KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

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DEPARTMENT OF CHEMISTRY

DETERMINATION OF BENZOIC ACID AND BENZENE IN SOFT DRINKS, FRUIT JUICES AND HERBAL PRODUCTS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY (MPHIL) DEGREE IN ANALYTICAL CHEMISTRY

BY

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DECLARATION

It is hereby declared that this thesis is the outcome of research work undertaken by James Kwame Kusi, the author. Any assistance obtained has been duly acknowledged. This work has not been previously presented for another degree elsewhere.

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DEDICATION

I dedicate this work to my wife, Josephine Osei and my son, Yaw Barimah Badu-Yeboah

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First of all, I want to give credit and thanks to the Almighty God, whose grace and power, has brought me this far. When the Lord defines work, He provides willing and capable hands to accomplish it. I therefore acknowledge with ineffable and profound gratitude the immense assistance given by Dr Osafo Acquaah, my project supervisor for his guidance, criticisms and suggestions. Special thanks also go to Mr. Elvis Baidoo and Mr. Gilbert Ofori for their support in the use of the HPLC instrument at the Chemistry Department, KNUST.

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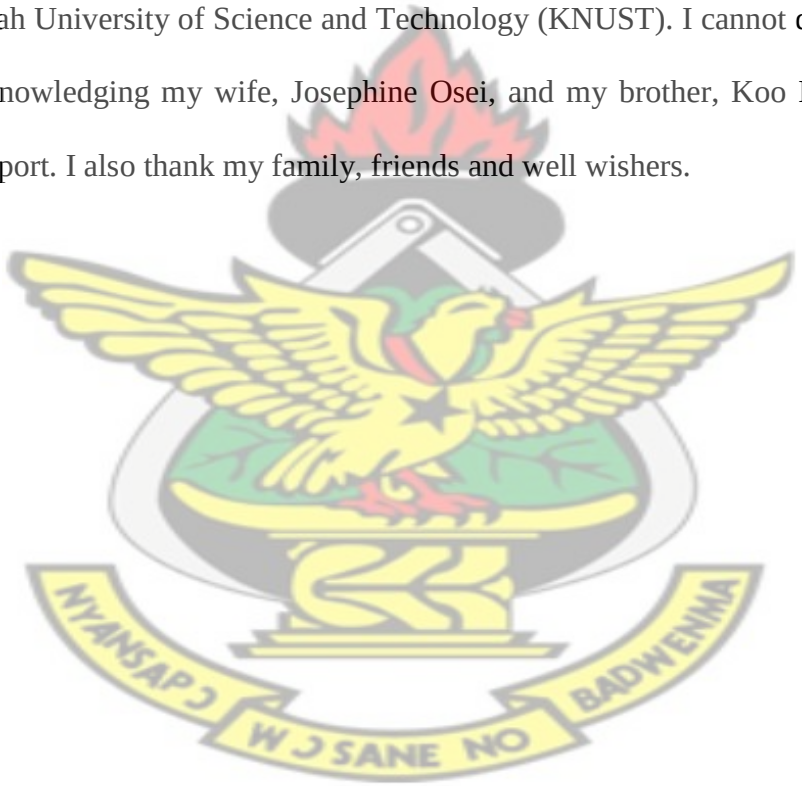

ABSTRACT

Benzoic acid is one of the most commonly used food preservatives. Benzoic acid and its salts are permitted food additives by international laws in processing in restrictive amounts, but their content must be declared and must not exceed the established limits by legislation. The level of benzoic acid in different brands of soft drinks, fruit juices herbal products available on the markets, stores and pharmaceutical shops in Kumasi, Ghana were determined by high performance liquid chromatography with a UV detector. Chromatographic separation was achieved with Phenomenex synergi 4μ polar – RP 80A 15 × 2 mm 4 micron column with ammonium acetate buffer (pH = 4.4) and acetonitrile (90:10) as the mobile phase and 0.4 mL/min as the flow rate for the benzoic acid determination. A mixture of methanol and water (70:30) was used as the mobile phase for the benzene analysis with a flow rate of 0.8 mL/min. The concentrations of benzoic acid and benzene in all the samples (soft and fruit drinks) were calculated by external standard method with a calibration of correlation coefficient of 0.9980 and 0.9933 respectively from the standards calibration curves. The range of concentrations were from below detection limit to 2004 mg/L. Thirty four (34) different brands of soft drinks, 16 different brands of fruit juices and 25 different kinds of herbal products were analysed. The herbal products had high concentrations of benzoic acid than the soft drinks and fruit juices. The ranges of concentration of benzoic acid were from below detection to 548.00 mg/L for soft drinks; below detection limit to 140.07 mg/L for fruit juices; and 0.10 to 2004 mg/L for herbal products. The estimated daily intake of benzoic acid for soft drinks and fruit juices for adults was 0.13 mg/L and 0.00072 mg/L respectively which were within the range of the acceptable daily intake (ADI) of benzoic acid (0-5 mg/L of body weight). Five of the soft drink samples contained levels of benzoic acid above the 150 mg/L which is the limit set by World Health Organization (WHO), while all the fruit juices were within the range. Benzene was not detected in any of the soft drinks and fruit juices. The

reproducibility of the method was good with a coefficient variation of 3.11%. The mean recoveries for the samples ranged from 107 to 110%. The limit of detection and quantification for benzoic acid analysis was 0.03 mg/L and 0.10 mg/L respectively and that for benzene analysis was 0.06 mg/L and 0.20 mg/L respectively.

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CHAPTER ONE

1.0 INTRODUCTION

Food additives have become increasingly important in modern food technology (Saad *et al*, 2005) as a result of the increase in the production of processed and convenience foods. Food additives have been used for centuries to enhance the quality of food products (Burdock & Carabin, 2004), with smoke, oil, vinegar, salt and spices being used to preserve food.

With the increase in technological development and better living standards, additive usage in foods significantly increased during the late 1950's (McWilliams, 2005). More than 2000 different chemicals were used in foods by the early 1960s. The demand for new, tasty convenient and nutritious foods continued to increase from then until today (Sloan, 2004). It is estimated that over 2500 different additives are currently being used in foods (Branen *et al*, 2002).

Food additives are used for various purposes, including preservation, colouring, and sweetening. The preservatives are added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes of foods and to prolong the shelf life and quality of foods. Benzoic acid is one of the oldest chemical preservatives used in food, cosmetics and drugs. Benzoic acid and its salts such as sodium, potassium and calcium benzoates are normally preferred and used as food preservatives. Other preservatives such as sorbic acid, methyl and propyl parabens are used in the food and beverage industry. They are normally represented by E- numbers; E210-benzoic acid, E211-sodium benzoate, E212-potassium benzoate and E213-calcium benzoate. Benzoic acid is used as food preservatives in beverages, fruit drinks, soft drinks, chemical leavened baked goods, condiments and canned foods. Benzoic acid inhibits the growth of mold, yeast and some bacteria. Benzoic acid is directly added or as its sodium, potassium or calcium salt. Sodium benzoate is use as preservative in the soft drink industry. Benzoic acid (sodium benzoate) is one of the

acceptable food preservatives that is allowed and used by the non-alcoholic beverage producers in Ghana by the Ghana Standard Authority. Other preservatives acceptable by Standard Authority are sulphur dioxide, orthophosphoric acid and sorbic acid and its salts. Benzoic acid is also used as preservative in pickles, sauces and fruit juices. Benzoic acid can be used in combination with salicylic acid (Whitfield's ointment) as a fungicidal treatment for ringworm. They are also used in other products, such as pharmaceuticals and herbal medicines preparation. The use of medicinal plants for treatment of different ailments is as old as human civilization. Africa, Australia and Asia are the three continents where traditional medicine became popular and ~80% of the people in the developing countries are still dependent on plant based medicines (TDR 2005; Newman *et al*, 2000). The medicines are termed as herbal medicines which are prepared either from aerial parts of herbs or the whole plant or parts of plants i.e., barks, leaves, roots, stems, flowers, fruits and seeds. Herbal products may contain a single herb or combination of several different herbs (Mosihuzzaman and Choudhary, 2008). In case of multiple plants it is believed that the efficacy is due to synergistic effects. Raw herbs are available on the market and the formulated products of the herbs are prepared following different pharmacopeia (Mosihuzzaman and Choudhary, 2008). In the recent times, African and Asian nations encourage traditional medicine as an integral component of their public health care programs as they are relatively inexpensive, locally available and are readily accepted by the local population.

Most of the herbal preparations have benzoic acid as a preservative agent. Although benzoic acid is generally recognized as safe (GRAS), short-term exposure to benzoic acid can irritate the eyes, the skin and the respiratory tract. And long-term exposure or repeated exposure may cause skin sensitization. Children are at higher risks as they have high energy intake per kg body weight and different dietary patterns and food preferences compared with adults. About 80% of benzoic acid and its salts exposure to children (youth) are from soft

drinks and prepackaged beverages. In fact, carbonated water-based flavoured drinks, soft drinks and fruit drinks are major contributors to the benzoic acid exposure in teenagers because of their high consumption levels of these products.

Adverse effects include asthma, urticaria, metabolic acidosis, convulsions and so on (Tfouni and Toledo, 2002) and (WHO, 2000). Again the development of Allergic reactions to benzoates in humans, lead to urticaria, non-immunological contact urticaria and asthma as has reported in some studies (Safford *et al*, 1990). Under certain conditions (high temperatures and the presence of Cu (II) or Fe (III) ions), benzene can form in beverages containing benzoic and ascorbic acids. The presence of benzene in food can be attributed to several potential sources. Since the early 1990s, concerns about benzene contamination of food, especially soft drinks and fruit juices have been raised. Several sources can contribute to the occurrence of benzene in foods. It may occur (at trace levels) in food naturally, as a result of process-induced changes to the food from high temperature transformations or cooking processes, from ionizing radiation, or through the migration or leaching of packaging materials. Benzene is one of the contaminants with the clearest evidence of carcinogenicity, and has been classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC, 1987). Active as well as passive smoking, automobile exhaust, and driving or riding in automobiles are postulated as the most important pathways of benzene exposure (Wallace, 1996).

The formation of benzene from benzoic acid and its salt in fruit juices and beverages is influenced by the presence of transition-metal catalysts (for example, Cu (II) or Fe (III) ions) and is dependent on pH, UV light or temperature (Gardener and Lawrence, 1993; McNeal *et al*, 1993; Barshick *et al*, 1995). In 2006, the US Food and Drug Administration (FDA) and the UK Food Standard Agency (FSA) conducted surveys on benzene in soft drinks available in their countries. Results showed that benzene was found in a small range of beverages

which contained either added or naturally occurring benzoates and ascorbic acid. About 5.0% of the samples taken by FDA and 2.6% of the samples taken by FSA were detected with a benzene level higher than 5 ppb and 10 ppb (parts per billion) respectively which is the acceptable limit of benzene in soft drinks (FDA, FSA, 2006). Benzoic acid and its salts are permitted food additives by international laws in processing in restrictive amounts, but their content must be declared and must not exceed the established limits by legislation.

The maximum accepted level of benzoates in beverages stipulated by national and European legislation is 150 ppm (Bennett *et al*, 2006; EPCD No 95/2/EC, 1995). In 1999, the Ghana Standards Authority pegged the maximum acceptable levels of benzoic acid and its salts in soft drinks at 200 ppm (GS 179, 1999). The objective of ensuring the quality of food products, the sanitary state and the commercial value of many products are established on the basis of chemical measurements. In order to maintain or improve the reliability of these determinations is a crucial aspect on which a great deal of effort is spent. The use of food additives is limited by specific regulations. Ghana, as many countries, follows the recommendations by the joint FAO/WHO Expert Committee on Food Additives on the safe use of food additive.

1.1 STATEMENT OF PROBLEM

In Ghana, there are several soft, fruit beverages and herbal prepared products on the market. These products are preserved with benzoic acid or its salts such as sodium, potassium and calcium and other food preservatives. Food preservation has always been of great importance. They are generally used to inhibit yeast and mold growth, being also effective against a wide range of bacteria.

The amount of this preservative (benzoic acid) has not been determined to check whether the right amount has been used. Since most of these manufactures do not have a well equipped

laboratories and again do not employ the right people. Again there are high levels of benzene metabolites that are frequently reported among children and non-smoking workers without occupational exposure, (Johnson *et al*, 2007) hypothesize that there may be significant sources of benzene, hitherto unidentified.

The importance of food preservatives to consumers has always been a health safety issue. Consumers and scientists have raised questions about the necessity and safety of these preservatives. Again the quality of food products, the sanitary state and the commercial value of many products are established on the basis of chemical measurements. Thus, maintaining or improving the reliability of these determinations is a crucial aspect on which a great deal of effort is spent. Not only is the importance of chemical measurements clear, but also the need to guarantee their quality to evaluate, as far as is possible, the economic and social consequences which may result from mistaken analytical measurements.

Legislation covering food additives, which is becoming stricter, obliges the analyst to develop new analytical methods and as a result to validate them, in order to identify the potential sources of error which may affect them. There is therefore the need to have a routine checkup of these preservatives especially benzoic acid and its salts in our soft and fruit drinks, herbal prepared products and the level of possible benzene that may occur as a result of the combination of benzoic acid and ascorbic acid in our processed soft drinks.

1.2 JUSTIFICATION

The use of food additives in a country is limited by specific regulations and legislation. Ghana, as many countries, follows the recommendation of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) on the safe use of food additives. According to JECFA, the safety in use of an additive can be expressed in terms of its acceptable daily

intake (ADI), which represents the amount of the substances that can be daily consumed, even for a lifetime, without health hazards.

The ADI is expressed in mg of the additive/kg body weight (WHO, 1987). The acceptable daily intakes of benzoic acid and its salts is 0 - 5 mg/kg of the body weight and this has been approved by JECFA (WHO, 1996) with the level of benzoic acid and benzoates in beverages stipulated by national and European legislation as 150 ppm.

For benzene, countries such as the US and EU have their regulatory guidelines and limits set for drinking water, bottled water, fruit juice and beverage ranging from the World Health Organization limit of 10 ppb, to the US EPA/FDA level of 5 ppb and the EU level of 1 ppb. However, in 2006 a consensus was reached among the EU member states and 10 ppb was approved as limit for benzene.

Several people have conducted a series of research in trying to determine the level of benzoic acid, benzoates and other food preservatives in their countries, (Tfouni and Toledo, 2002; Harry M. *et al***,** 2000; Bahruddin et al, 2004; Cornelia and Elena, 2009 and others). In mid-February 2006, the US Food and Drug Administration (FDA) reported the results of tests showing that some soft drinks were contaminated with the chemical benzene at levels above the World Health Organization limit for drinking water of 10 parts per billion (ppb).

Again in early 2006, Health Canada initiated a survey of benzene in soft drinks as well as in some low alcohol drinks and cocktail mixes in order to assess benzene levels in products available in Canada (Cao *et al*, 2007). Samples of 118 products were analyzed for benzene. Benzene was found in samples of four of the products at levels above the Canadian guideline of 5 µg/L benzene in drinking water. In these samples, average benzene concentrations ranged from 6.0 - 23.0 µg/L.

Ghana as a country lacks literature on the levels of benzoic acid and benzene in our foods as compared to other countries. With the increase in the availability of locally manufactured flavoured carbonated soft drinks and fruit juices on the market, there is the need to look at the levels of the preservative (benzoic acid) being used and the possibility of benzene in them since most of these products are sold in the open market. The role of preservatives has become more prominent with the increase in production of processed and convenience foods. For these reasons, a rapid and accurate testing method is desired on routine basis to check the levels of these preservatives in the drinks on the Ghanaian market.

1.3 SPECIFIC OBJECTIVES

1. To determine the concentration of benzoic acid in soft and fruit drinks on the Ghanaian market using the High-Performance Liquid Chromatography (HPLC).
2. To determine the concentration of benzoic acid in some herbal medicines on the Ghanaian market using the High Performance Liquid Chromatography
3. To determine the concentration of benzene in some soft and fruit drinks on the Ghanaian market using the High performance Liquid Chromatography

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FOOD PRESERVATION

A food preservative is a natural or synthetic chemical that is added to foods or pharmaceuticals to retard spoilage, whether from microbial growth, or undesirable chemical changes. The early methods of preservation are brewing, baking, and winemaking which created products that allowed trades and commerce. Storage of grains added tetracyclines to the diets, which probably helped resist many diseases and infections. The preserved products also introduced proteins and vitamins into the diets.

2.1.1 History of Preservation

Preserving food is a normal household activity. The need to preserve food is not new, and it has been employed since humans existed. But earlier, the need was less as people never stored food with them, as much as we do today. They used to hunt for the food in the required amount. But slowly, as humans started living in groups with increased in population they started becoming agrarian, they felt the need of storing and preserving food. Food preservation history dates back to this era of ancient civilization. But, the dramatic changes and advancement in food preservation started taking place from 18th century. The progress of science in 18th and 19th century heavily influenced the food preservation methods and processes (Stuart, 1986).

During the 18th century, drying, curing, sugaring, pickling and cold storage were some of the most common methods of preserving food, but when the population and food quantity started increasing, people needed something more than natural food preservatives. And this was the time, a not so trained scientist known as Nicolas Appert came up with a new method. He designed a new process to preserve food, which involved cooking the food and then sealing

them inside sterile bottles or cans. This process is today known as canning. This process was introduced to America by William Underwood after the civil war was over. Later, in 1858, John L. Mason designed a canning jar with a rubber gasket and metal cap, which were produced on a mass scale (Stuart, 1986).

The problem of removing air out of the container was later solved by Louise Pasteur. His studies show that particles in the air can also spoil the food. After few years of studies and experiments he designed a swan neck flask to exclude air from the preservation containers. Using his unique design, he boiled yeast soups in airless flasks and observed no contaminants. He discovered that heating foods at high temperature kills microbes and unwanted flavours. This process is today known as pasteurization (Stuart, 1986).

The process of freezing was discovered by Clarence Birdseye at his home in Canada. He stored some foods in ice and realized that they were fresh and no change in the flavour or quality occurred. Then soon he designed a method for freezing foods. He soaked metal plates in calcium chloride brine and made them cold. The food was stored between these cool plates. It was considered as one of the healthy ways to preserve food. In 1930, the first Birdseye freezer was launched in Massachusetts (Stuart, 1986).

