, S.; Genotoxicity of five food preservatives tested on root tips of Allium cepa L. *Mutat Res***. 2007**,626(1-2), 4–14.
9. Zengin, N.; Yuzbasioglu, D.; Unal, F. Yilmaz, S.; and Aksoy, H. The evaluation of the genotoxicity of two food preservatives: sodium benzoate and potassium benzoate. *FoodChemToxicol*. **2011**,49(4), 763–9.
10. Hong, H.; Liang, X.; and Liu, D. Assessment of benzoic acid levels in milk in china. *Food Control*. **2009**, 20(4), 414–8.

11. Safari, R.; and Saeidi, A.M. The effect of nisin A and sodium benzoate on behavior of listeria monosytogenes and some microbial and chemical parameters in silver carp (hypophtalmichtys molitrix) fillet stored at 4° c. *J Food Hygiene*. **2011**,1(3),9–13.
12. D Beyoglu and JR Idle, The glycine deportation system and its pharmacological consequences, PharmacolTher.135(2), **2012**,151– 67.
13. Vernole, P.; Caporossi, D.; Tedeschi, B.; Porfirio, B.; Melino, G.; and Bonmassar, E. Cytogenetic effects of 1-p-(3-methyltriazeno) benzoic acid potassium salt on human lymphocytes in vitro. *Mutat Res Genet Toxicol*. **1987**,189(3), 349–56.
14. El-Ziney, M.G.; GC-MS analysis of benzoate and sorbate in Saudi dairy and food products with estimation of daily exposure. *J Food Technol*. **2009**, 7(4), 127–34.
15. Freedman, B.J.; Asthma induced by Sulphur dioxide, benzoate and tartrazine contained in orange drinks. *Clin. Allergy*. **1977**, 7, 407.
16. WHO; Principles for the safety assessment of food additives and contaminants in food. *Environmental Health Criteria*. 1987, 70.
17. Cheung, P.C.K.; Food Preservatives, Food and nutritional sciences. *The Chinese University of Hong Kong*. **2003**,1- 53.
18. Tfouni, S.A.V.; and Toledo, C.F.; Determination of Benzoic and sorbic acids in Brazilian Foods. *Food control*. **2002**, 13 (2), 117-123.
19. Hannuksela, M.; andHaahtela, T.; Hypersensitivity reactions to food additives. *Allergy*.**1987**,42 (8), 561–575.
20. Venu, L. N.; Austin, A. Study and Quantification of Preservative (E211) In Carbonated Soft Drink Samples. IOSR Journal of Applied Chemistry.**2019**, *12* (4), 17–23.
21. Ochia, N.; Sasamoto, K.; Takino, M.; ·Yamashita, S.; Daishima, S.; Heiden, A.C.; Hoffmann, A.; Simultaneous determination of preservatives in beverages, vinegar, aqueous sauces, and quasi-drug drinks by stirbarsorptive extraction (SBSE) and thermal desorption GC–MS. *Analytical Bioanalytical Chemistry*.**2002** ,373, 5663
22. Abedi, A.S.; Mohammadi, A.; Azadniya, E.; Mortazavian, A.M.; Khaksar, R.; Simultaneous determination of sorbic and benzoic acids in milk products using an optimized micro extraction technique followed by gas chromatography. *Food Additives and Contaminants Part A*,**2014**, 31, 21-28