2.1.2 Food Preservation Methods

The preservation of harvested and prepared food for future consumption is one of the oldest practical arts, a necessity that developed from the sheer need to survive in a hostile environment where fresh food was not always available. The techniques for drying foods date back to ancient times, when fruits and vegetables were dried in the sun or on an open stove. The historical methods of preservation were salting, sugaring, drying, prickling and cold storage which were used to preserve fruits, vegetables meat and fish. The emergence of food science technology has led to several modern methods of food preservation. These include

CANNING: It is the process of preserving food by heating and sealing it in containers for storage. The microorganisms & autolytic enzymes are destroyed. It helps in preserving food for times of need. Canning prevent the risk of botulism poisoning, botulism bacteria is most resilient to heat due to endosperm coating. However, water-soluble nutrients can be lost into liquid in can. In Ghana, canning is employed in many industries such as meat and milk.

FREEZING: Prevents microbial growth by low temperature & unavailability of water. It involves creating the environment where bacteria cannot grow. Although bacteria on food are still there, they are just dormant and upon thawing, bacteria will resume replicating. Freeze foods last months to years. Freezing is commonly use in the preservation of fish and meat (Stuart, 1986).

HIGH HEATING PROCESSING (PASTEURISITION): This inactivates autolytic enzymes and also leads to the destruction of microorganisms. However, it also leads to loss of heat-sensitive nutrients (Stuart, 1988). In Ghana, this method is employed by the fruit juice industry. Most of the fruit industries use pasteurization as preservative method.

DEHYDRATION: Dehydration modern food processes take their cue from ancient actually drying process to preserve food. The basic assumption is that water content in food is reduced to a certain extent to prevent them from cultivating microbial growth. Water actually helps increase the rate of deterioration in food. Dehydration leads to longer storage, small size and weight.

IONIZING IRRADIATION: The food is exposed to a controlled amount of radiation to destroy organisms responsible for spoilage. Irradiation sterilizes foods (such as spices) whose flavour would change with heating. It helps food maintain chemical make-up after exposure which ensures that microorganisms are not reproduced or make toxins. Increased radiation also increases preservation effect (Farkas, 2011).

CHEMICAL PRESERVATIVES: Growth of microorganisms in a food material can also be inhibited by adding certain chemical substances. It prevents microbial growth with no loss of nutrient. Common food preservatives act as either antioxidants or antimicrobials. Antioxidants prevent the oxidation of constituents in food, keeping it from becoming rancid or from developing black spots when exposed to oxygen (like an opened cut apple). Antimicrobial preservatives kill or prevent the growth of bacteria and fungi. However, the chemical substance should not be harmful to the human beings. There are lot of chemical food preservatives used in the food industry. A survey conducted in some of the locally manufacturers of soft drinks in Kumasi showed that most of them use benzoic acid as a preservative. They claim it is the most readily available preservative on the market and also not expensive. In Ghana, the Ghana Standards Authority allows preservatives such as benzoic acid and its salts, sulphur dioxide, orthophosphoric acid and sorbic acid to be used in the non-alcoholic beverage industry.

2.2 HARMFUL EFFECTS OF FOOD PRESERVATIVES

Although preservatives have been used for centuries, there has always been some concern regarding the safety of consuming food additives used in foods (Branen *et al*, 2002). The first real concern regarding food additives was expressed by Wiley (Bennion, 1980). His work led to the first regulations to control the use of food additives (Branen *et al*, 2002).

They are harmful to the body and can cause a number of health problems, especially if consumed in large amounts. Effects of food additives may be immediate or may be harmful in the long run if there have been a constant exposure. Immediate effects may include headaches, change in energy level, and alterations in mental concentration, behaviour, or immune response. Long-term effects may increase risk of cancer, cardiovascular disease and other degenerative conditions. Some common food preservatives used in the United States

have been banned in some other countries because of the harm they can cause. Some common food preservatives, their harmful effects, and foods commonly used include:

Benzoic acid (Benzoates): Many soda manufacturers use sodium benzoate to prevent mold. This chemical can damage mitochondria in cells, which leads to neuro-degenerative diseases. Professor Andrew Kemp from the University of Sydney mentions in an editorial for the May 24, 2008, edition of the "British Medical Journal" that children "were significantly more hyperactive after they ate a mixture of food colourings and a preservative [sodium benzoate]."

Nitrates and Nitrites: Sodium nitrate, which is found in many packaged meats like hot dogs, sausages and bacon, is used as a curing agent. The curing of meats reduces the possibility for botulism-causing bacteria. Studies have link pancreatic and lung cancers to an increased consumption of meats containing sodium nitrate

BHA and BHT: (butylated hydroxyanisole and butylated hydroxytoluene) are the two most widely used synthetic antioxidants in foods. These preservatives have caused cancers in rats; however, some studies show they may actually protect against the development of cancers. BHA can be found in meats, baked goods, cereals, snack foods, and beer. BHT is often found in pre-packaged food.

Glutamates: Almost all convenience foods contain this harmful preservative. Glutamates may cause headaches, palpitations, dizziness, and cancer. Almost all convenience foods contain this harmful preservative.

Mono- and Di-glycerides: These preservatives are found in many foods, such as cookies, cakes, pies, bread, peanut butter, dry roasted nuts, vegetables packaged with sauce, shortening, and margarine. Mono- and di-glycerides can cause birth defects and cancer.

*Propyl gallate***:** This preservative is mostly found in meat products, vegetables packaged with sauce, pickles, vegetable shortening and oils, and even in chewing gum. Propyl gallate can cause birth defects and damage the liver.

*Sulfites***:** are also common food preservatives and may cause headaches, joint pain, heart palpitations, allergies, and cancer. Foods include fruit, dried fruit, jarred olives and peppers, corn syrup, cornstarch, wine vinegar, and wine.

Potassium Bromate: Many bread companies still use potassium bromate to strengthen bread dough. Although countries like Britain and Canada banned the preservative as a carcinogen because significant evidence shows that it causes cancerous kidney and thyroid tumors in rats, it is still found in some baked goods in the United States. Potassium bromate is especially dangerous if the food product is not baked long enough at a high enough temperature.

*Mono sodium Glutamate (MSG)**:*** MSG is an amino acid used as a flavor enhancer in soups, salad dressings, chips, frozen food, and restaurant food. MSG is known as an excitotoxin, a substance which overexcites cells to the point of damage or death. It can cause headaches and nausea, and animal studies link it to damaged nerve cells in the brains of infant mice

*Parabens***:** They are a class of chemicals widely used as preservatives by cosmetic, pharmaceutical and food industries. Parabens are effective preservatives in many types of formulas. These compounds, and their salts (methylparaben, propylparaben and ethylparaben), are used primarily for their bactericidal and fungicidal properties. Parabens have been found in breast cancer tumors (an average of 20 nanograms/g of tissue). Parabens have also displayed the ability to slightly mimic estrogen (a hormone known to play a role in the development of breast cancer (Harvey and Everett, 2004). A visit to some of the local manufacturers of soft drinks showed that parabens are not common on the Ghanaian market.

2.3 EFFECTS OF FOOD PRESERVATIVES ON CHILDREN

Food additives have long been suspected to be associated with increased hyperactivity in children. The use of preservatives continued to increase and spread to too many foods. In the 1970s a US allergist Dr Ben Feingold suggested that certain additives could contribute to hyperactivity in children (Feingold, 1975). After 30 years of controversy, Dr Feingold's research was vindicated. In the study, undertaken by researchers at the University of Southampton in the UK and published in the Lancet on 8 September, the behaviour of two sets of children (three year olds, and eight to nine year olds), including those who had been diagnosed with Attention Deficit Hyperactivity Disorder (ADHD), was monitored before and after consuming one of two drinks containing different mixtures of commonly used artificial colours and the preservative sodium benzoate.

2.4 BENZOIC ACID

Benzoic acid (CAS No. 65-85-0; $C_7H_6O_2$; C_6H_5COOH), can also be called benzene carboxylic acid or phenyl carboxylic acid and usually represented as E210 on the labels of foodstuffs (.Wibbertmann et al, 2005). Benzoic acid is a colourless white crystalline solid (Fig 2.1). It melts at 122 °C, boils at 249 °C and starts to sublime at 100 °C, It has a solubility in water (2.9 g/litre at 20 °C), and its solution in water is weakly acidic (dissociation constant at 25 °C = 6.335×10^{-5}; pKa 4.19). It is soluble in ethanol and slightly soluble in benzene and acetone. In the 19th century, benzoic acid was synthesized from coal tar (the acid found naturally in Siam benzoic resin, was produced synthetically for the first time in the 1860 using coal tar). Today it is manufactured from toluene, a petroleum byproduct. Benzoic acid is produced exclusively by the liquid phase oxidation of toluene (Fig 2.2) (Srour, 1998). According to Srour (1998), the estimated global production capacity of benzoic acid is 638,000 tonnes per year, although over half of this is converted directly to phenol. The new estimated global production capacity for benzoic acid is about 6,000,000

tonnes per year from (Indrajit *et al*, 2011). The major producers of benzoic acid are the Netherlands (220 000 tonnes per year) and Japan (140 000 tonnes per year), followed by the USA (125 000 tonnes per year). Another reference gives the total European capacity as less than 153 000 tonnes (SRI, 1998). Benzoic acid occurs naturally in many plants and animals. It is therefore a natural constituent of many foods, including milk products.

Figure 2.1. Photograph of benzoic acid

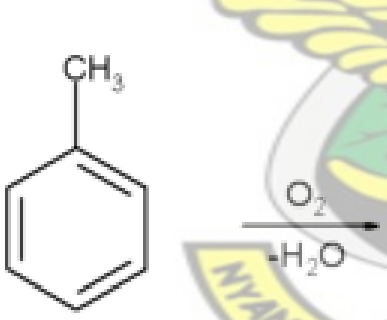

Figure 2.2 The oxidation of Toluene to produce Benzoic acid

2.5 REACTIONS AND SYNTHESIS OF BENZOIC ACID

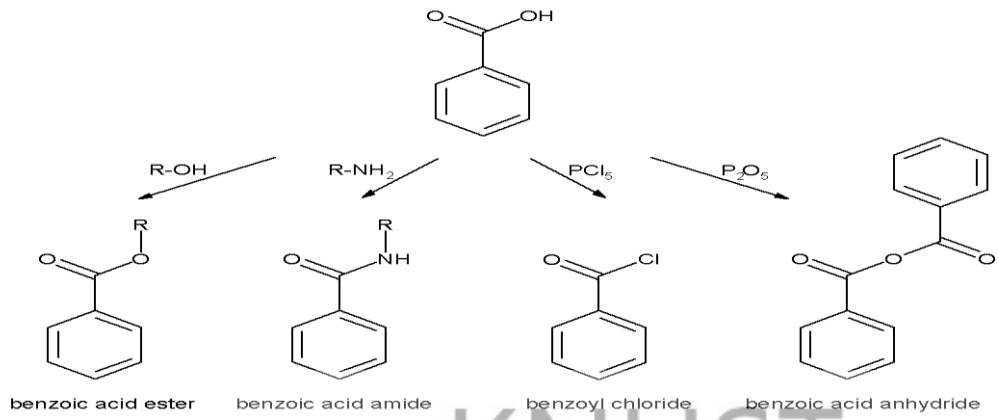

Figure 2.3 Reactions of benzoic acid

The chemical properties of benzoic acid are based on its molecular structure. In particular, the reactions of benzoic acid can involve modifications of the carboxyl group or the aromatic ring. Figure 2.3 shows how benzoic acid reacts with alcohols to produce esters. For example, with ethyl alcohol (C_2H_5OH), benzoic acid forms ethyl benzoate, an ester ($C_6H_5CO\text{-}O\text{-}C_2H_5$). Some esters of benzoic acid are plasticizers.

Benzoic acid react with phosphorus pentachloride (PCl_5) or thionyl chloride ($SOCl_2$) to form benzoyl chloride (C_6H_5COCl), which is classified as an acid (or acyl) halide. Benzoyl chloride is highly reactive and is used to form other products. For example, it reacts with ammonia (NH_3) or an amine (such as methylamine, $CH_3\text{-}NH_2$) to form an amide (benzamide, $C_6H_5CONH_2$).

Other reactions of benzoic acid include: Salt formation where the carboxyl group of benzoic acid reacts with a base to form a salt. For example, it reacts with sodium hydroxide (NaOH) to produce sodium benzoate, an ionic compound ($C_6H_5COO\text{-}$ Na+). Both benzoic acid and sodium benzoate are used as food preservatives; Sulfonation, which involves benzoic acid with fuming sulfuric acid (H_2SO_4) that leads to sulfonation of the aromatic ring, in which the

functional group SO_3H replaces a hydrogen atom on the aromatic ring. The product is mostly meta-sulfobenzoic acid (SO_3H-C_6H_4-COOH). The prefix "meta" indicates that the functional group is attached to the third carbon atom relative to the point of attachment of the carboxyl group; Benzoic acid reacts with concentrated nitric acid (HNO_3), in the presence of sulfuric acid as catalyst, leading to nitration of the ring. The initial product is mostly meta-nitrobenzoic acid (NO_2-C_6H_4-COOH), in which the functional group NO_2 is attached to the ring at the meta position relative to the carboxyl group. In the presence of a catalyst such as ferric chloride ($FeCl_3$), benzoic acid reacts with a halogen such as chlorine (Cl_2) to form a halogenated molecule such as meta-chlorobenzoic acid (Cl-C_6H_4-COOH). In this case, a chlorine atom is attached to the ring at the meta position relative to the carboxyl group.

Benzoic acid can also be synthesized through: Bromobenzene can be converted to benzoic acid by "carbonation" of the intermediate phenylmagnesium bromide.

$$C_6H_5MgBr + CO_2 \rightarrow C_6H_5CO_2MgBr$$

$$C_6H_5CO_2MgBr + HCl \rightarrow C_6H_5CO_2H + MgBrCl$$

Benzoic acid (C_6H_5COOH) can be synthesized by oxidation of benzyl chloride in the presence of alkaline $KMnO_4$.

$$C_6H_5CH_2Cl + 2\ KOH + 2\ [O] \rightarrow C_6H_5COOK + KCl + H_2O$$

The base-induced disproportionation of benzaldehyde, the Cannizzaro reaction, affords equal amounts of benzoate and benzyl alcohol; the latter can be removed by distillation.

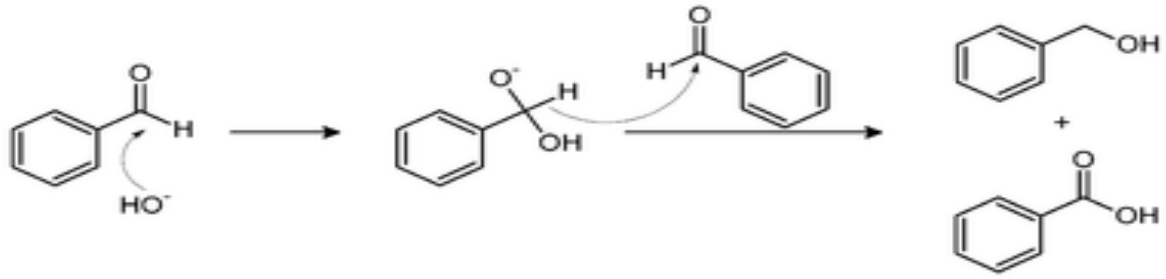

Figure 2.4 The Cannizzaro reaction showing the production of benzoate and benzyl alcohol

Benzyl alcohol is refluxed with potassium permanganate or other oxidizing reagents in water. The mixture is hot filtered to remove manganese dioxide and then allowed to cool to afford benzoic acid.

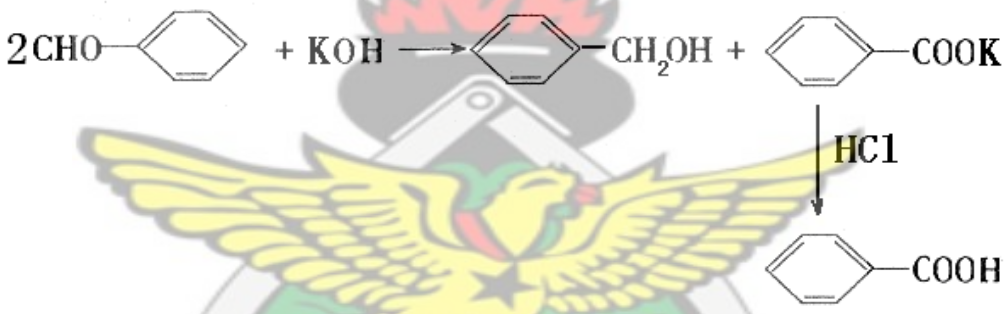

Figure 2.5 The formation of benzoic acid from benzyl alcohol.

2.6 USES OF BENZOIC ACID

2.6.1 FEEDSTOCK

In 1988, of the benzoic acid produced in Europe, about 60% was further processed to phenol and 30% to caprolactam (for nylon fibres). Five per cent was used for the production of sodium and other benzoates, 3% for benzoyl chloride, and the rest for alkyd resins (a polyester modified by the addition of fatty acids and other components), benzoate esters, such as methyl benzoate, and various other products (Srour, 1989). These percentages are still approximately correct today (Srour, 1998). Caprolactam seems to be produced only by

European companies (Srour, 1998). Benzoic acid is increasingly used in the production of diethylene and dipropylene glycol dibenzoate plasticizers in adhesive formulations (about 40 000 tonnes in 1997). It is also used to improve the properties of alkyd resins for paints and coatings and as a “down hole” drilling mud additive in secondary oil production. Its use as a rubber polymerization retarder is diminishing (Srour, 1998).

2.6.2 MEDICINE

Benzoic acid is a constituent of Whitfield's ointment which is used for the treatment of fungal skin diseases such as tinea, ringworm, and athlete's foot. As the principal component of benzoin resin, benzoic acid is also a major ingredient in both tincture of benzoin and Friar's balsam. Such products have a long history of use as topical antiseptics and inhalant decongestant. Sodium benzoate is used in the treatment of patients with urea cycle enzymopathies (i.e., hyperammonaemia due to inborn errors of urea synthesis) in order to facilitate an alternative pathway of nitrogen excretion. The therapeutic dose given over several years is in the range of 250–500 mg/kg body weight per day (Feillet & Leonard, 1998)

2.6.3 FOOD PERSERVATIVE

Benzoic acid and its salts are used as a food preservative, represented by the E-numbers; E210, E211, E212, and E213 representing benzoic acid, sodium benzoate, potassium benzoate and calcium benzoate respectfully. Benzoic acid inhibits the growth of mould, yeast and some bacteria. Benzoic acid is either added directly or created from its reaction with sodium, potassium or calcium salt. Benzoic acid is used as food preservatives and is most suitable for foods such as fruit juices and soft drinks that are naturally in an acidic pH range (Indrajit, *et al*, 2011). The mechanism starts with the absorption of benzoic acid into the cell. If the intracellular pH changes to 5 or lower, the anaerobic fermentation of glucose through phosphofructokinase is decreased by 95%. The efficacy of benzoic acid and benzoate is thus

dependent on the pH of the food. Although undissociated benzoic acid is the more effective antimicrobial agent for preservation purposes, sodium benzoate is used preferably, as it is about 200 times more soluble than benzoic acid. About 0.1% is usually sufficient to preserve a product that has been properly prepared and adjusted to pH 4.5 or below (Chipley, 1983). A major market for sodium benzoate and benzoic acid as a preservative is in the soft and fruit drink industry, as a result of the demand for high-fructose corn syrup in carbonated beverages. Benzoic acid and sodium benzoate are used as antimicrobial agents in edible coatings (Baldwin *et al*, 1995).

Benzoic acid is widely used in preserving nonalcoholic beverages, since nonalcoholic beverages are high in water activity and some are rich in vitamins and minerals, they are an attractive environment for microbes. However, the usually low pH of beverages, due to carbonation, the sugar content in some of them and the addition of preservatives help inhibit the growth of microbes. The type of chemical preservative that can be used in beverages depends on the chemical and physical properties of both the antimicrobial preservative and the beverage. The pH of the product, the presence of vitamins, the packaging and the storage conditions will determine whether preservatives are necessary and what type should be used to prevent microbial growth. The main preservatives allowed and used in nonalcoholic beverages are sorbic and benzoic acids and their salts. (Directive 95/2/EC(1995) Benzoic acid occurs naturally, notably in cranberries, cinnamon, plums and currants, and it has long been used to inhibit microbial growth in many products, including nonalcoholic beverages. Benzoate salts are more stable than the acid form and more soluble in water, making benzoates a favorable choice for the beverage industry. The salts are particularly well suited for use in carbonated, nonalcoholic and juice beverages because they work best between pH levels of 2 and 4 (Gordana *et al*, 2012).

2.7 HUMAN EXPOSURE

The main route of exposure of the general population to benzoic acid or sodium benzoate is likely through foodstuffs that contain the substances naturally or added as antimicrobial agents. Benzoic acid is produced by many plants as an intermediate in the formation of other compounds (Goodwin, 1976). Benzoic acid therefore occurs naturally in many foods, including milk products (Sieber *et al,* 1989, 1990). According to JECFA, (Joint FAO/WHO Expert Committee on Food Additives, 2005) the safety in use of an additive can be expressed in terms of its acceptable daily intake (ADI), which represents the amount of the substances that can be daily consumed, even for a lifetime, without health hazards. The ADI is expressed in mg of the additive/kg body weight (WHO, 1987). 0 – 5 mg/kg of body weight have been established by JECFA as the ADI for benzoic acid and benzoates salts (WHO, 1996). Because diets differ among countries, the foods that contribute to benzoate intake would be expected to vary. The food category that contributed most to benzoate intake was soft drinks (carbonated, water-based, flavoured drinks) for Australia/New Zealand, France, the United Kingdom, and the USA. To assure the security of consumers the intake of certain food additives, among which benzoic acid is included, has been evaluated by JECFA. Nine member states (Australia, China, Finland, France, Japan, New Zealand, Spain, the UK and the USA) provided JECFA information on benzoates intake for assessment during its 51st meeting. This Committee noted that mean intake estimates based on maximum limits specified in national standards were below the ADI for benzoates. However, when the intake estimates were based on the maximum limits and the range of uses specified in the draft General Standard for Food Additives (GSFA), the ADI was exceeded. The Committee observed that benzoates are not likely to be used in all foods for which their use is permitted, concluding that information on these aspects could be used to revise both the intake assessment and the maximum limits specified in the GSFA. The Committee also concluded

that further information on levels of benzoates in food at the time of consumption was needed (WHO, 2000).

2.8 KINETIC AND METABOLISM IN LABORATORY ANIMALS AND HUMANS

After oral ingestion of benzoic acid and sodium benzoate, there is a rapid absorption (of undissociated benzoic acid) from the gastrointestinal tract in experimental animals or humans (US FDA, 1972a, 1973). After oral and dermal uptake, benzoate is metabolized in the liver by conjugation with glycine, resulting in the formation of hippuric acid (US FDA, 1972a; WHO, 1996; Feillet & Leonard, 1998).

The rate of biotransformation in humans is high: after oral doses of 40, 80 or 160 mg sodium benzoate/kg body weight, the transformation to hippuric acid was independent of the dose. Hippuric acid is rapidly excreted in urine. In humans, after oral doses of up to 160 mg/kg body weight, 75–100% of the applied dose is excreted as hippuric acid within 6 h after administration, and the rest within 2–3 days (Fujii *et al*, 1991; Kubota & shizaki, 1991). The limiting factor in the biosynthesis of hippuric acid is the availability of glycine. The utilization of glycine in the detoxification of benzoate results in a reduction in the glycine level of the body. Therefore, the ingestion of benzoic acid or its salts affects any function or metabolic process in which glycine is involved; for example, it leads to a reduction in creatinine, glutamine, urea, and uric acid levels (US FDA, 1972a, 1973; WHO, 1996).

2.9 HAZARD CHARACTERISATION

JECFA has evaluated benzoic acid and its salts several times. Generally speaking, benzoic acid is low in acute and chronic toxicity.

ACUTE EFFECTS

Based on the available animal data, benzoic acid is low in acute oral toxicity. The acute oral LD_{50} (the lethal dose administered that kills half of the experimental animals) of benzoic acid and sodium benzoate in rodents is more than 1940 mg/kg bw (body weight).

CHRONIC EFFECTS

In a four-generation study, no effects on life span, growth rate, fertility, lactation or organ weight were observed in rats fed a diet containing up to 1% benzoic acid (500 mg/kg body weight/day).

GENOTOXICITY AND CARCINOGENICITY

The International Agency for Research on Cancer (IARC) has not evaluated the carcinogenicity of benzoates. However, there is no evidence of carcinogenicity for benzoic acid and its salts. Nevertheless, genotoxic tests on sodium benzoate with mammalian cells gave consistently positive results. Positive result was also obtained in an *in vivo* study (dominant lethal assay in rats). Based on the available evidence, the genotoxic activity of sodium benzoate could not be ruled out entirely.

2.10 REPORTED EFFECTS ON HUMANS

In a 5-day human volunteer study ingesting up to 2500 mg/day benzoic acid and sodium benzoate, signs of discomfort and malaise including nausea, headache, weakness, burning and irritation of oesophagus were reported. However, symptoms including urticaria, asthma, rhinitis, or anaphylactic shock which appeared shortly after low doses exposure to benzoic acid and sodium benzoate were also reported as both substances are known to cause non-immunological contact reactions (pseudoallergy) in sensitive persons (Lahti *et al*, 1995; Anderson, 1996; Bindslev-Jensen, 1998; Coverly *et al*, 1998). However, adverse health impact regarding the intake of benzoic acid has aroused public concern recently. Media

reported that scientists had produced further evidence suggesting the ability of sodium benzoate to cause DNA damages and neuro-degenerative diseases such as Parkinson's disease (Piper, 2007).

2.11 FORMATION OF BENZENE FROM BENZOIC ACID AND ASCORBIC ACID

Benzene forms at very low level (ppb level) in some beverages containing both benzoates and ascorbic acid. Exposure to heat and light further stimulate the reaction (FDA, 2006). In 2006, the US Food and Drug Administration (FDA) and the UK Food Standard Agency (FSA) conducted surveys on benzene in soft drinks available in their countries. Results showed that benzene was found in a small range of beverages which contained either added or naturally occurring benzoates and ascorbic acid. About 5.0% of the samples taken by FDA and 2.6% of the samples taken by FSA were detected with a benzene level higher than 5 ppb and 10 ppb (parts per billion) respectively. Affected products had subsequently either been reformulated by the manufacturers or removed from sale. In follow-up samplings, all reformulated products were found to have a benzene level less than 1 ppb. Both surveys concluded that the benzene levels found in soft drinks and other beverages to date do not pose a safety concern for consumers (FDA, 2006; FSA, 2006).

2.12 MECHANISM OF BENZENE FORMATION

Ascorbic acid (vitamin C) is a natural component of many foods and its often added to foods and beverages as a vitamin supplement or fortifier and promoted as an antioxidant. Transition metals, example, Cu(II) and Fe(III) can catalysed the one-electron reduction of O_2 by ascorbic acid to produce the superoxide anion radical, which undergo spontaneous disproportionation to produce hydrogen peroxide. Subsequent metal-catalysed reduction of H_2O_2 by ascorbic acid can generate the hydroxyl radical (Lalita and Glen, 1993). The equation below shows how the process occur

$$Cu^{2+} + H_2Asc \rightarrow Cu^{+} + HAsc^*$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-}$$

$$2O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^*$$

The hydroxyl radical generated then attacks benzoic acids ions and decarboxylate (removal of CO_2) to produce the benzene. Industry testing soft drinks 15 years ago is thought to get point that exposing to temperatures of 30°C and UV light for several hours were enough to increase a rate of more than triple benzene residues in some drinks. Testing soft drinks to reflect the effects of storage and transport conditions will be crucial to realistically monitor benzene formation in different drinks, (Food Standards Agency, October 2003). Leaving soft drinks in warm conditions, such as a car boot, garage or the open place under the sun, can significantly increase the chance of benzene forming in the drink (Gardner and Lawrence 1993; McNeal *et al*, 1993; Chang and Ku 1993; and Barshick *et al*, 1995).

2.13 BENZENE

Benzene is an organic chemical compound with the molecular formula C_6H_6. Benzene is a colourless and highly flammable liquid with a sweet smell. Benzene is used mainly as an intermediate to make other chemicals. About 80% of benzene is consumed in the production of three chemicals, ethylbenzene, cumene, and cyclohexane. Its most widely produced derivative is ethylbenzene, precursor to styrene, which is used to make polymers and plastics. Cumene is converted to phenol for resins and adhesives. Cyclohexane is used in the manufacture of nylon. Smaller amounts of benzene are used to make some types of rubbers, lubricants, dyes, detergents, drugs, explosives, and pesticides. Exposure to benzene increases the risk of cancer and other illnesses. Benzene is a notorious cause of bone marrow failure. Substantial quantities of epidemiologic, clinical, and laboratory data link benzene to aplastic anemia, acute leukemia, and bone marrow abnormalities. The specific hematologic

malignancies that benzene is associated with include: acute myeloid leukemia (AML), aplastic anemia, myleodysplastic syndrome (MDS), acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia. Human exposure to benzene is a global health problem. Benzene targets liver, kidney, lung, heart and the brain and can cause DNA strand breaks and chromosomal damage. There are several exposures of benzene, soft drinks exposure is one of them which is as a result of the combination of benzoic acid/sodium benzoate and ascorbic acid as preservatives under certain conditions.

2.14 PREVENTION OF BENZENE IN SOFT AND FRUIT DRINKS

The International Council of Beverages Associations (ICBA) has developed and approved a guidance document to mitigate the potential for benzene formation in beverages which has been adopted by the many national beverages councils and associations, and been made available to beverage manufacturers around the world. The ICBA guidance document (ICBA, 2006) summarizes factors which may mitigate the formation of benzene in beverages containing benzoic acid sources and ascorbic acid based on experience in the beverage industry and experiments that have been carried out on the formation of benzene. In particular, evidence indicates that nutritive sweeteners (sugar, high fructose corn or starch syrup), where permitted, may delay or inhibit the reaction by reacting with and inactivating hydroxyl radicals, as the formation of benzene seems most noticeable in diet beverages containing intense sweeteners (Cao *et al*, 2007 and Nyman *et al*, 2008). However, the longer a product is in the market (shelf-life), the greater the potential for benzene formation if its precursors are present. Evidence also suggests that chelating agents, such as calcium disodium ethylenediaminetetraacetic acid (EDTA) or diethylenetriamine pentaacetic acid (DTPA), where permitted, may mitigate the formation of benzene in products containing benzoates and ascorbic acid, possibly by complexing metal ions that may act as catalysts (Gardner and Lawrence, 1993). However, the effectiveness of EDTA as a chelating agent is

not always obvious and the degree of mitigation may be lessened in products containing calcium or other minerals, such as mineral fortified products (Nyman *et al*, 2008). It is also suggested that sodium poly (or hexameta) phosphate, may mitigate benzene formation.

The ICBA Guidance Document also provides advice and strategies to beverage manufacturers on ways to minimize the potential for benzene formation in beverages through formulation control. These strategies include: reviewing existing products and new formulations (including their ingredients, such as fruit juices, flavor emulsions, colours, clouding agents that may contain preservatives or antioxidants, either naturally or added intentionally for a technological effect, and storage and shelf-life considerations) for their potential to form benzene in consideration of the current knowledge on factors contributing to benzene formation; looking at alternative ingredients (the replacement/reduction of benzoates with sorbates or other preservative systems, and/or ascorbic acid with attendant challenges addressed); or reviewing manufacturing processes that are available to prevent the formation of benzene. Advice is also provided on: storage tests of products to determine the potential for benzene formation; reformulating affected products in which benzene may be present; confirming that new formulations and reformulations are effective in minimizing the potential for benzene formation through market sampling and analytical procedures for the determination of benzene. It is clear that several factors may be interacting to increase or decrease the formation of benzene in beverages including: the order of added ingredients, the specific formulation and precursors that may be present within the beverage, as well as storage conditions experienced throughout the life of the product. Reformulated products have been shown to contain reduced benzene levels, where levels are either not detected or detected at very low levels, generally below 1 ug/L (Cao *et al*, 2007).

2.15 SAFETY REFERENCE VALUE

JECFA re-evaluated and maintained an ADI of 0 - 5 mg/kg body weight for benzoic acid and its salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate, expressed as benzoic acid equivalents, in 1996 (WHO, 1996) and the maximum accepted level of benzoic acid and benzoates in beverages stipulated by national and European legislation is of 150 ppm. The maximum accepted level of benzene in soft drinks and drinking water is also 10 ppb. In Ghana, the food additive and contaminants regulations are based on Codex Alimentarius standards (vol. 1, 1991 pages 49-179) in its assessment of food safety.

2.16 BENZENE OCCURRENCE AND SURVEYS

Heikes and colleagues found benzene in 28 of 234 table-ready foods from the US FDA total diet study (TDS), ranging from 9.49 - 283 ppb, with the highest level being reported in sauerkraut (a kind of sausage). A survey on the occurrence of benzene in 60 beverages available on the Italian market showed the presence of benzene at levels ranging from 1-3.8 ppb (Fabiette *et al*, 2001).

In March 2006 the Food Safety Authority of Ireland (FSAI) conducted a survey in conjunction with the Galway Public Analysts Laboratory in order to establish the levels of benzene present in 76 samples of soft drinks, squashes and flavoured waters available on the Irish market (FSAI, 2006). Only 7 beverages contained benzene above the detection limit and of those, 2 were above the WHO guideline for benzene in drinking water of 10 ug/L. In a follow-up survey, 63 samples of the same beverage types were analyzed for benzene. Of those, 9 were above the limit of detection and 2 were above the WHO guideline for benzene in drinking water (FSAI, 2008).

In 2006, the United Kingdom Food Standards Agency (FSA) collected 150 drinks from supermarkets and independent shops from four regions in the UK (UK FSA, 2006). The samples consisted mainly of concentrates (squashes), carbonated drinks, and ready-to-drink still drinks (noncarbonated drinks with less than 25% juice content). The majority of drinks contained benzoates and ascorbic acid. Some of the carbonated drinks contained benzene levels ranging from 1 ppb to 5 ppb.

Reda *et al*, 2012 conducted a survey on the probability of benzene forming in Egyptian non-alcohol carbonated soft drinks. 75 samples (five brands) of carbonated soft drinks were sampled on the Egyptian market. The range of detection was between 0.52 ng/ mL and 20.0 ng/ mL. Robert Frey *et al*, 2007 conducted a research to determine the level of benzene in carbonated beverages by purge and trap thermal desorption GC/MS. Three brands of orange flavoured beverages were purchased and analysed. All three beverages showed the presence of benzene by this technique. Benzene levels were found to be between 0.14 pg/µl and 10.1 pg/µl.

2.17 BENZOIC ACID OCCURRANCES AND SURVEYS

Cornelia *et al*, 2009 conducted a survey on the level of benzoic acid in soft drinks in Romania. A total of forty liquid soft drinks samples were obtained from the Romanian market. The levels of benzoic acid obtained ranged from 0 to 110 mg/L.

Simultaneous determination of preservatives (benzoic acid, sorbic acid, methylparaben and propylparaben) in foodstuffs by Bahruddin *et al*, 2004 showed the range of benzoic acid levels from not detected to 1260 mg/L. 67 foodstuffs (mainly imported), comprising soft drinks, jams, sauces, canned fruits/vegetables, dried vegetables/fruits and others were analysed in Malaysia.

In Portugal 2003, a total of Eighty-seven commercial brand foods were analysed including table olives, jams , jellies , spreadable fats, sauces, fruit juices and table wines for levels of benzoic and sorbic acids by Fernando et al, 2003. The range of benzoic acid in the fruit juices was from not detected to 153 mg/L.

Khosrokhavar *et al*, 2010 conducted a research on the simultaneous determination of preservatives (sodium benzoate and potassium sorbate) in soft drinks and herbal extracts in Iran. A total of 50 soft drinks and 55 herbal extracts were obtained from the supermarkets and two herbal companies respectively. The range of sodium benzoate as preservative was from not detected to 2477 mg/L. The study also showed that the concentration of sodium benzoate in soft drink samples were higher even for ADI of normal consumers

Tfouni and Toledo, 2002 in Brazil conducted a survey on the determination of benzoic and sorbic acids. 56 samples analysed included soft drinks, fruit juices, margarine, cheese and yougurt. Levels of benzoic acid ranged from not detected to 804 mg/L in the samples. The survey concluded that the utilization of benzoates is significantly lower than the maximum authorized levels.

In 2007, the center of food safety and food and environmental hygiene department, Hong Kong conducted a research on the dietary exposure of benzoic acid from prepackaged non alcoholic beverages which included soft drinks and fruit juices. A total of 211 samples were analysed and the results showed that benzoic acid was detected in 36 (17.1%) beverage samples, including soft drink and fruit juice, with concentrations ranging from 51 to 580 mg/kg.