23. Rahmawati, K.R.; Effendi, N.; &Ismayani, N.; Analisis Kadar Pengawet Natrium BenzoatPadaProdukMinumanBerkarbonasiDenganMetode HPLC. *As-SyifaaJ Farmasi,***2014**,6(2), 112-117.
24. Third, T. H. E.; Conference, I.; Sciences, O. N. B.; Applications, T. Spectrophotometric Determination of Sodium Benzoate in Some Soft Drinks Collected from Some Local Markets in El-Bieda City - Libya. No. 2, 67–76.
25. Jurnal, F.; Dan, F.; Kesehatan, I.; Astuti, E. J.; Fakhri, R.; Ilham, N.; Rahman, J. Validation Method for Determining Sodium Benzoate in Fruit Juice Drinks in Malang. **2019**, *4* (1), 19–23.
26. Zhong, Y.; Wu, L.; Chen, X.; Huang, Z.; Hu, W. Effects of Food-Additive-Information on Consumers' Willingness to Accept Food with Additives. **2018**
27. Karunaratne, N.; Pamunuwa, G. Introductory Chapter: Introduction to Food Additives World' s Largest Science, Technology & Medicine Open Access Book Publisher. **2017**, No. September.
28. International Food Information Council (IFIC) and U.S. Food and Drug Administration. *Overview of Food Ingredients, Additives & Colors*. **2010**.
29. Al-Kharosi, A. S.; Al-Sabari, W. A.; AL-Shidhani, F. H.; Alfarsi, S. A.; Taha, S. M. A Simple Method for Simultaneous Determination of Commonly Used Artificial Food Colors and Preservatives in Soda, Jam, and Yogurt by HPLC-PDA. *Brazilian J. Anal. Chem.* **2020**, *7* (26), 18–26.
30. Boca, F. L.; Smoley. C. K.; U.S. Food and Drug Administration. *CRC Press, Inc., New York*. **1993**,171pp.
31. Branen, A.; Haggerty. R.J.; Food additives (2nd Ed). New York Basel. ISBN: 0-8247-9343-9. Food Additives: Functions, Effects, Regulations, Approval and Safety Evaluation **2002**
32. Al-Shammari. B, R.; Khan. S.; and Shankity. I.; The effect of preservatives and flavour additive on the production o. oxygen-free radicals by isolated human neutrophils. *Int. J. Nutrit. Food Sci.* **2014**, 3(3),210-215.
33. European Union legislation. Food additive approved by the EU (E-number list by category)**2016**.
34. Food Additives and Ingredients Association. E numbers. [Internet] **2017**.

35. Ann Lawrence; Understanding food Additives. *FAIA*.**1998**,1-128
36. Rahman, S.M.; Hand book of preservation,2nded.; CR_press:**2007**.
37. Gherezgihier, B. A.; Mahmud, A.; Admassu, H.; Shui, X. W.; Fang, Y.; Tsighe, N.; Mohammed, J. K. Food Additives: Functions, Effects, Regulations, Approval and Safety Evaluation. *J. Acad. Ind. Res.* **2017**, *6* (4), 62–68
38. Bruna, G. O. L.; Thais, A. C. C.; Lígia, A. C. C. Food Additives and Their Health Effects: A Review on Preservative Sodium Benzoate. *African J. Biotechnol.* **2018**, *17* (10), 306–310.
39. Thomas, O.E.; Adegoke, O.A.; Toxicity of food colours and additives: *A review. Afr. J. Biotechnol*. 9(36):900-914.
40. Krishna, G. V. Review on the Role of Food Preservatives and Its Efficacy. **2017**, *2* (2), 1–12
41. Sunitha, J.; Preethi. R.; FDST Food Additives. *Acharya N. G. Ranga Agricultural University* .**2000**.
42. Abdulmumeen1,.A.H; Risikat.N.A.; and. Sururah.R.A.; Food: Its preservatives, additives and applications. IJCBS.**2012,**1,36-47).
43. Samal, D.; Industries, S. A.; Gouda, S. Food Preservatives and Their Uses: A Short Report Food Preservatives and Their Uses: A Short Report. **2017**, No. January
44. Amponsah, D. Determination of the Amount of Caffeine and Benzoic Acid in Selected Soft Drinks. *Int. J. Sci. Eng. Res.* **2014**, *5* (6), 1375–1378
45. Lennerz.; Vafai, S.B.; Delaney, N.F.; Clish, A.B.; Deik. A.A.; Pierce. K.A.; Ludwig. D.S.; Mootha, V.K.; Effect of sodium benzoate, a widely used food preservative, on glucose homeostasis and metabolic profiles in humans. *Mol. Genet. Metab*. **2015**, 114(1),73-79
46. Yadav, A.; Kumar. A.; Das.M.; Tripathi, A.; Sodium benzoate, a food preservative, affects the functional and activation status of splenocytes at non cytotoxic dose. *Food Chem. Toxicol*. **2016**, 88,40-47.
47. Tsay, H. J.; Wang, Y.H.; Chen. W.L.; Huang. M.Y.; Chen.Y.H.; Treatment with sodium benzoate leads to malformation of zebrafish larvae. Neurotoxicology. Teratol.**2007**,29(5),562-569
48. Zhang, G.; M.Y.; Spectroscopic studies on the interaction of sodium benzoate, a food preservative, with calf thymus DNA. *Food Chem*. **2013**, 141(1),41-47.