In August 2006, two soft drink manufacturers agreed to settle a class-action lawsuit that had been filed by a group of parents in the District of Columbia Superior Court. The two companies, Zone Brands Inc., maker of "BellyWashers" products, and Talking Rain Beverage

Co, denied that their products were harmful, but agreed to change the ingredients in their drinks (Libby Q, 2006). Coca-Cola announced that it would be phasing out sodium benzoate from many of its drinks, but not Fanta and Dr Pepper (Martin H., 2008). As of January 2010, Coca Cola Zero still contains benzoate (added as potassium salt).

2.18 ANALYTICAL METHODS

Analytical methods for the determination of benzoic acid include spectrophotometric methods, which need extensive extraction procedures and are not very specific; gas chromatographic (GC) methods, which are more sensitive and specific but need lengthy sample preparation and derivatization prior to determination; and high-performance liquid chromatography (HPLC), which has a high specificity and minimum sample preparation and does not require derivatization (Wibbertmann, 2005). HPLC is also the most common analytical procedure for the detection and quantification of these preservatives in foods and beverages (Castellari, 1997; Chen, 1995; Mannino 1996; Montano *et al*, 1995). When benzoic acid is used as a preservative in soft drinks and fruit drinks, other additives, colouring agents and other acids (e.g, sorbate) may interfere with its analysis. Liquid chromatographic methods were developed to overcome this (e.g., Bennett & Petrus, 1977; Puttemans *et al*., 1984; Tyler, 1984). Methods for determination of benzene in food usually apply GC/MS and various sample clean-up techniques, such as liquid–liquid extraction (Carrillo-Carrion *et al*. 2007), static or purge and trap headspace sampling (Nicholas, 2002; Page *et al*, 1992; Gardener and Lawrence, 1993; McNeal *et al*, 1993; Barshick *et al*, 1995; Fabietti *et al*, 2001; Cao *et al*, 2007) or headspace solid-phase dynamic extraction (Ridgway et al, 2007). Headspace solid-phase microextraction (HS-SPME) in combination with HPLC was proposed for determination of benzene in fruit juices (Classadonte *et al*, 1997). Owning to high specificity and minimum sample preparation, HPLC has been the most common

method for the analysis of benzoic acid and its salt in foods (Jr and Grether, 2000; Saiz *et al*, 2001; Tfouni and Toledo, 2002).

2.18.1 LIQUID-LIQUID EXTRACTION

It is a process of transferring a solute from one liquid phase to another immiscible or partially miscible liquid in contact with the first. The two phases are chemically quite different, which leads to a separation of the components according to their distribution or partition between the two phases, normally one organic and one water. This is different from distillation, in which the liquid is partially vaporized to create another (vapor) phase, but the two phases are similar chemically. Liquid-liquid extraction, also known as solvent extraction and partitioning chromatography, is employed to separate compounds based on their relative solubility's in two different immiscible liquids

2.18.2 STATIC OR PURGE AND TRAP HEADSPACE SAMPLING

Headspace analysis is a means of separating the volatile materials from a liquid or solid prior to analysis by GC/MS analysis. There are two basic means of collecting volatile compounds for analysis. They are dynamic headspace and static headspace.

Dynamic headspace (DHS): Is a form of headspace analysis that utilizes a "purge and trap" method to collect and concentrate outgassed materials for analysis by GC/MS. In this method, the sample is purged with ultra pure nitrogen while being heated in a Teflon vessel. As the nitrogen stream exits the vessel it passes through a thermal desorption tube filled with an adsorbent material. The outgassed products are collected onto the adsorbent material. Following the predetermined collection time, the tubes are transferred to a thermal desorption unit which is in line with the gas chromatograph and mass selective detector (GC/MS).

Static Headspace (SHS): In static headspace analysis, a liquid or solid sample is placed into a vial, sealed, and heated to a specific temperature. All of the components that are volatile at or

below the pre-set temperature escape from the sample to form a gaseous "headspace" above the sample. The term "static headspace" refers to the sealed environment in which the outgassed products are collected. After a certain period of time, the headspace gas is extracted from the vial and injected into a gas chromatograph which separates the various components of the sample based on size and/or polarity. The separated components then go into a mass selective detector. Static headspace analysis is an ideal choice for volatile compounds, such as residual solvents or low molecular weight additives (Nicholas HS *et al*, 2002).

2.18.3 HEADSPACE SOLID-PHASE MICROEXTRACTION

Headspace solid-phase microextraction (HS-SPME) is a solvent-free sampling technique based on the sorption characteristics (adsorption or absorption) of fiber coating materials.The analytes (volatiles or semi volatiles) from gaseous, liquid, or solid matrices are first released from the matrices and sorbed onto a fiber coated with an adsorbent or absorbant polymer introduced into the headspace. Following sorption, analytes are either thermally desorbed onto a gas chromatographic (GC) inlet or solvent desorbed into a high-performance liquid chromatographic (HPLC) inlet. Originally developed by Pawliszyn and co-workers in the early 1990s, applications for SPME have extended from environmental analyses to food/beverage analyses (Da-Mi J *et al*, 2003).

2.18.4 HEADSPACE SOLID-PHASE DYNAMIC EXTRACTION

HS-SPDE is a technology that allows you to constantly extract samples due to the fact that it uses significantly high amounts of sorbent material. It also has a large surface area with fast sample flow (turbulent flow) over the active coating, thereby ensuring short extraction times. Chromtech (Idstein, Germany) introduced SPDE to improve the adsorption capacity of SPME by coating the inside of a 5 cm long stainless steel needle with a 50 µm thick film of PDMS

containing 10% activated carbon. No extra thermal desorption system and mechanical and thermal stress are needed (Ron S, 2005).

2.19 CHROMATOGRAPHIC METHODS FOR SEPARATION OF COMPOUNDS

The separation and purification of a mixture of compounds is mainly carried out using one or other or a combination of chromatographic techniques; Thin Layer Chromatography (TLC), Column Chromatography (CC), High Performance Thin layer Chromatography (HPTLC), Optimum Performance Laminar Chromatography (OPLC), High Performance Liquid Chromatography (HPLC), Supercritical-fluid Chromatography, Gas Liquid Chromatography (GLC) and Electrophoresis. Chromatography is a technique used to separate molecules based on their size, shape, or charge. It is employed to analyse and isolate a variety of macromolecules. During chromatography molecules in some kind of buffer or solvent move through a solid phase that acts as sieving material. As the molecules move through the molecular sieve, they are separated. Three well-known types of chromatography include paper chromatography, thin-layer chromatography and column chromatography.

Few methods of chemical analysis are truly specific to a particular analyte. It is often found that the analyte of interest must be separated from the myriad of individual compounds that may be present in a sample. Separation on paper (paper chromatography) or thin layers (thin layer chromatography) are the earliest of chromatographic techniques to perform and require simple apparatus. They readily provide qualitative information and with careful attention to detail, it is often possible to obtain qualitative data. Chromatography is one method used to ascertain the amino acid composition of a protein. A sample is digested with proteases. The resulting amino acid mixture is run on a chromatograph. The amino acids travel up the paper to different heights. The distance they travel as compared to the solvent is measured and reported as Rf value. Each amino acid has a characteristic Rf for a given solvent. By

determining the Rf values for the sample, we can learn which amino acids are found in a given protein.

2.20 MODES OF CHROMATOGRAPHIC SEPARATIONS

Chromatographic separations are a result of the interactions between the analyte and the two phases (stationary and mobile phases). In general, there are five types of interactions: Adsorption, Partition, Ion-Exchange, Affinity and Size-Exclusion.

Adsorption Chromatography

This is also known as displacement, liquid/solid chromatography. It is based on interactions between the solute and fixed active sites on the stationary phases. Stationary phases are normally a solid adsorbent packed in a column, spread on a plate or on a porous paper. The mobile phase is usually a liquid solvent. The active sites of the stationary phase interact with the functional groups of the compounds to be separated by non-covalent bonds, non-polar interactions, Van der Waals forces and hydrophobic interactions. The less tightly bound compounds will be eluted out by the mobile phases at earlier time, 23 classes of compounds can be separated. Example is the separation of alcohols from hydrocarbons using silica gel. Silanol groups on the gel interact with the polar functional groups on the alcohols.

Partition chromatography

During a partition separation, solute particles interact between two non miscible liquid phases according to their relative solubility. This process is also referred to as liquid/liquid chromatography. The stationary phase is a film of liquid that is strongly adsorbed to an inert support and the mobile phase is a different liquid with different polarity. In general, i.e. normal phase chromatography, the stationary liquid is polar and the mobile phase is non polar. Examples are separation of polar compounds such as amino acids, carbohydrates and water-soluble plant pigments. In reverse-phase chromatography, the stationary liquid is non-

polar and the mobile phase is polar. Example is separation of non-polar compounds such as lipids and fat-soluble pigments. Partition chromatography is a very useful technique because it can resolve minute differences in the solubility of the solutes. It is well suited for separating homologues and isomers.

Ion-Exchange chromatography

This process enhances the separation of ions and polar molecules based on the electrical properties of the molecules. The stationary phase is a resin or gel matrix which contains covalently bound positive or negative functional groups. The cation exchange column carries negatively charged groups. Anion exchange carries positively charged groups. The mobile phase is a buffered aqueous solution which carries a counter-ion whose charge is opposite and in equilibrium with the total charge of the resin. Once the charged analytes are attached to the exchange groups in the column, they must be eluted out using a buffer with a higher ionic strength or a different pH in order to weaken the electrostatic interactions between the analytes and the exchangers.

Affinity Chromatography

Seperations are based on specific interactions between interacting pairs of substances such as macromolecules and its substrate, cofactor, allosteric effector or inhibitor. The stationary phase is a gel matrix to which a specific ligand is attached. The mobile phase is a buffered solution. During an affinity chromatography, a mixture of substances is applied to the column. Substances that have no affinity for the ligand are washed through with the buffer used and the desired compound is bound to the ligand. A buffer with a different pH or an increased ionic strength is used to elute the desired compound out. The choice of ligand is important in affinity chromatography. The ligand must interact specifically and reversibly with the molecule of interest. In addition, it must be suitable for coupling to a matrix.

Size exclusion chromatography

This is also referred to as gel filtration, gel permeation chromatography and molecular sieve chromatography. In this process, there are no chemical attractions or interaction which occurs between the solutes and the stationary phase. The molecules are separated according to their size. Higher molecular weight molecules ranging between 2000 to 25,000,000 daltons can be separated. The stationary phase is made up of inert material such as gel or a porous glass or porous silica beads. The mobile phase is water or an aqueous solution that solely serves as a carrier for the analyte. The degree of retention is dependent on the size of the solvated solute molecule relative to the size of the pore. Smaller molecules will permeate the smaller pores, intermediate sized molecules will permeate some pores and larger molecules are eluted at earlier time. By knowing the relative elution time, molecular weights of unknown compounds can be estimated (Raaman, 2006).

2.21 THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) was developed because of a specific need for a rapid method which would separate small amounts of compounds. Thin-layer chromatography is one of the most popular and widely used separation techniques. Thin-layer chromatography is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents. TLC is a quick, inexpensive micro-scale technique that can be used to determine the number of components in a mixture, verify a substance's identity, monitor the progress of a reaction, determine appropriate conditions for column chromatography, and analyze the fractions obtained from column chromatography.

Although alumina and silica are the most common stationary phases used for TLC, there are many different types including the following (arranged in increasing order of polarity);

Reverse Phase (hydrocarbon-coated silica e.g. C-18), Paper, Cellulose, Starch, Calcium sulfate, Silica (silica gel), Florisil (Magnesium silicate) Magnesium oxide, Alumina (aluminum oxide; acidic, basic or neutral),Activated carbon (charcoal; Norit pellets). Thin layer chromatography is a separation method in which uniform thin layer of sorbent or selected media are used as carrier medium. The sorbent is applied to a backing as a coating to obtain a stable layer of suitable size. The most common support is a glass plate, but other supports such as plastic sheets and aluminium foil are also used. The four sorbents mostly commonly used are silica gel, alumina, kieselguhr (diatomaceous earth), and cellulose. Silica gel (silica acid) is the most popular layer material. It is slightly acidic in nature. In order to hold the silica gel firmly on the support, a binding agent such as plaster of Paris (calcium sulphate hemihydrates) is commonly used.

Two ultraviolet (UV) indicators, which aid in the location of separated substances, are also incorporated, either singly or together, in silica gel or other layer materials. Zinc silicate fluoresces when exposed to ultraviolet light of 254nm wavelength, so that substances adsorbing this wavelength will contrast sharply by appearing dark through quenching of the greenish-yellow fluorescing background.

Alumina (aluminium oxide) is chemically basic and for a given layer of thickness it will not separate quantities of material as large as can be separated on silica gel. Alumina is more chemically reactive than silica, and care must be exercised with some compounds and compound classes to avoid decomposition or rearrangement of these substances during sample application, storage before development or development. Diatomaceous earth (kieslghur) is a chemically neutral sorbent that does not separate or resolve as well as either alumina or silica gel. Precoated TLC plates are commercially available. Compared to paper chromatography, the special advantages of thin layer chromatography are the versatility, speed of separation and sensitivity. TLC is used for the separation of substances in a wide

variety of fields. Amino acids from protein, hallucinogenic alkaloids from plant, steroids from the urine of a newborn infant, morphine in the blood of an overdose victim and pesticides from soil may be separated by TLC, with sensitivities of 1μg or less (Touchstone, 1992).

2.21.1 Diagnostic/ Qualitative TLC

The aim of diagnostic or qualitative TLC is to determine the number of components in a system and if possible to learn what they are without isolating them. This technique can be used to monitor the components of eluents in column chromatography and subsequently combining fractions with similar components.

2.21.2 Quantitative or Analytical TLC

This deals with how much of each component present in the sample mixture. The thickness of the adsorbent layer is typically around 0.1 – 0.25 mm for analytical purposes (Raaman, 2006). In order to use TLC as a quantitative method of analysis, it is important to quantify the spots along with definition for all of the usual parameters (specificity, range of the domain of linearity and precision). This is done by placing the plate under the lens of a densitometer (or scanner) that can measure either adsorption or fluorescence at one or several wavelengths. This instrument produces a pseudo-chromatogram that contains peaks whose areas can be measured. In fact it is an isochronic image of the separation at the final instant. In TLC a spot is usually detectable if it corresponds at least to a few ng of a compound UV absorbent (Rouessac. and Rouessac, 2007).

2.21.3 Preparative TLC

TLC can be used on a microscale to monitor a reaction and determine if the product or products were successfully produced using only microgram quantities of materials. It is difficult to separate gram quantities using TLC and therefore column chromatography is used

at this scale. However, larger TLC plates, called a Preparative Plates, can be used for separations of milligram quantities of materials because they are coated with thick layers (1-3mm) of stationary phase. Sample is applied to the plate as a thin even layer horizontally to and just above the solvent level. When developed with solvent the compounds separate in horizontal bands rather than horizontally separated spots. Each band (or a desired band) is scraped off the backing material. The adsorbent material is then extracted with a suitable solvent and filtered to give the isolated material upon removal of the solvent.

The "Chromatotron" is a novel and highly convenient piece of equipment for preparative thin-layer separations. Basically it consists of a slanted circular glass plate which is spun about a central shaft by means of an electric motor. The glass plate carries the adsorbent layer of 1.2 or 4mm thickness. The plate is spun during loading and solvent development and the sample and separated components are as radial bands (Furniss *et al*, 1989).

2.21.4 Visualization of spots on TLC plates

Any compounds have to be visualized after TLC separation in order for the compounds to be seen. There are a number of visualization reagents which can be used to detect any number of compounds either by reacting with a specific chemical structure (a ketone, aldehyde or carboxylic acid) or by forming a complex with a double bond, or finally by oxidizing to visible intermediates or if carried to the extreme, to carbon itself. Among the most spraying reagents employed include; Aluminium chloride for Flavonoids, Anisaldehyde and Sulfuric acid for steroids, terpenes, sugars, Bromothymol blue for lipids, HCl for indole derivatives, Diphenylamine for glycolipids, Potassium hydroxide for coumarins and Dragendorff's solution I and II for alkaloids (Raaman, 2006).

2.22 COLUMN CHROMATOGRAPHY

Chromatography is based on differential migration rates of components of a liquid or gas as it moves past adsorptive materials. Some combination of adsorbent, conditions, and carrier fluid allows application of a mixture of materials to a column of adsorbent and to flush so that differential migration rates separate the materials before they exit. In column Chromatography the stationary phase (which can be solid or liquid) and a mobile phase (usually liquid or gas) are both placed in a column container.

The conventional technique of liquid-solid column chromatography employs the continuous passage of a single eluting solvent through the column under gravity or under pressure applied to the top of the solvent reservoir (Flash column chromatography).

If the desired compound is coloured or strongly fluorescent under ultraviolet light, their location in selected eluent fractions presents no problems. Hence suitable fractions are combined and concentrated to recover the purified material. Also, each of the individual fractions collected could be examined directly by TLC (employing one of the non- selective detecting agents e.g. iodine vapour) (Furniss et al, 1989).

The most common adsorbents used in column chromatography include; aluminium oxide (alumina), silica gel, cellulose, Charcoal, Dextran, Agarose, Polyacrylamide, Polystyrene.

The Solvent system (Eluent) must be significantly less polar than the components of the mixture. If the solvent is more polar and strongly adsorbed, then the components of the extract will remain in the mobile phase and little separation will take place. It is also essential that the mixture is soluble in the solvent; otherwise it will remain permanently adsorbed on the adsorbent. Polar solvent are used to elute strongly adsorbed components while non-polar solvents are used for weakly adsorbed components of a mixture. The eluotropic series serves

as a guide to selection of solvent or mixture of solvents based on their polarity. Elutrophic series ranks solvents by their relative abilities to displace solute from a given adsorbent.

2.23 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC represents the modern culmination of the development of liquid chromatography. The user begins by placing samples on a tray for manual/ automatic injection into the column. Solvent is continually pumped through the column, and the separated compounds are continuously sensed by a detector as they leave the column. The resulting detector signal plotted against time is the chromatogram.

The basic information attained from the Chromatogram includes:

- The number of peaks, which appear, indicates the level of complexity of the sample.
- Qualitative information about the sample is obtained by comparing peak positions with those of standards.
- Quantitative assessment of the relative concentration of components is obtained from peak area comparisons (Raaman, 2006)

This technique is the prime analytical method for compounds which are involatile or thermally unstable. They include the natural products (carbohydrates, steroids, alkaloids, peptides and amino acids, antibiotics, nucleosides, etc.), and the synthetic and naturally occurring compounds arising from research in the pharmaceutical, agricultural and food industries. Analytically useful information obtainable from the chromatogram can show whether a given sample is pure or not. Compared to other separation procedures, HPLC is exceptional in terms of the following characteristics:

• Almost universal applicability; few samples are excluded from the possibility of HPLC separation.

• Remarkable assay precision (±0.5% or better in many cases).

• A wide range of equipment, columns, and other materials is commercially available, allowing the use of HPLC for almost every application (Snyder *et al*, 2010).

2.23.1 SEPARATION MODES IN HPLC

The modes of HPLC are classified into two. They are:

Normal-Phase Chromatography (NPC)

In normal-phase chromatography, the stationary phase is more polar than the mobile phase. The stationary phase is a polar adsorbent such as bare silica or silica to which polar nonionic functional groups such as alcoholic hydroxyl, nitro, cyano (nitrile), or amino-have been chemically attached (e.g., R = $-CH_2CH_2CH_2NH_2$, aminopropyl). The mobile phase is made of nonpolar solvent, such as hexane, to which is added a more polar modifier, such as methylene chloride, to control solvent strength and selectivity. Normal-phase can be used for compounds that are too hydrophobic or hydrophilic for separation using reversed-phase. Compounds that are not soluble in water or that may decompose in water are candidates for normal-phase chromatography. One of the main uses of normal-phase chromatography is for the separation of isomers. It can also be used for separating compounds that are highly soluble in organic solvents, such as fat-soluble vitamins and phospholipids

Reversed-Phase Chromatography (RPC)

Reversed-phase chromatography is the term used to describe the state in which the stationary phase is less polar than the mobile phase. Chemically bonded octadecylsilane (ODS), an n-alkane with 18 carbon atoms, is the most frequently used stationary phase. C8 and shorter alkyl chains and also cyclohexyl and phenyl groups provide other alternatives. Phenyl groups are more polar than alkyl groups.

Water is often described as the strongest elution medium for chromatography, but in fact this is only true for adsorption processes. Water may interact strongly with the active centers in silica and alumina, so that adsorption of sample molecules becomes highly restricted and they are rapidly eluted as a result. Exactly the opposite applies in reversed-phase system; water cannot wet the non-polar (hydrophobic¼water-repellent) alkyl groups and does not interact with them in any way. Hence it is the weakest mobile phase of all and gives the slowest sample elution rate. The greater the amount of water in the eluent, the longer is the retention time.

The better the Sample compounds are retained by the reversed-phase surface the less water soluble (i.e. the more non-polar) they are. The retention decreases in the following order: Aliphatics > induced dipoles (e.g. CCl4) > permanent dipoles (e.g. CHCl3) >weak Lewis bases1 (ethers, aldehydes, ketones) > strong Lewis bases (amines)> Weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids) (Meyer, 2004). The advantage of RPC is that this technique is perhaps the most efficient of all the HPLC separation modes. RPC has a high peak capacity and is particularly effective for separating small molecules, peptides, nucleotides and restriction fragments.

2.23.2 ELUTION MODES IN HPLC

Gradient Elution

It is the continuous change of solvent composition to increase eluent strength. The mobile phase has to become steadily stronger as the separation proceeds. The strength of the mobile phase is characterized by the solvent strength which is specific for a given stationary phase. Gradient elution are used for complex sample mixtures with a wide retention range, biopolymers whose retention changes markedly for small changes in the mobile phase composition and samples which contain a variable and/or unknown composition (tool for

screening). Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower and taller peaks for most components

Isocratic Elution

A separation in which the mobile phase composition remains constant throughout the analysis is termed isocratic (meaning constant composition). In isocratic elution, peak width increases with retention time linearly according to the equation for N, the number of theoretical plates. This leads to the disadvantage that late-eluting peaks get very flat and broad. Their shape and width may keep them from being recognized as peaks.

2.23.3 HPLC INSTRUMENTATION

The main components of an HPLC system are a high-pressure pump, a column and an injector system as well as a detector (figure 2.6). The system works as follows: The eluent is filtered and pumped through a chromatographic column, the sample is loaded and injected onto the column and the effluent is monitored using a detector and recorded as peaks.

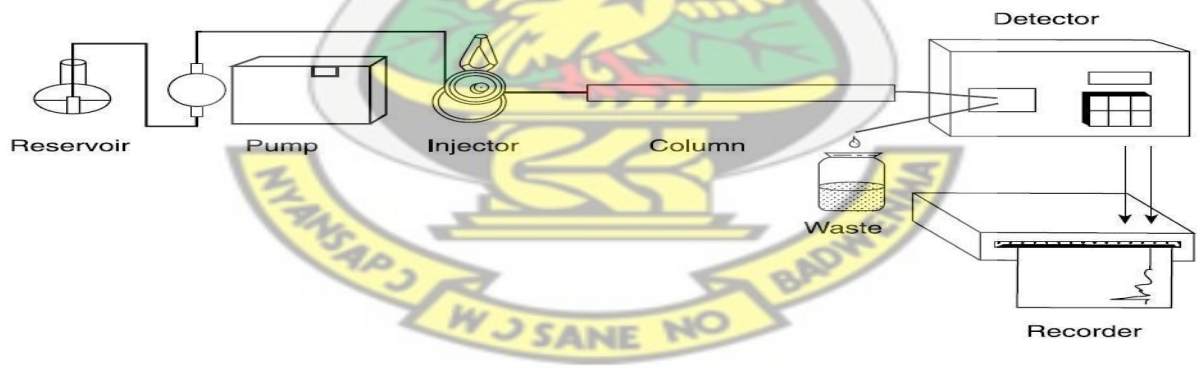

Figure. 2.6 A Schematic Diagram of HPLC

The Chromatogram and Its Purport

The eluted compounds are transported by the mobile phase to the detector and recorded as Gaussian (bell-shaped) curves. The signals are known as peaks (Figure 2.7) and the whole entity is the chromatogram.

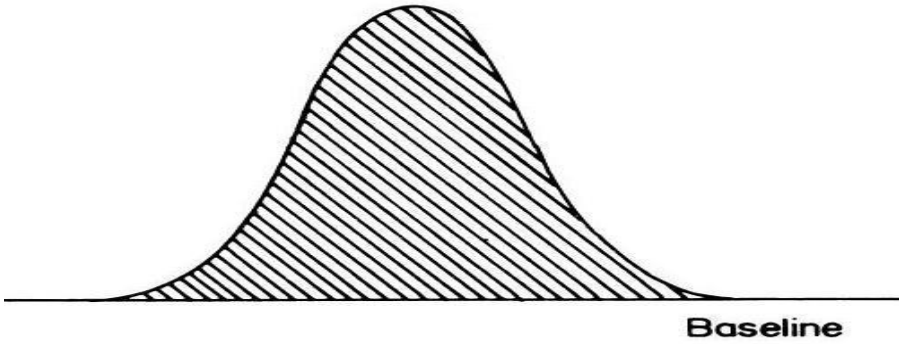

Figure 2.7 Diagram showing an HPLC peak

The peaks give qualitative and quantitative information on the mixture in question:

Qualitative: the retention time of a component is always constant under identical chromatographic conditions. The retention time is the period that elapses between sample injection and the recording of the signal maximum. The column dimensions, type of stationary phase, mobile phase composition and flow velocity, sample size and temperature provide the chromatographic conditions. Hence, a peak can be identified by injecting the relevant substance and then comparing their retention times.

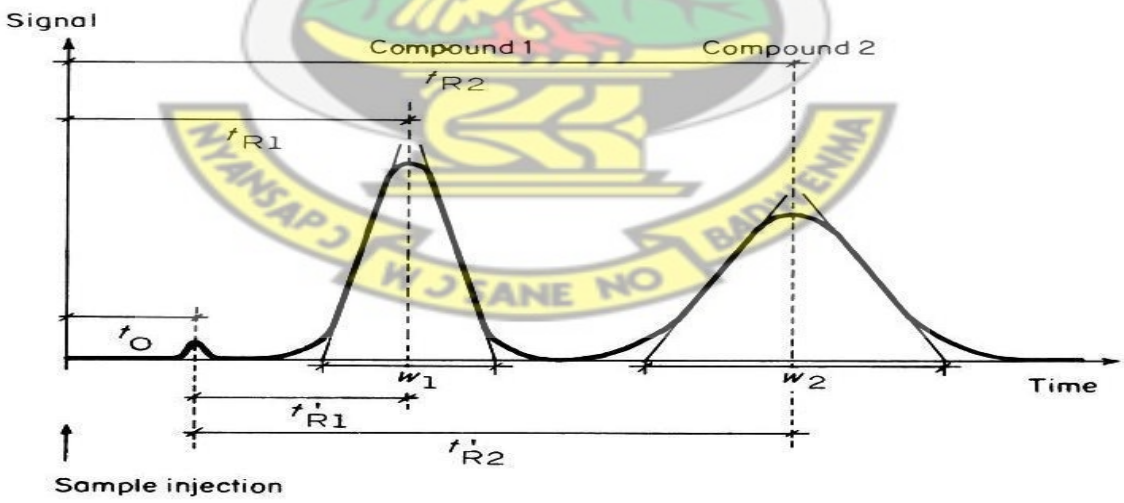

Figure 2.8 Diagram showing a typical HPLC chromatogram

Quantitative: both the area and height of a peak are proportional to the amount of a compound injected. A calibration graph can be derived from peak areas or heights obtained for various solutions of precisely known concentration and a peak-size comparison can then be used to determine the concentration of an unknown sample.

The chromatogram can be used to provide information on separation efficiency

Here:

w = peak width at the baseline, to = dead time or retention time of an unretained solute, i.e. the time required by the mobile phase to pass through the column (also called the breakthrough time).

Hence the linear flow velocity, **U**, can be calculated as;

U=L/to

Where **L**= length of the column.

tR = the retention time; this is the period between sample injection and recording of the peak maximum.

Two compounds can be separated if they have different retention times.

t'R = Net retention time or adjusted retention time.

Figure above shows that **tR= to** + **t'R**.

to is identical for all eluted substances and represents the mobile phase residence time. **t'R** is the stationary phase residence time and is different for each separated compound. The longer a compound remains in the stationary phase, the later it becomes eluted.

Retention time is a function of mobile phase flow velocity and column length. If the mobile phase is flowing slowly or if the column is long, then **to** is large and hence so is **tR**; **tR** is

therefore not suitable for characterizing a compound. Therefore the retention factor or **k** value (formerly known as the capacity factor, ko) is preferred:

$$k = \frac{t'_R}{t_0} = \frac{t_R - t_0}{t_0}$$

k is independent of the column length and mobile phase flow-rate and represents the molar ratio of the compound in the stationary and the mobile phase (Meyer, 2004).

Resolution

The aim of chromatography is to separate components in a mixture into bands or peaks as they migrate through the column. Resolution, R, provides a quantitative measure of the ability of a column to separate two analytes.

The resolution of two neighbouring peaks is defined by the ratio of the distance between the two peak maxima, i.e. the distance between the two retention times, **tR**, and the arithmetic mean of the two peak widths, **w.**

$$R = 2\frac{t_{R2} - t_{R1}}{w_1 + w_2} = 1.18\frac{t_{R2} - t_{R1}}{w_{1/2_1} + w_{1/2_2}}$$

Where *W*1/2 is the peak width at half-height

2.23.4 QUANTIFICATION OF AN ANALYTE USING HPLC TECHNIQUE.

Peak height or peak area can be measured, either manually or with electronic devices. Peak height measurements have the advantage of simplicity but are sensitive to changes in peak shape; hence it is advised that peak areas should be used where peaks are broad and tailing.

For a given system, a calibration graph must be constructed for each compound to be analysed because the detector response to each will be different. This graph of peak height (or

area) against the standard concentration can then be used to quantify the unknown sample by extrapolation. Such external calibration requires careful control of the injection volumes and valve injection should be used. However, external calibration is susceptible to errors arising from fluctuations in column performance and the internal standard technique gives better precision. This involves the addition of a fixed amount of a substance (internal standard) to the sample before injection. Quantification is carried out using peak height (or area) ratios of drug to internal standard. The general assumption used in quantitation of analyte using HPLC technique is that the peak area/ratio, **A,** is proportional to the concentration, **c,** of the analyte under the same chromatographic conditions.

A α **c**

A =k **c**

So to quantify a sample X, a known concentration of a reference sample, cs is prepared and run under the same chromatographic conditions as sample X and their peak areas compared as shown below;

As/Ar =cs/cr. Hence, concentration of sample X, **cs**, can be calculated.

Where**;**

As= peak area (or ratio) of sample **Ar**= peak area (or ratio) of the reference sample.

2.23.5 COLUMN EFFIECIENCY

A chromatographic column is divided into **N** theoretical plates. A thermodynamic equilibrium of the analytes between the mobile phase and stationary phase occurs within each plate. The efficiency of the column is expressed as the number of theoretical plates, **N**.

$$N = 16\left(\frac{t_R}{w}\right)^2$$

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$$

Poor column efficiency results in band/peak broadening.

The height of a theoretical plate, **H,** is readily calculated provided the length of the column **(L)** is known;

$$H = \frac{L}{N}$$

2.23.6. COMMON HPLC DETECTORS

*Refractive Index (RI)***:** It is a universal analyte detector in which the solvent remains the same throughout separation and very temperature sensitive. It is sometimes difficult to stabilize the baseline. The detection principle involves measuring of the change in refractive index of the column effluent passing through the flow-cell. The greater the RI difference between sample and mobile phase, the larger the imbalance will become. Thus, the sensitivity will be higher for the higher difference in RI between sample and mobile phase. On the other hand, in complex mixtures, sample components may cover a wide range of refractive index values and some may closely match that of the mobile phase, becoming invisible to the detector. RI detector is a pure differential instrument, and any changes in the eluent composition require the rebalancing of the detector. This factor is severely limiting RI detector application in the analyses requiring the gradient elution, where mobile phase composition is changed during the analysis to effect the separation.

*Fluorescence (FD)***:** Fluorescence detectors are probably the most sensitive among the existing modern HPLC detectors but not popular. It is possible to detect even a presence of a single analyte molecule in the flow cell. Typically, fluorescence sensitivity is 10 -1000 times higher than that of the UV detector for strong UV absorbing materials. Fluorescence detectors are very specific and selective among the others optical detectors. This is normally used as an

advantage in the measurement of specific fluorescent species in samples. Analytes involve must have a fluorophore group.

*UV-Ultraviolet Light (UV/VIS)***:** Beam of the electromagnetic radiation passed through the detector flow-cell will experience some change in its intensity due to this interaction. Measurement of this change is the basis of the most optical HPLC detectors. UV/VIS detection is more rugged than many other detection systems. Radiation absorbance depends on the radiation wavelength and the functional groups of the chemical compound. Electromagnetic field depending on its energy (frequency) can interact with electrons causing their excitation and transfer onto the higher energy level, or it can excite molecular bonds causing their vibration or rotation of the functional group. The intensity of the beam which energy corresponds to the possible transitions will decrease while it is passing through the flow-cell. According to the Lambert-Bear law absorbance of the radiation is proportional to the compound concentration in the cell and the length of the cell. UV/VIS detectors can be used for the analysis of many organic compounds, that is why it is so popular and almost all published HPLC analysis use this detector.

Photodiode array detector (PDA)

A photodiode array (PDA) also known as diodearray detector (DAD) is a linear array of discrete photodiodes on an integrated circuit (IC) chip. For spectroscopy it is placed at the image plane of a spectrometer to allow a range of wavelengths to be detected simultaneously. In this regard it can be thought of as an electronic version of photographic film. Array detectors are especially useful for recording the full uv-vis absorption spectra of samples and to some extent IR that are rapidly passing through a sample flow cell, such as in an HPLC detector. Thus the advantages are higher sensitivity and measure the entire absorption range (gives a scan of an entire spectrum). PDAs work on the same principle as simple photovoltaic detectors. Light creates electron-hole pairs and the electrons migrate to the nearest PIN

junction (p-type, n-type and insulating). After a fixed integration time the charge at each element is sequentially read with solid-state circuitry to generate the detector response as a function of linear distance along the array. PDAs are available with 512, 1024, or 2048 elements with typical dimensions of ~ 25 µm wide and 1-2 mm high. Photodiode array detectors facilitate peak identification and they are the best detectors for method development.

2.23.7 HPLC-COUPLED TECHNIQUES

Coupled and complementary methods of analysis in HPLC are employed to bring about separation of constituents of a mixture and other analytical methods of analysis for detection and identification of species. This has developed into what is referred to as hyphenated methods of analysis.

Hyphenation of liquid chromatographic (LC-hyphenation) methods primarily began with the advent of diode array detector (DAD) or Photodiode array detector (PAD). Mass spectrometry (MS) has been coupled to liquid chromatography, as have Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR), and inductively coupled plasma (ICP) spectroscopies. Also, different types of chromatographies have been coupled such as LC-GC.

In LC-MS hyphenated system, the chromatographic technique separates the components of a mixture, while MS provides structural information with regards to each of the eluting molecular species (Ewing, 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CHEMICALS

- Benzoic acid, extra pure, Gatt Koller (Germany).
- Benzene, 99%, Scientific laboratory reagent, Fissions scientific equipment (London).
- Acetonitrile, G Chromasolv, HPLC super gradient grade, Sigma Aldrich (Germany).
- Ammonium acetate, 97%, Alfa Aesar, Johnson Mathey Company (UK).
- Acetic acid, Analytical reagent, GFS Chemicals, Inc, Columbus.
- Methanol, Chromasolv for HPLC, Sigma Aldrich (Germany)

3.2 EQUIPMENT

- Varian Proster High Performance Liquid Chromatography, Galaxie chromatograph data system. Varian Inc, USA
- Centrifuge machine (Heraus Christ) RPM × 1000
- pH Meter, LTG 1/24, 800366 probe, Phywe measure
- Electronic balance, Sartorius AG Germany, CPA623S
- Magnetic stirrer, Heidolph, Mr Hei-Standard

3.3 INSTRUMENTATION

The component of the Varian Proster Chromatograph include; Varian Proster 210-218 SD-1 pumps, Phenomenex synergi 4µ polar – RP 80A 150×2mm 4 micron column with Proster 325 UV detector (Fig 3.1).

Figure 3.1 The HPLC used for the analysis.

3.4 SAMPLE COLLECTION

34 different soft drinks, 16 fruit juices and 25 herbal prepared products were purchased in three batches at the open market and pharmacy shops in the city of Kumasi, Ghana (Fig 3.2).