49. Pongsavee, M.; Effect of sodium benzoate preservative on micronucleus induction, chromosome break, and Ala40Thr superoxide dismutase gene mutation in lymphocytes. *Biomed. Res. Int*. **201**5,1-5
50. Ren, L.; Meng, M. Wang, P.; Xu. Z.; Eremin, S.A.; Zhao. J.; Yin, Y.; Xi. R.; Determination of sodium benzoate in food products by fluorescence polarization immunoassay. *Talanta*.**2014**,121,136-143.
51. European food Safety Authority (EFSA) Regulation .**2017**.
52. Food and Drug Administration of the United States of America (FDA).**2017**.
53. Pongsavee, M.; Effect of sodium benzoate preservative on micronucleus induction, chromosome break, and Ala40Thr superoxide dismutase gene mutation in lymphocytes. *Biomed. Res. Int.* **2015,**1-5.
54. Bridges, J.W.; French, M.R.; Smith., R.L; Willians, R.T.; The fate of benzoic acid in various species. *Biochem. J*.**1970** ,118(1,47-51.
55. Akintonwa, A.; Awodele, O.; Emeka, P.M.; Osajare, O.; The mutagenic potentials of potassium bromate and some commonly used food additives. *Afr. J. Biotechnol*. **2007**, 6(8),1004-1006.
56. Benzoic Acid and Sodium Benzoate. *Concise International Chemical Assessment* .**2000,** Document 2**.**
57. Maki., T.; Suzuki, Y.; Benzoic acid and derivatives. *In: Ullmann's encyclopedia of industrial chemistry*. **1985**, 3. 555–568
58. SCCNFP (Scientific Committee on Cosmetic Products and Non-food Products. Cosmetic Ingredients). *Amended final safety assessment: benzyl alcohol, and benzoic acid and its salts and benzyl este*r. **2011**,1-38.
59. FAO/WHO (Food and Agriculture Organization of the United Nations and the World Health Organization). Benzoic acid and sodium benzoate. *Concise International Chemical Assessment Document*.**2000 ,**26,1-48
60. CIR (The Cosmetic Ingredient Review). Expert Panel final report on the safety assessment of benzaldehyde. *Int. J. Toxicol*.**2006.** 25:11-27.
61. EU Regulation 528/2012. Evaluation of active substances. Benzoic acid. Product-type 03 (Veterinary hygiene). *Assessment report finalized in the Standing Committee on Biocidal Products***.2013**,1-74