Figure 3.2 Some soft drink samples

Figure 3.3 Some herbal products

3.5 SAMPLE PREPARATION

All the samples were centrifuged at 3000 rpm for 25 min and the supernatant was filtered and kept in sample vials for analysis (Tfouni and Toledo 2002, Bennet and Petrus 1977, Argoudelis 1984, Williams 1986, Bui and Copper 1987, and Veerabhadrarao *et al 1987*). All the samples were diluted and if the concentration of the preservative in the soft drinks and herbal products are higher than the largest one used to build the calibration curve or very concentrated (thick), the drink and herbal samples were further diluted with water.

Figure 3.4 Filtration of Samples

3.6 MOBILE PHASE PREPARATION

The mobile phase consisted of 90% ammonium acetate buffer with 10% HPLC-grade acetonitrile (Inmaculada, 2003). The ammonium acetate buffer was prepared by dissolving 7.6 g of the salt in double distilled water and making it up to 2000 mL in a volumetric flask . The pH was adjusted to 4.4 with the addition of acetic acid drop wise. 1800 mL of the ammonium acetate buffer was measured into a 2000 mL volumetric flask, 200 mL acetonitrile was also measured and added to the buffer. It was well shaken, filtered with Whiteman filter paper and was kept in the fridge for the analysis.

For the benzene analysis in the soft and fruit drinks, methanol (HPLC grade) and water (double distilled) was used as the mobile phase. The methanol constituted 70% and the water 30% (Abdulrahman *et al*, (2011). Both methanol and water were kept in separate containers and the mixing was done by the instrument.

3.7 STANDARDS PREPARATION

An amount of 0.25 g of extra pure benzoic acid was weighed into a 250 mL volumetric flask. It was dissolved in water with the help of the magnetic stirrer. Water was toped to the mark and the solution was filtered. From the stock of 250 mg/L, working standards for the analysis was prepared. 75 mg/L, 50 mg/L, 25 mg/L, 15 mg/L and 5 mg/L were prepared by using the dilution formula; $C_1V_1 = C_2V_2$.

The density of benzene was used to calculate the volume of pure benzene to be use in preparing the standards. In preparing 0.5 ppm, the density of benzene (0.876 g/mL) is considered as the initial concentration and the final volume is the 100 mL volumetric flask. Using the dilution formula, the initial volume was then calculated. Standards of 0.4, 0.3, 0.2 and 0.1 ppm were prepared from this procedure.

3.8 OPTIMAZATION

This was done to know the conditions especially the wavelength before the analysis of the samples. The UV-spectrophotometer was used to determine the wavelength of the stock benzoic acid prepared and the stock benzene. The determined wavelength for the analysis was 238 nm for benzoic acid and 264 nm for benzene analysis. Several runs were made to determine the retention time for the analysis. The retention time for the benzoic acid and the time for the analysis were determined for the soft drinks and the herbal products. The retention time for benzene and the analysis time were also determined.

3.9 CHROMATOGRAPHIC CONDITIONS

The analytical separation was carried out on a Varian Proster HPLC unit using a Phenomenex synergi 4µ polar-RP 80A column (150×2mm 4 micron) at room temperature. The detector used was Proster 325 UV detector set at 238 nm for the analysis of benzoic acid in soft drinks and herbal products and 264 nm for the analysis of benzene with 20 µL as the volume of standards and samples injected. The separation was achieved with isocratic elution (a separation in which the mobile phase remain constant throughout the analysis) at a flow rate of 0.4 mL/min for benzoic acid analysis and 0.8 mL/min for the benzene analysis.

3.10 CALIBRATION CURVE

The external standard plot method was used. The standard concentration range from 1 mg/L to 50 mg/L and 20 µL each of the standards was injected. 0.1 mg/mL to 0.5 mg/mL was the range for the benzene. The peak areas where measured and those of the analytes (y) were plotted against the concentration (mg/l) of the benzoic acid (x). Least square linear regression analysis was used to determine the slope, y-intercept and the correlation coefficients of the standards plots.

3.11 QUANTIFICATION

The Varian Proster HPLC is equipped with Galaxie chromatograph data system as the software and this was used for the calibration curve and the quantification of the samples. The quantification of the samples was based on comparison of the peaks of the standards with those of the samples.

3.12 LIMIT OF DETECTION AND QUANTIFICATION

The limit of detection (LOD) is defined as the smallest peak detected with a signal height three times that of the baseline while the limit of quantitation (LOQ) refers to the lowest level of analyte which can be determined with an acceptable degree of confidence. In this work the limit of detection and quantitation were determined by the continual decrease of the concentration of a known standard and injecting into the instrument until no appreciable peak is observed. The concentration was multiplied by 3 and 10 to obtain the limit of detection and quantitation respectively.

3.13 RECOVERY STUDY

In order to verify the accuracy and precision of the analytical procedure, recovery studies were carried out by spiking some samples with very low levels of benzoic acid (< 0.4 mg/L) with a known standard. In this work 2 mL of each sample mixture and 2 mL of 25 mg/L standard was taken mixed together and injected. Due to the dilution the actual concentration becomes 12.5 mg/L. The observed concentrations and the known concentration are divided and multiply by 100 to obtain the recoveries.

3.14 REPRODUCIBILITY

The reproducibility of the method was carried out by injecting the mid standard of benzoic acid everyday during the analysis. The relative standard deviation was obtained to check the reproducibility of the method.

3.15 STATISTICAL ANALYSIS

A descriptive analyses encompassing means, median, standard errors and coefficient of variation were computed. A one way analysis of variance (ANOVA) was carried out using SPSS version 17, at a significance level of 5%. The Least Significant Difference (LSD) test was used to locate differences in means.

3.16 ASSESSMENT OF POTENTIAL DAILY INTAKES OF BENZOIC ACID

The daily intakes of benzoic acid can be estimated based on the mean national food consumption data on soft and fruit drinks and the mean on the analytical determinations. The estimated daily intake was calculated by multiplying the consumption per capita of the soft and fruit drinks by the average concentration determined. It was then divided by the average weight of 60 kg for adults and 10 kg for children (Tfouni and Toledo, 2002). The consumption per capita used in the calculation was taken from Codex Alimentariuus Commission (2012) and Business and Financial Times (B&FT, 2011).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 BENZOIC ACID DETERMINATIONS

The concentration of benzoic acid in the soft drinks, fruit juices and herbal products ranged from below the detection limit to 2004 mg/L. The calibration curve for benzoic acid was plotted on the basis of peak areas of chromatograms obtained for various concentrations of working standard solutions, prepared from the stock solutions. A very good linearity for benzoic acid in the range of 1-50 mg/L concentrations was obtained, with a regression factor (r2) of 0.9980. From the characteristic HPLC chromatogram of benzoic acid it can be observed that a good resolution of the chromatographic peak was achieved. The retention time was 7.50 ± 0.27 min and 10 min was used for each analysis.

A regression factor (r^2) of 0.9933 was obtained for benzene in the range of 0.1 – 0.5 mg/L. The retention time was 1.14 ± 0.04 and 6 min was allowed for each sample.

4.2 BENZOIC ACID IN SOFT DRINKS

Thirty four (34) different brands soft drinks were analysed for benzoic acid. The concentration of benzoic acid in the samples ranged from below the detection limit to 564.00 mg/L (Table 4.2). Thirty three (33) samples showed levels of benzoic acid which shows that benzoic acid is the readily available preservative on the market which also confirms a survey conducted on the most preservatives used by local soft drinks manufacturers. Bottled Coca cola (CC) showed no level of benzoic acid which confirms with literature (Martin H., 2008) which says Coca Cola Company is phasing out sodium benzoate in some of its products. The highest concentration of benzoic acid was 564.00 mg/L which was recorded in Hita orange flavour (HOF) and the lowest concentration of benzoic acid was 0.2 mg/L which was also recorded in Malta Guinness (MG), deco cola (DC) and club soda (CS). Out of the 34 samples analysed only one had levels below the detection limit, 6 samples (Hita orange flavour,

Klassy orange flavour, Hita cola flavour, Klassy cola, Anabe fun cola and royal orange flavour with levels from 201.07 to 548.00 mg/L) showed levels above the set standard for benzoic acid or benzoates in soft drinks (150 mg/L) with the rest being within the range. Alvaro pineapple, Africa cola, B-Cool, Kalah cola, Alvaro passion fruit, Alvaro pear, Africa fun orange, Africa fun lemonade and club muscatalla showed levels of benzoic acid from 25.87 to 40.03 mg/L. Fanta, Decco cola, Malta guinness Fresh orange flavour, Vanda cool, Sil mixed fruit, U-fresh coke also showed levels of benzoic acid from 0.27 to 5.43 mg/L. The mean concentration for all the samples was 70.20 mg/L which was below the mean concentration recorded by Tfouni *et al*, 2002 in Brazil (259.2 mg/L) and Khosrokhavar *et al*, 2010 in Iran (163.8 mg/L). The standard deviation, the standard error of mean and the coefficient of variation were 119.00, 11.78 and 169.52% respectively. The high value of the coefficient of variation was due to the fact that the samples used for the analysis were produced from different companies. Each company has its own way of production and therefore the precision varies from company to company. However, the calculated coefficient of variation from Alvaro pineapple (ALP), Alvaro pear (ALPE) and Alvaro passion fruit (ALPF) which are from one company was 1.52% and suggests that the company uses the same procedure in the production. Table 4.1 shows the mean concentrations of the samples analysed. There was no significant difference between Fanta (FTA), Club soda (CS), Deco cola (DC), Master Cai Cocktail (MCC) and Malta Guinness (MG). However, there was a significant difference between Master Cai Pineapple (MCP) and Alvaro pineapple (ALP) and between Hita cola flavour (HICF) and Orange Vika (OV) at $P < 0.05$

Table 4.1 Levels of benzoic acid in soft drinks

Sample	Level of Benzoic acid (mg/L)			Mean(mg/L)	Standard Deviation
	1	2	3		
ALP	28.2	28.1	27.4	27.90[h]	0.44
AFC	28.2	28.2	27.3	27.90[h]	0.52
B-C	25.9	25.1	24.3	25.10[h]	0.80
MCP	1.4	1	1	1.13[b]	0.23
TOF	26.5	25.7	25.4	25.87[h]	0.57
DPF	13.7	13.1	12.6	13.13[f]	0.55
VC	3.3	3.6	3.5	3.47[c]	0.15
SP	65.4	65.4	63	64.60[l]	1.39
AFO	111	108	110	109.67[g]	1.53
FTA	0.7	0.6	0.6	0.63[a]	0.06
KOF	326	321.5	305.5	317.67[r]	10.77
CO	7.8	7.4	7	7.40[e]	0.40
DC	0.3	0.2	0.3	0.27[a]	0.06
FCF	35.7	34.8	34.2	34.90[k]	0.75
ANFC	235	232.5	227.5	231.67[q]	3.82
HOF	564	550.5	529.5	548.00[s]	17.39
ROF	199.6	202.8	200.8	201.07[n]	1.62
KAC	27.5	27.7	28	27.73[h]	0.25
AFFO	30.2	30.9	29.9	30.33[i]	0.51
FOF	1.8	2	1.8	1.87[b]	0.12
HICF	251.4	249	246.2	248.87[p]	2.60
VANC	4.8	4.95	4.35	4.70[c]	0.31
CS	0.2	0.2	0.4	0.27[a]	0.12
KC	242.5	243	234	239.83[m]	5.06
ALPF	28.2	28.2	27.9	28.10[h]	0.17
UFC	4.8	4.9	4.6	4.77[c]	0.15
CC	nd	nd	nd	nd	nd
SMF	50.4	49.9	51	50.43[t]	0.55
AFFL	35.1	34.8	33	34.30[j]	1.14
CM	41.5	40	38.6	40.03[k]	1.45
MCC	0.8	0.9	0.7	0.80[a]	0.10
ALPE	28.1	28.5	29	28.53[h]	0.45
MG	0.2	0.4	0.4	0.33[a]	0.12
OV	5.7	5.6	5.4	5.57[d]	0.15

Numbers in rows followed by different superscript are significantly different at $P < 0.05$

Table 4.2 Descriptive statistics of soft drinks

Statistic	Number of samples used	Mean Conc of Benzoic acid (mg/L)	Variance	Standard deviation	SEM	CV (%)	Min. level of Benzoic acid	Max. level of Benzoic acid
Soft drink	34	70.20	14160.51	119.00	11.78	169.52	nd	564

SEM= Standard error of mean

CV= Coefficient of variation

Min= Minimum

Max= Maximum

nd= Not detected

Conc = Concentration

4.3 BENZOIC ACID IN FRUIT JUICES

Sixteen (16) samples of fruit juices were analysed for the levels of benzoic acid. The concentration of the samples ranged from below the detection limit to 148 mg/ (Table 4.3). 5 star apple juice (5SAJ) recorded the highest concentration of 148 mg/L while kalyppo apple (KA) and kalyppo guava (KG) recorded the lowest concentration of 0.2 mg/L. 5 Star strawberry juice, Tampico, Fandango and Sunfruits had levels of benzoic acid from 60.75 to 93.70 mg/L. Kalyppo pineapple, Kalyppo guava, Pina juice, Maslenda pineapple juice, Lotti fruity, Kalyppo orange and Kalyppo apple also had levels of benzoic acid from 0.27 to 8.23 mg/L. The mean concentration was 31.00 mg/L which was below the mean concentrations recorded by Tfouni *et al*, 2002 in Brazil (533.6 mg/L) and Fernando *et al*, 2003, Porto, Portugal (165.5 mg/L). The standard deviation, the standard error of the mean and the coefficient of variation were 43.62, 6.32 and 140.70 respectively. The high percentage of coefficient variation from the 16 fruit juices was observed because the fruit juices were produced from different companies and therefore each company has its own production practices. Table 4.4 shows the mean concentrations of the fruit juices that were analysed.

There were no significant differences between levels of benzoic acid for kalyppo pineapple (KP), pina juice (PIJ), maslenda pineapple juice (MPJ), kalyppo guava (KG) and lotti fruity (LFTY). However, there was a significant difference between kalyppo fruitmix (KFM) and kalyppo pineapple (KP) and between 5 star apple juice (5SAJ) and Tampico (TAM) at $P < 0.05$. Most of the fruit juices with labels designated 'no preservatives' actually had some levels of benzoic acid in them with the exception of fruity (FTY) which had no detected levels of benzoic acid.

Table 4.3 Level of benzoic acid in fruit juices

Sample	Level of Benzoic acid (mg/L)			Mean(mg/L)	Standard Deviation
	1	2	3		
KP	0.3	0.2	0.3	0.27[a]	0.06
Pe	2.7	2.8	2.7	2.73[b]	0.06
FTY	nd	nd	nd	nd	nd
KFM	7.5	7.6	7.4	7.50[c]	0.10
5SSJ	60.6	62.25	59.4	60.75[e]	1.43
SUNF	95.25	93.15	92.7	93.70[h]	1.36
NAP	14.7	13.9	14.8	14.47[d]	0.49
PIJ	0.7	0.6	0.8	0.70[a]	0.10
MPJ	1.2	0.9	0.8	0.97[a]	0.21
LFTY	0.9	1.1	1	1.00[a]	0.10
KG	0.3	0.2	0.5	0.33[a]	0.15
5SAJ	148	134.5	141.5	141.33[i]	6.75
TAM	74.55	68.8	65.25	69.53[f]	4.69
FDG	87	86.2	85.6	86.27[g]	0.70
KA	8.4	8.4	7.9	8.23[c]	0.29
KO	8.5	7.8	8.2	8.17[c]	0.35

Numbers in rows followed by different superscript are significantly different at $P < 0.05$

Table 4.4 Descriptive statistics of fruit juices

Statistic	Number of samples used	Mean Conc of Benzoic acid (mg/L)	Variance	Standard deviation	SEM	CV (%)	Min. level of Benzoic acid	Max level of Benzoic acid.
Fruit juice	16	31.00	1902.62	43.62	6.32	140.70	nd	148

SEM = standard error of mean

CV = coefficient of variation

Min = minimum

Max = maximum

nd = not detected

Conc = Concentration

4.4 BENZOIC ACID IN HERBAL PRODUCTS

Twenty five (25) herbal products were analysed for the level of benzoic acid. The concentration levels ranged from 0.10 to 2004 mg/L (Table 4.6). Onyame ama mixture (OAM) recorded the highest concentration mean of 1932.33 mg/L while Tete wobika garlic bitters (TWGB) recorded the lowest concentration mean of 0.23 mg/L. The mean concentration for all the samples was 226.66 mg/L which was far below the mean concentration from herbal extract conducted by Khosrokhavar *et al*, 2010 carried out in Tehran, Iran (2312.7 mg/L). This could be due to the different herbs (trees and plants) used and the manufacturing processes since all the extracts from Khosrokhavar *et al* were produced from one company. The standard deviation, the standard error of the mean and the coefficient variation were 393.20, 45.20 and 173.47 respectively. The coefficient of variation was high because the 25 samples were from different manufacturing centers and there each manufacturing center has its way of preparation. Table 4.5 shows the mean concentrations of benzoic acid in the 25 analysed samples. There was no significant difference between

Ebetoda bitters (EB), Alafia bitters (AB), Kingdom garlic bitters, Kwasu ooko aduro (KOA), Spanish garlic bitters (SGB), Adom koo mixture (AKM) and Agbevo tonic (AT). However, there was a significant difference between Elephant herbal mixture (EHM) and Adutwumwaa bitters (ADB) and between Alafia bitters (AB) and Living bitters tonic (LBT) at P<0.05. All the labels on the herbal products did not include any preservative on it.

Table 4.5 Level of benzoic acid in herbal products.

Sample	Level of Benzoic acid (mg/L)			Mean(mg/L)	Standard Deviation
	1	2	3		
EHM	328.2	329.3	319.2	325.57^{k}	5.54
ADB	64.5	64.2	66.8	65.17^{d}	1.42
AB	0.3	0.3	0.35	0.32^{a}	0.03
YM	33	32	33	32.67^{b}	0.58
OAM	2004	1910	1883	1932.33^{p}	63.52
EB	0.8	0.8	0.85	0.82^{a}	0.03
LBT	53.9	53.65	54.1	53.88^{c}	0.23
TFS	285.3	284.5	286.1	285.30^{j}	0.80
KGB	0.9	0.8	0.4	0.70^{a}	0.26
DE	156.6	157.1	156	156.57^{g}	0.55
TWGB	0.3	0.1	0.3	0.23^{a}	0.12
FTM	248.3	247.2	234.41	243.30^{i}	7.72
ADT	102.5	97.9	98.4	99.60^{e}	2.52
PHB	429	441	402	424.00^{m}	19.97
AWGM	373	378.3	380.8	377.37^{l}	3.98
THM	701.2	689.8	683.5	691.50^{n}	8.97
NB	385	381	380	382.00^{l}	2.65
KOA	1.6	0.9	1.4	1.30^{a}	0.36
SGB	2	1.6	1.1	1.57^{a}	0.45
AKM	2.2	1.3	1.6	1.70^{a}	0.46
TIHM	266.6	249.2	227.5	247.77^{i}	19.59
AHM	117.4	119.9	117.2	118.17^{f}	1.50
AT	1	1	1.1	1.03^{a}	0.06
MCT	0.5	0.6	0.5	0.53^{a}	0.06
FKB	226.3	223.8	219.2	223.10^{h}	3.60

Numbers in rows followed by different superscript are significantly different at P<0.05

Table 4.6 Descriptive statistics for herbal prepared products

Statistic	Number of samples used	Mean Conc of Benzoic acid (mg/L)	Variance	Standard deviation	SEM	CV (%)	Min. level of Benzoic acid.	Max. level of Benzoic acid
Herbal product	25	266.66	154603.64	393.20	45.20	173.47	0.10	2004

SEM= standard error of mean

CV= coefficient of variation

Min= minimum

Max= maximum

Conc = Concentration

A visit to the herbal department, KNUST showed that there is no standard limit set for the herbal prepared products for benzoic acid or its salts as a preservative. However, during interactions with the herbalists and herbal products producers, they have been advised to use 0.1% of the benzoic acid during preservation. The high concentrations of benzoic acid are shown because these locally manufacturers do have standard laboratories and therefore just fetch any amount during their preparations and therefore fetching more mean longer preservation since this preservative is not costly.

COMPARISON OF THE THREE PRODUCTS

Fig. 4.1 shows the mean concentrations of benzoic acid in the fruit juices, herbal products and flavoured carbonated drinks (soft drinks) analysed. The concentration of product varies and therefore there was a significant difference between the three products analysed at $P < 0.05$. The coefficient of variation for fruit juice, herbal products and soft drinks were 140.70%, 173.47% and 169.52% respectively. This shows that the precision for the analysis of fruit juices was better than the soft drink analysis and herbal analysis.

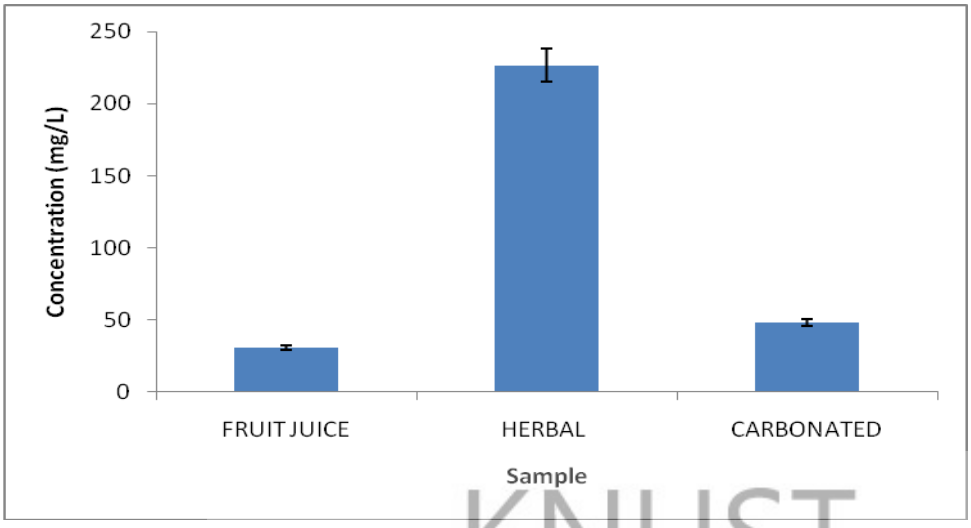

Figure. 4.1 Mean concentrations of herbal, fruit juice and carbonated drinks

4.5 BENZENE DETERMINATION

All the fruit juices and soft drinks were analysed for benzene. All the samples analysed showed no detection for benzene. This means there was no benzene in the samples or the benzene levels were below the detection limit of the HPLC (Table 4.9). This can also be to the sensitivity of the HPLC equipment since all work in the literature available used the head space GC-MS and GC – FID.

4.6 ANALYTICAL METHODS

The precision of the method was also evaluated (Table 4.8). The coefficient of variation of 3.11% was found, which not only indicate the high reproducibility of the method but also indicates that the preservative is stable under the conditions of the measurement.

Table 4.7 Reproducibility of benzoic acid

Statistic	Number screened	Mean Conc of Benzoic acid (mg/L)	Median Conc of Benzoic acid (mg/L)	Variance	Standard deviation	SEM	CV (%)	Min. level of Benzoic acid.	Max. level of Benzoic acid
Standard	6	26.43	26.74	0.68	0.82	0.34	3.11	25.08	27.17

SEM= standard error of mean

CV= coefficient of variation

Min= minimum

Max= maximum

Conc = Concentration

BA = Benzoic acid

The recovery for benzoic acid ranged from 107% to 110% (Table 4.9).

Table 4.8 Results for recoveries of spiked standards to various samples

Sample	Mean recovery (%)
Ebetoda bitters	107.8 ± 2.60
Alafia bitters	109.28 ± 1.13
Kalyppo guava	108.88 ± 4.30
Pina juice	110.98 ± 0.68
Malta Guinness	110.20 ± 0.85
Coca cola	107.68 ± 2.60

The detection limit for benzoic acid and benzene was 0.03 mg/L and 0.06 mg/L respectively and the limit of quantification was also 0.10 mg/L for benzoic acid and 0.2 mg/L for benzene (Table 4.10).

Table 4.9 Analytical characteristics of HPLC method

Parameter	Benzoic acid	Benzene
Limit of detection (mg/L)	0.03	0.06
Limit of quantification (mg/L)	0.10	0.20

4.7 ESTIMATED DAILY INTAKE OF BENZOIC ACID

Table 4.10 shows the estimated daily intake of benzoic acid from the soft drinks and fruit juices analysed. Since there is no data on the consumption per capita of soft drinks and fruit juices in the country, the codex (2012) report on the consumption of soft drinks per day and a report by the Business and Financial Times, a News Paper in the country was used in the estimations. The total estimate of intake of benzoic acid for soft and fruit drink for adults and children was 0.19 and 1.14 mg/L respectively. Soft drinks contributed substantially to the intake of benzoic acid or benzoates with 0.19 mg/L for adults and 1.14 mg/L for children. The intake of benzoic acid for fruit juice was 0.00072 and 0.0043 mg/L for adults and children respectively. This also shows that the intake of benzoic acid is higher in soft drinks than fruit juices. This is because most of the fruit juices producers use pasteurization as the method in preserving their products. The low level of the per capita consumption of fruit juice shows that Ghanaians do not take in much of fruit juice. This may be due to the abundance of natural fruits in the country. The potential daily intake of benzoic acid calculated are within the range of 0 – 5 mg/L body weight per day by JECFA and WHO, 1987. The estimated daily intake of benzoic acid calculated for the soft drink and fruit juice

was below that of Tfouni and Toledo, 2002 for the same product (soft drinks and fruit juice) who conducted a survey of the daily estimates of benzoic acid in Brazilian foods.

Table 4.10 Estimated daily intake (EDI) of benzoic acid

Food category	Analytical concentration (mg/L)	Consumption product (mg/person day^{-1})	EDI[a] (mg/L)	EDI[b] (mg/L)
Soft drink	70.20	0.162	0.19	1.14
Fruit juice	31.00	0.0014	0.00072	0.0043
		Total	0.19072	1.1443

[a] Average body weight (adult) = 60 kg

[b] Average body weight (children) = 10 kg

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

This work is based on a simple, selective and rather fast HPLC method for the determination of the levels of benzoic acid in soft drinks, fruit juices and herbal product in Ghana. The results showed that benzoic acid is a common preservative used by producers in the non-alcoholic industry and in herbal medicine and that the benzoic acid concentration varied between different kinds of soft drink, fruit juices and herbal products. The herbal products had very high levels of benzoic acid. Six of the soft drinks samples; Hita orange flavour, Klassy orange flavour, Hita cola flavor, Klassy cola, Anabe fun cola and Royal orange flavour had levels higher than the maximum values established by national and international legislation. Their mean level of benzoic acid were 548.00 mg/L, 317.67 mg/L, 248.87 mg/L, 239.83 mg/L, 231.67 and 201.07 respectively.

The products that declared "no preservatives" especially with the fruit juices were not in accordance with their label claims. Lotti fruity, Maslenda pineapple juice and Pinea juice showed small levels of benzoic acid (1 mg/L, 0.97 mg/L and 2.73 mg/L respectively). The highest level of benzoic acid concentration among the sampled fruit juices was 141.33 mg/L which was recorded in 5 Star apple juice.

In the case of the herbal products, all the samples showed detections of benzoic acid. All the labels on the herbal products did not have benzoic acid as part of the stipulated ingredients or as a preservative or additive. Although the labels on the herbal products did not have benzoic acid as part of the stipulated ingredients, they showed high levels of benzoic acid as much as 2004 mg/L in one of the herbal products. The total estimate of intake of benzoic acid for soft and fruit drinks for adults and children was 0.19 and 1.14 mg/L, for the fruit juices it was 0.0072 mg/L for the adults and 0.0043 mg/L for the children. These values were low and

within the range of 0 – 5 mg/L body weight per day by JECFA and WHO, 1987. Soft drinks contributed substantially to the intake of benzoic acid or benzoates for adults and children than the fruit juices by Ghanaians. None of the soft drinks and fruit juices analysed showed levels of benzene, and are safe for consumption.

5.2 RECOMMENDATIONS

1. The use of benzoic acid and benzoate should be regulated and used only as a means to control yeast and bacteria at concentrations not exceeding the actual need. For products like Hita orange flavour, Klassy orange flavour, Hita cola flavor, Klassy cola, Anabe fun cola and Royal orange flavour whose level of benzoic acid exceeded the legislation limit, it is recommended that whenever possible the addition of additives be refined by a more precise approach using analytical data by the manufacturers to ensure that the right amount is used.
2. The manufacturers should also consult or employ the chemist to help them at the production stage.
3. For the herbal manufacturers since there is no documentation on the limit of preservatives to use, the Food and Drugs Board and the Ghana Standards Authority should educate the local producers on how to use the benzoic acid (benzoates) in their products. They should be educated that not too much of the preservative is needed to preserve their products. This is important because some of the herbal products are for the treatment of asthma and if this benzoic acid or the benzoates is more it could lead to worsening the situation of the patient.
4. The government and for that matter the Food and Drugs and the Ghana Standards Authority should have a routine check up on the various soft drinks, fruit juices, herbal products and other consumable products on the market to check the level of

this preservative and other preservatives to check whether their levels are within the stipulated limits.

5. Also this study was on the determination of benzoic acid only, other preservatives like sorbic acids and parabens can also be determined simultaneously. With the benzene determination, the head space GC-MS should be tried if available

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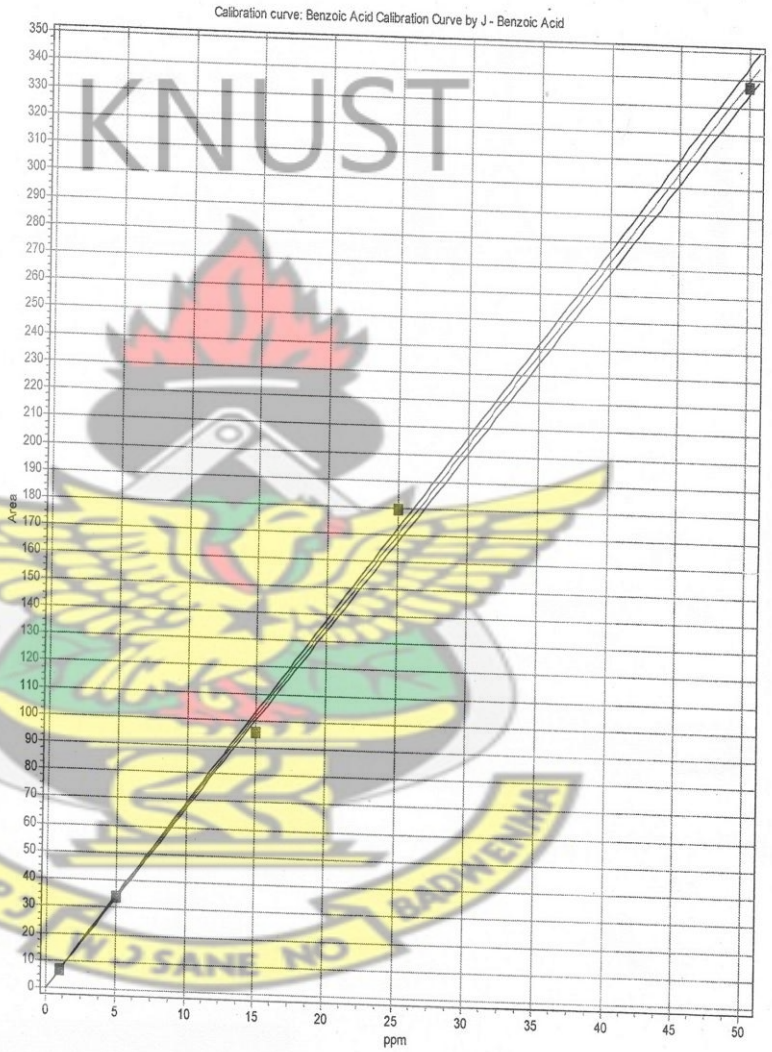
APPENDICES

APPENDIX A Calibration Curve

Calibration Report :
File : Benzoic Acid Calibration Curve by J

Component : Benzoic Acid
Polynom : y = b x +a
a = 0
b = 6.79887
Correlation Coef. : 0.9980
Weighting : None
Force zero : Yes



Calibration table : Benzoic Acid

#	Used	Name	ppm	Area	RF	Chromatogram	Date	Area (recalc)	Res %	time [Min]	Level
1	1	Point 1	1.00	6.45	0.15	Std 1 ppm_14_07_2012 16_43_08 (1)	16/08/2012 12:16:39	6.80	5.35	7.33	1
2	1	Point 2	5.00	34.02	0.15	std 5 ppm_09_07_2012 14_09_53 (1)	16/08/2012 12:17:29	33.99	0.07	7.52	2
3	1	Point 3	15.00	95.44	0.16	std 15 ppm (1)	16/08/2012 12:17:55	101.98	6.86	7.51	3
4	1	Point 4	25.00	179.41	0.14	std 25 ppm (1)	16/08/2012 12:18:08	169.97	5.26	7.51	4
5	1	Point 5	50.00	337.19	0.15	std 50 ppm (1)	16/08/2012 12:18:21	339.94	0.82	7.46	5

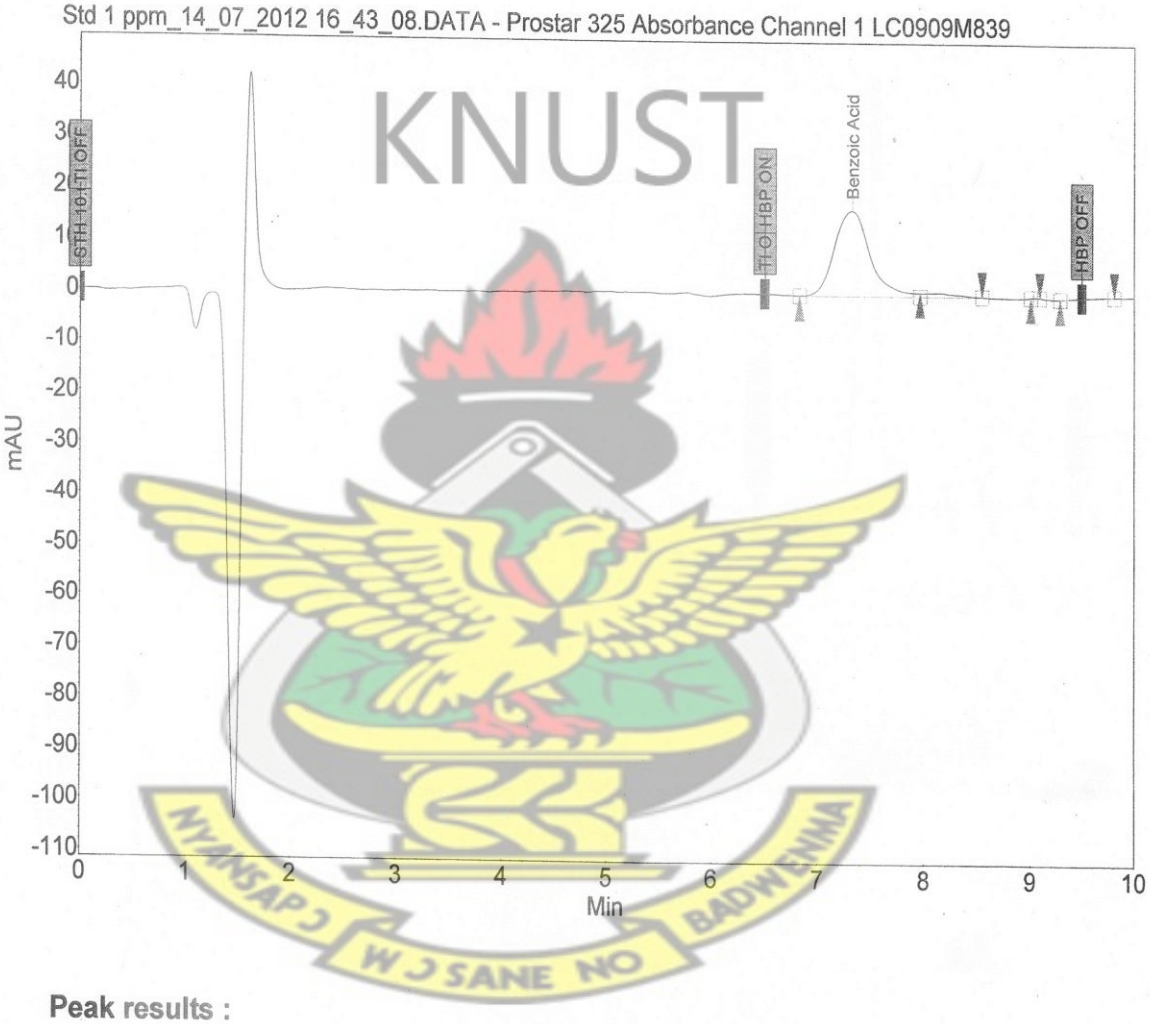
APPENDIX B Benzoic Acid Standards

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 14/07/2012 16:43:31
Processed : 16/08/2012 12:16:39
Printed : 16/08/2012 13:03:36