62. Liao, C.; Liu, F.; and Kannan, K..; Occurrence of and dietary exposure to parabens in foodstuffs from the United States. *Environ. Sci. Technol*. **2013**., 4,3918-3925
63. FAO/WHO (Food and Agriculture Organization of the United Nations and the World Health Organization). (2006). Evaluation of certain food additives and contaminants. 67th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). 20-29 June **2006**, Rome, Italy. pp 1-49
64. European Commission. Scientific Committee on consumer products. Opinion on benzoic acid and sodium benzoate. SCCP/0891/05. 21 June **2005**. pp. 1-30
65. Sudareva, N. N.; Chubarova, E. V.; Time-dependent conversion of benzyl alcohol to benzaldehyde and benzoic acid in aqueous solutions. *J. Pharm. Biomed. Anal***2006**. 41:1380-1385
66. Becker, L. C.; Bergfeld, W. F.; Belsito, D. V.; Hill, R. A.; Klaassen, C. D.; Liebler, D.; Marks, J. G.; Jr., Shank, R.C., Slaga, T. J.; Snyder, P. W.; Andersen, F. A..; Safety assessment of alkyl benzoates as used in cosmetics. *Int. J. Toxicol*. **2012,**31,342S-372S
67. Iammarino, M., Di Taranto, A., Palermo, C. and Muscarella, M. Survey of benzoic acid in cheeses: contribution to the estimation of an admissible maximum limit. *Food Addit. Contam. Part B.***2011** 4,231-237
68. Tremblay, G. C.; and Qureshi, I. A.; The biochemistry and toxicology of benzoic acid metabolism and its relationship to the elimination of waste nitrogen. *Pharmacol.Ther*. **1993** 60,63-90.
69. Egger, J.; Graham, P. J.; Carter, C. M.; Gumley, D.; and Soothill, J. F.; Controlled trial of oligoantigenic treatment in the hyperkinetic syndrome. *The Lancet***1985** 32,540-545
70. Tuormaa, T. E.; The adverse effects of food additives on health: a review of the literature with special emphasis on childhood hyperactivity. *J. Orthomol*. *Med*. **1994,**9,225-243.
71. McCann, D.; Barrett, A.; Cooper, A.; Crumpler, D.; Dalen, L.; Grimshaw, K..; Kitchin, E.; Lok, K..; Porteous, L; Prince, E.; Sonuga-Barke, E.; Warner, J. O.; Stevenson, J.; Food additives and hyperactive behavior in 3-year-old and 8/9-year-old children in the community: *a randomized, double-blinded, placebo-controlled trial*. *Lancet*. **2007**, 370:1560-1567.
72. EMA (European Medicines Agency). Science medicines health. Questions and answers on benzoic acid and benzoates in the context of the revision of the guideline on excipients

in the label and package leaflet of medicinal products for human use" (CPMP/463/00). 23 January **2014**. pp. 1-7

73. Islam, S.Md.; Zahan, N.; Hossain, S.Md.; Rouf, S.S.As. Determination of Preservatives in Fruit Juice Products Available in Bangladesh by a Validated RP-HPLC Method. J. Pharm. Sci. **2019,**18(2): 195-208.
74. Onwordi C.T.; Olanrewaju A.J.; Wusu A.D.; Oguntade B.K. Levels of Benzoic Acid, Sulphur (IV) Oxide and Sorbic Acid in Carbonated Drinks Sold in Lagos, Nigeria. American Journal of Food Science and Technology. 2017, 5(2), 38-44.
75. Magomya, M.A; Yebpella, G.G.; Okpaegbe, C.U.; Oko, J.O.; Gambo, B.S. Analysis and Health Risk Assessment of Sodium Benzoate and Potassium Sorbate in Selected Fruit Juice and Soft Drink Brands in Nigeria. *International Journal of Pharmacy and Chemistry*, **2020**,6(5),54-59.
76. lic, B.; Titration: ph indicator, Thermometer Titration, Nonaqueous Titration, Equivalence point, Acid-Base Titration, Amperometry Titration. General *Books LLC*.**2010**
77. Jacobsen, J.J.; Jetzer, K.H.; Patani, N.; Zweerink, G.; Zimmerman, Ti*tration Techniques. Chem.Eduv*.**1995**,72,612.
78. Harris, D.C. Quantitative Chemical Analysis.8$^{th}$edn, W.H. Freeman,**2010**
79. Mattock, G.; Taylor, G.R.; and Paul, M.A. pH Measurement and Titration.*J.Electrochem.Soc*.**1963**,110
80. Lenghor, N.; Jakmunee, J.; Vilen, M.; Sara, R.; Christian, G.D.; Grudpan, Sequential injection Redox or Acid-Base Titration for determination of weak acid.**2002**,58,1139-1144.
81. Mitchell, P.; Moyle, J.; Smith, L.; Bromothymol Blue as a pH indicator in Mitochondrial Suspensions.*Eur.J. Biochem*.**1968**,4,9-19
82. Mehmood, Y.; Saleem, N.; Mahmood, R. K.; Riaz, H.; Raza, S. A. Analytical Method Development and Validation of Pheniramine Maleate Injection. *Int. J. Drug Dev. Res.* **2016**, *8* (1), 44–48
83. All, C.; Sections, R.; No, I. F.; No, I. Department of Chemistry, Cambridge. **2015**, *2015* (June), 1–2.
84. Ermer, J.; Validation in pharmaceutical analysis. Part I: An integrated approach. Journal of pharmaceutical and biomedical analysis**2001**, 24: 755–767.

85. Kislik, S. V.; Solvent Extraction: Classical and Novel Approaches (1st Ed). Givat Ram, Jerusalem. Casali Institute of Applied Chemistry:**2002.**
86. Stalikas, C. D. Review Extraction, Separation, and Detection Methods for Phenolic Acids and Flavonoids. **2007**, 3268–3295.
87. Romero, E. B. Laboratory Manual. **2014**.
88. Rahardian, G. W.; Monica, S.; Wasito, H.; Susilowati, S. S. Chemometrics-Assisted Spectrophotometry for Simultaneous Determination of Sodium Benzoate and Citric Acid in Beverage Products. *Pharmaciana* **2018**, 8 (1), 1
89. FSSAI.Manual of methods of analysis of foods. *Food additives* .**2015**.
90. Mehmood, Y.; Development and Validation of UV-VIS Spectrophotometric Method for the Estimation of Diclofenac Sodium Injections
91. Lensa, K. Method Development and Validation for Determination of Doxycycline Hyclate in Capsule Formulation High Performance Thin Layer Chromatographic (HPTLC) Method Development and Validation for Determination Of. **2019**
92. Ermer, J.; Validation in pharmaceutical analysis. Part I: An integrated approach. Journal of pharmaceutical and biomedical analysis**2001**, 24: 755–767.
93. Elgailani, I. E. H.; Elkareem, M. A. M. G.; Noh, E. A. A.; Adam, O. E. A.; Alghamdi, A. M. A. Comparison of Two Methods for The Determination of Vitamin C (Ascorbic Acid) in Some Fruits. *Am. J. Chem.* **2017**, *2* (1), 1–7.
94. Giraldo, J. D.; Rivas, B. L. Determination of Urea Using P-N, N-Dimethylaminobenzaldehyde: Solvent Effect and Interference of Chitosan. *J. Chil. Chem. Soc.* **2017**, *62* (2), 3538–3542
95. Pourmohammad, M..; Faraji, M.; Jafarinejad, S. Extraction of chromium (VI) in water samples by dispersive liquid–liquid microextraction based on deep eutectic solvent and determination by UV–Vis spectrophotometry. *International Journal of Environmental Analytical Chemistry*.**2019**.
96. Ahmed, S.; Mustaan, N.; Sheraz, M. A.; Nabi, S. A. A. un; Ahmad, I. Validation of a UV Spectrometric Method for the Assay of Tolfenamic Acid in Organic Solvents. *J. Pharm.* **2015**, *2015*, 1–8.

## 7. Appendix

### 1.Study area


### 2.ANOVA

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| M1 | Between Groups | 9953.105 | 1 | 9953.105 | 77176.891 | .000 |
| | Within Groups | .516 | 4 | .129 | | |
| M2 | Between Groups | 41.020 | 1 | 41.020 | 351.642 | .000 |
| | Within Groups | .467 | 4 | .117 | | |
| M3 | Between Groups | 228.006 | 1 | 228.006 | 1399.605 | .000 |
| | Within Groups | .652 | 4 | .163 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| M4 | Between Groups | 927.261 | 1 | 927.261 | 9709.524 | .000 |
| | Within Groups | .382 | 4 | .096 | | |
| M5 | Between Groups | 83051.817 | 1 | 83051.817 | 2321323.621 | .000 |
| | Within Groups | .143 | 4 | .036 | | |
| M6 | Between Groups | 3012.747 | 1 | 3012.747 | 6014.725 | .000 |
| | Within Groups | 2.004 | 4 | .501 | | |
| M7 | Between Groups | .002 | 1 | .002 | .067 | .808 |
| | Within Groups | .141 | 4 | .035 | | |
| T1 | Between Groups | 2565.925 | 1 | 2565.925 | 20676.864 | .000 |
| | Within Groups | .496 | 4 | .124 | | |
| O1 | Between Groups | 5737.398 | 1 | 5737.398 | 613482.283 | .000 |
| | Within Groups | .037 | 4 | .009 | | |
| G1 | Between Groups | 7676.349 | 1 | 7676.349 | 765262.745 | .000 |
| | Within Groups | .040 | 4 | .010 | | |
| S1 | Between Groups | 8554.596 | 1 | 8554.596 | 55610.803 | .000 |
| | Within Groups | .615 | 4 | .154 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| S2 | Between Groups | 48108.911 | 1 | 48108.911 | 471112.262 | .000 |
| | Within Groups | .408 | 4 | .102 | | |
| S3 | Between Groups | 14781.511 | 1 | 14781.511 | 159303.842 | .000 |
| | Within Groups | .371 | 4 | .093 | | |

### 3. Pearson Correlation

| | | M1 | M2 | M3 | M4 | M5 | M6 | M7 | T1 | O1 | G1 | S1 | S2 | S3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M1 | Pearsc | 1 | | | | | | | | | | | | |
| M2 | Pearsc | -.994* | 1 | | | | | | | | | | | |
| M3 | Pearsc | .998** | -.996* | 1 | | | | | | | | | | |
| M4 | Pearsc | 1.000* | -.994* | .999** | 1 | | | | | | | | | |
| M5 | Pearsc | -1.000 | .994** | -.999* | -1.000 | 1 | | | | | | | | |
| M6 | Pearsc | 1.000* | -.996* | 1.000* | 1.000* | -1.000 | 1 | | | | | | | |
| M7 | Pearsc | 0.12 | -0.2 | 0.18 | 0.15 | -0.13 | 0.15 | 1 | | | | | | |
| T1 | Pearsc | 1.000* | -.993* | .999** | 1.000* | -1.000 | 1.000* | 0.13 | 1 | | | | | |
| O1 | Pearsc | -1.000 | .995** | -.999* | -1.000 | 1.000* | -1.000 | -0.13 | -1.000 | 1 | | | | |
| G1 | Pearsc | -1.000 | .994** | -.999* | -1.000 | 1.000* | -1.000 | -0.13 | -1.000 | 1.000* | 1 | | | |
| S1 | Pearsc | -1.000 | .994** | -.998* | -1.000 | 1.000* | -1.000 | -0.13 | -1.000 | 1.000* | 1.000* | 1 | | |
| S2 | Pearsc | -1.000 | .994** | -.998* | -1.000 | 1.000* | -1.000 | -0.13 | -1.000 | 1.000* | 1.000* | 1.000* | 1 | |
| S3 | Pearsc | -1.000 | .995** | -.999* | -1.000 | 1.000* | -1.000 | -0.13 | -1.000 | 1.000* | 1.000* | 1.000* | 1.000* | 1 |