Std 1 ppm_14_07_2012 16_43_08.DATA - Prostar 325 Absorbance Channel 1 LC0909M839



Peak results :

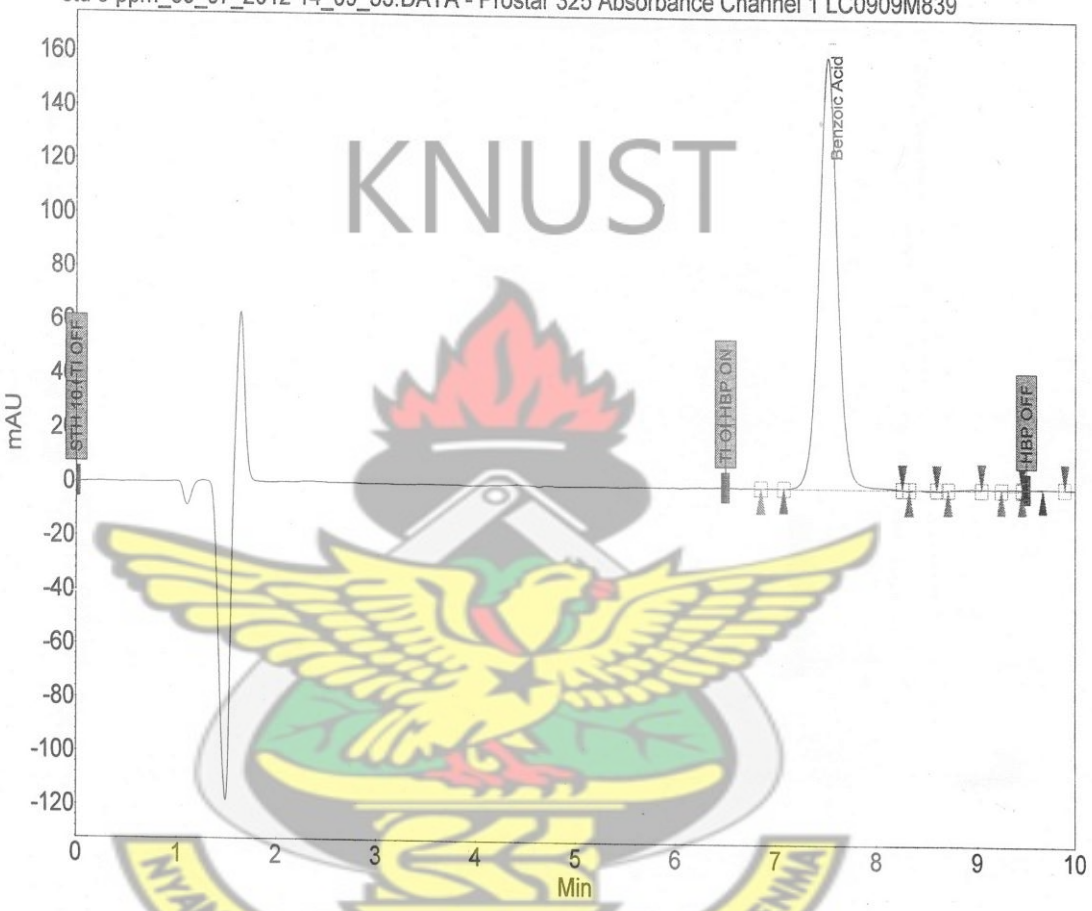
Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	Benzoic Acid	7.33	1.00	16.5	6.5	95.262
Total			1.00	17.5	6.8	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 09/07/2012 14:10:14
Processed : 16/08/2012 12:17:29
Printed : 16/08/2012 13:15:00

std 5 ppm_09_07_2012 14_09_53.DATA - Prostar 325 Absorbance Channel 1 LC0909M839



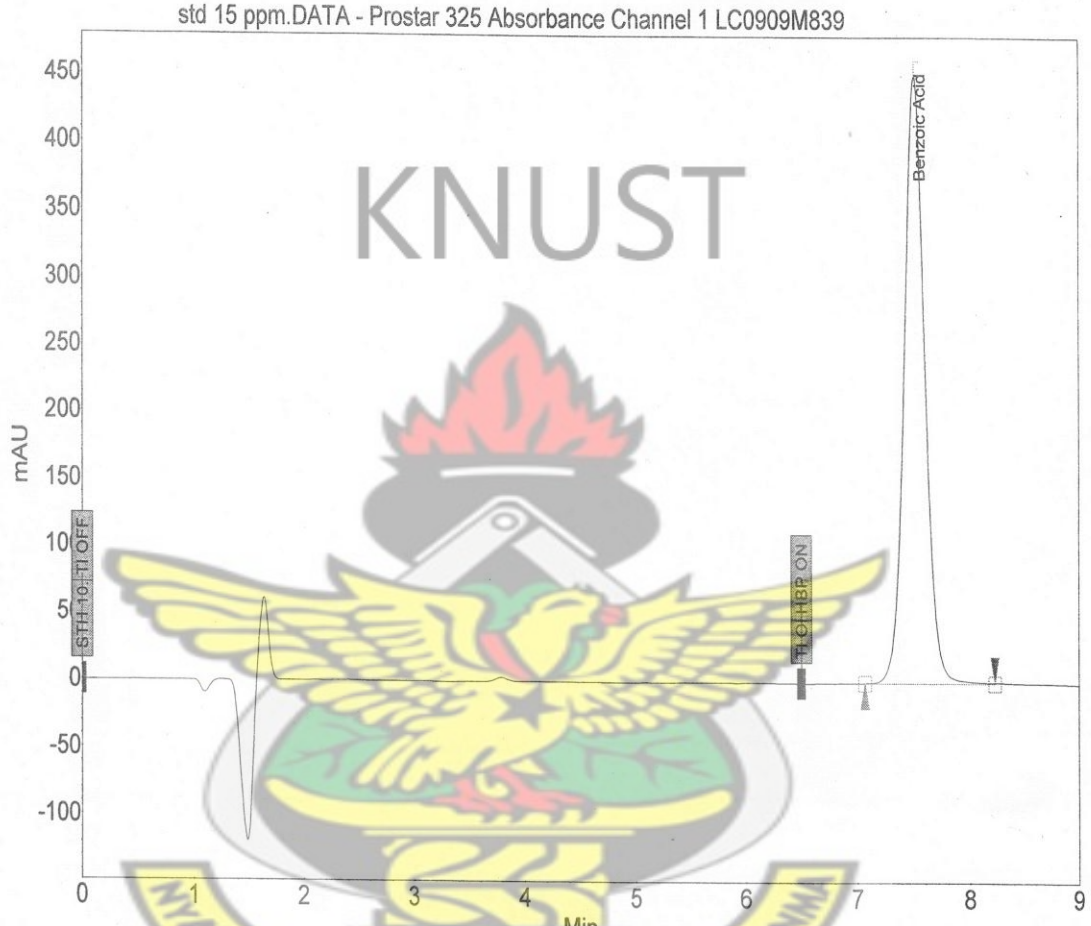
Peak results :

Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
2	Benzoic Acid	7.52	5.01	160.1	34.0	99.034
Total			5.01	162.0	34.3	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 09/07/2012 14:21:50
Processed : 16/08/2012 12:17:55
Printed : 16/08/2012 13:01:29



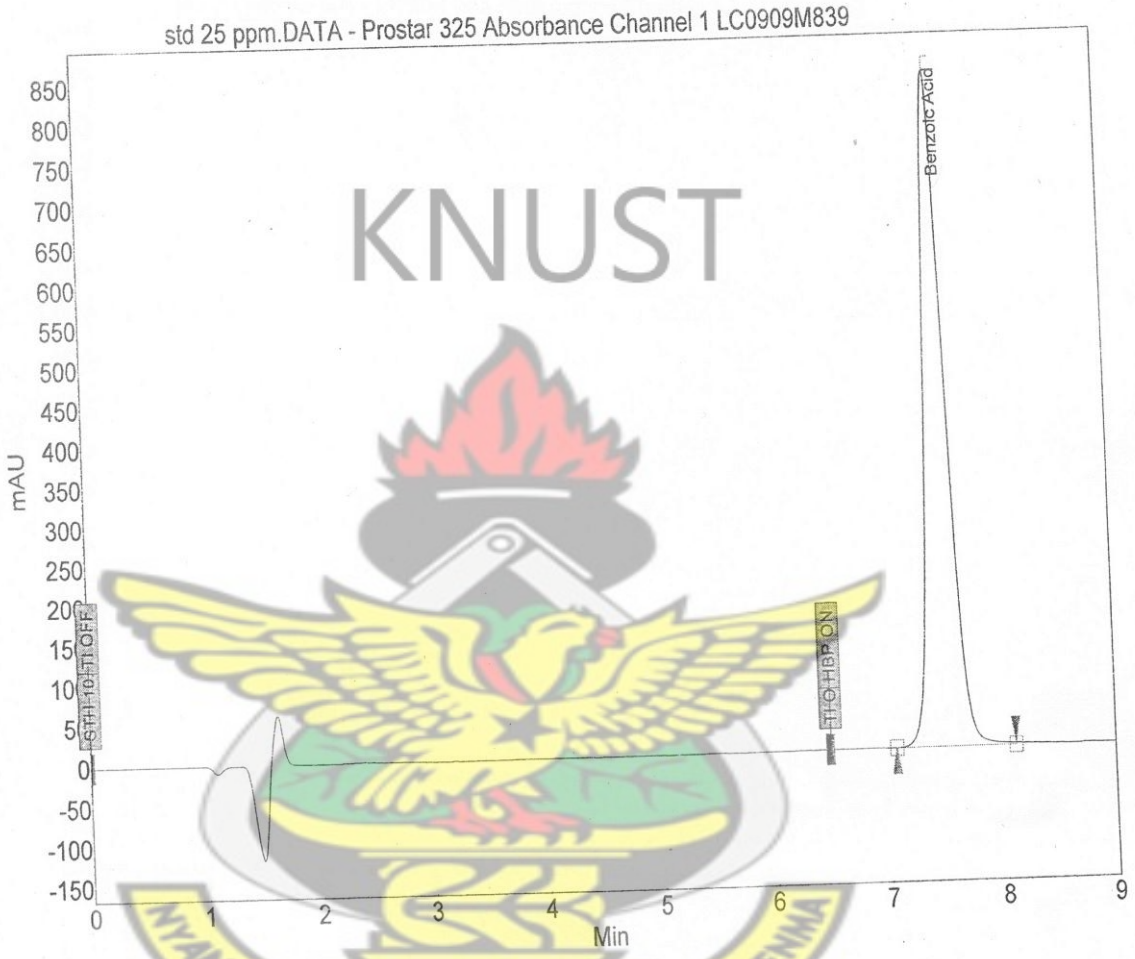
Peak results :

Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	Benzoic Acid	7.51	14.90	450.6	95.4	100.000
Total			14.90	450.6	95.4	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 09/07/2012 14:33:28
Processed : 16/08/2012 12:18:08
Printed : 16/08/2012 13:01:34



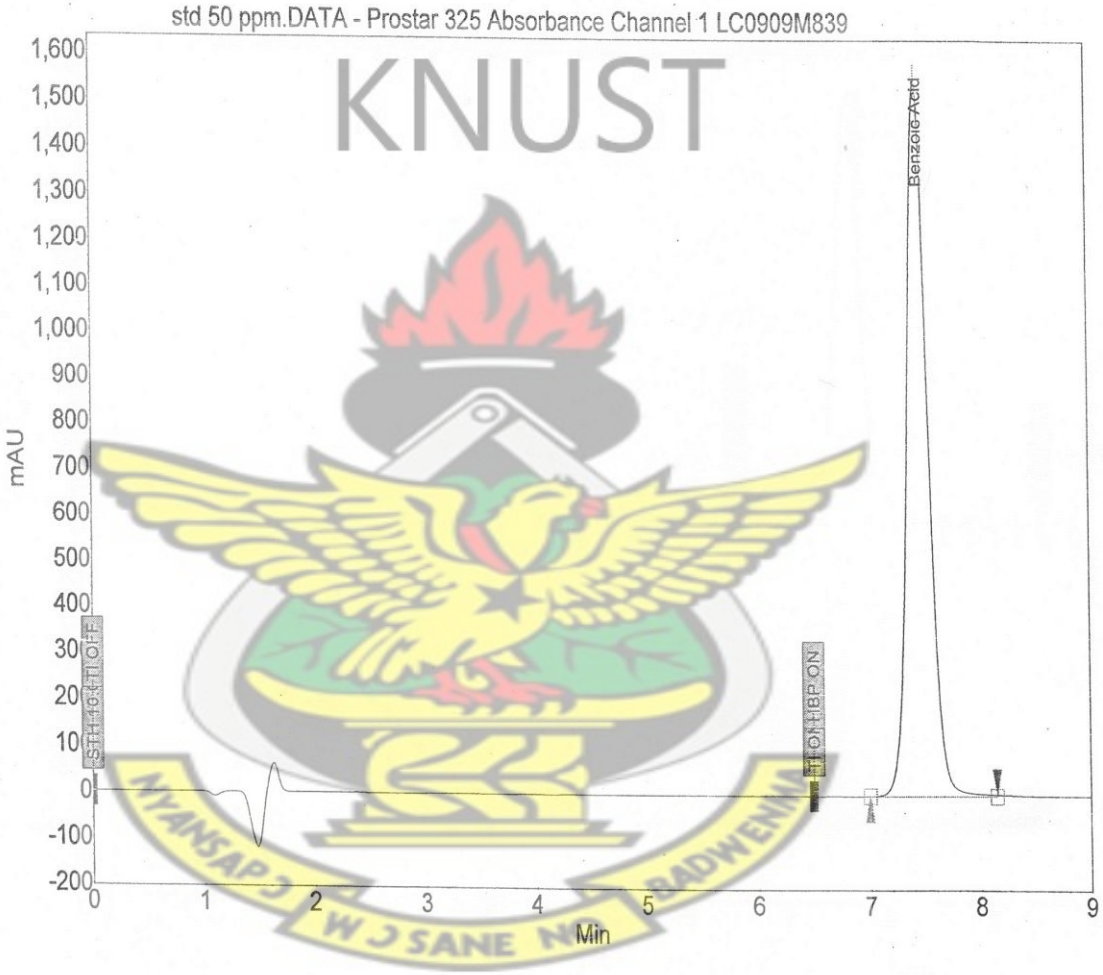
Peak results :

Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	Benzoic Acid	7.51	25.79	845.5	179.4	100.000
Total			25.79	845.5	179.4	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 09/07/2012 14:44:04
Processed : 16/08/2012 12:18:21
Printed : 16/08/2012 13:01:38



Peak results :

Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	Benzoic Acid	7.46	49.60	1551.6	337.2	100.000
Total			49.60	1551.6	337.2	100.000

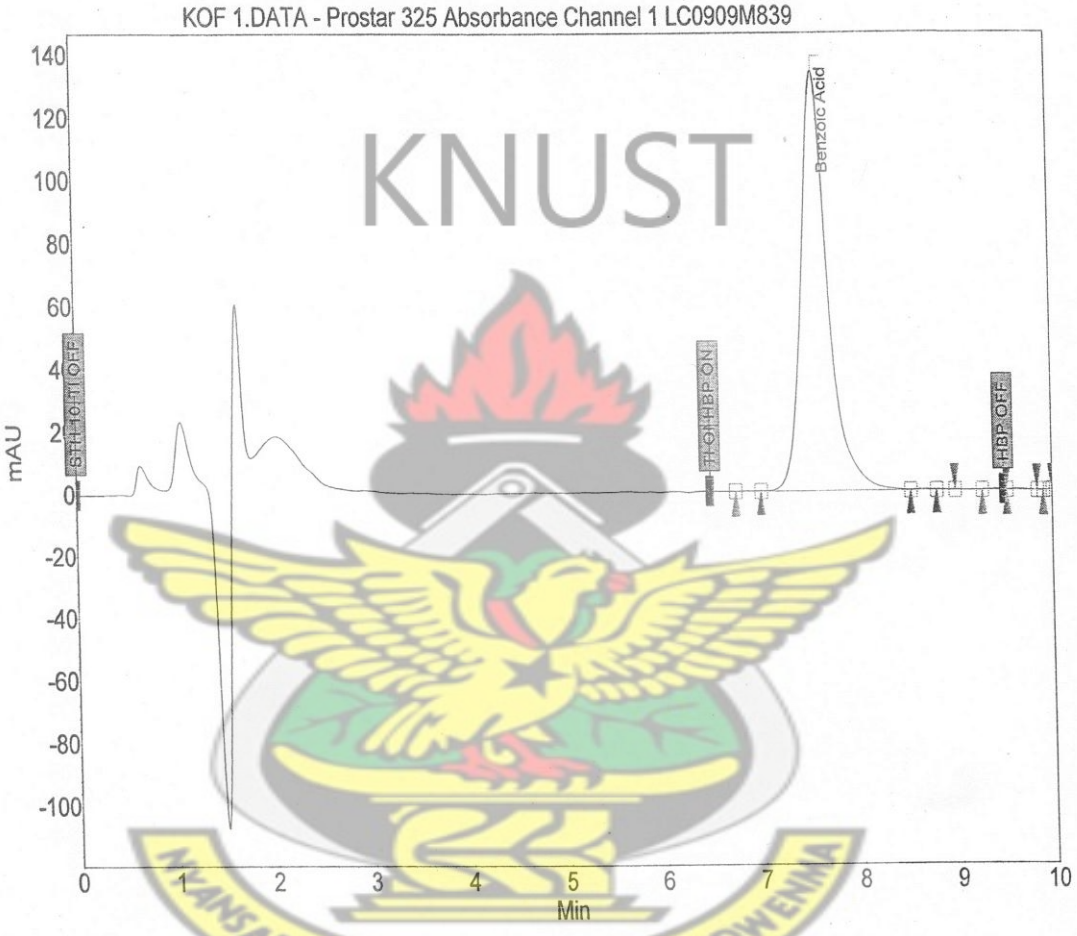
APPENDIX C Chromatogram of samples

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 12/07/2012 14:58:30
Processed : 16/08/2012 13:05:16
Printed : 16/08/2012 13:08:34

KOF 1.DATA - Prostar 325 Absorbance Channel 1 LC0909M839



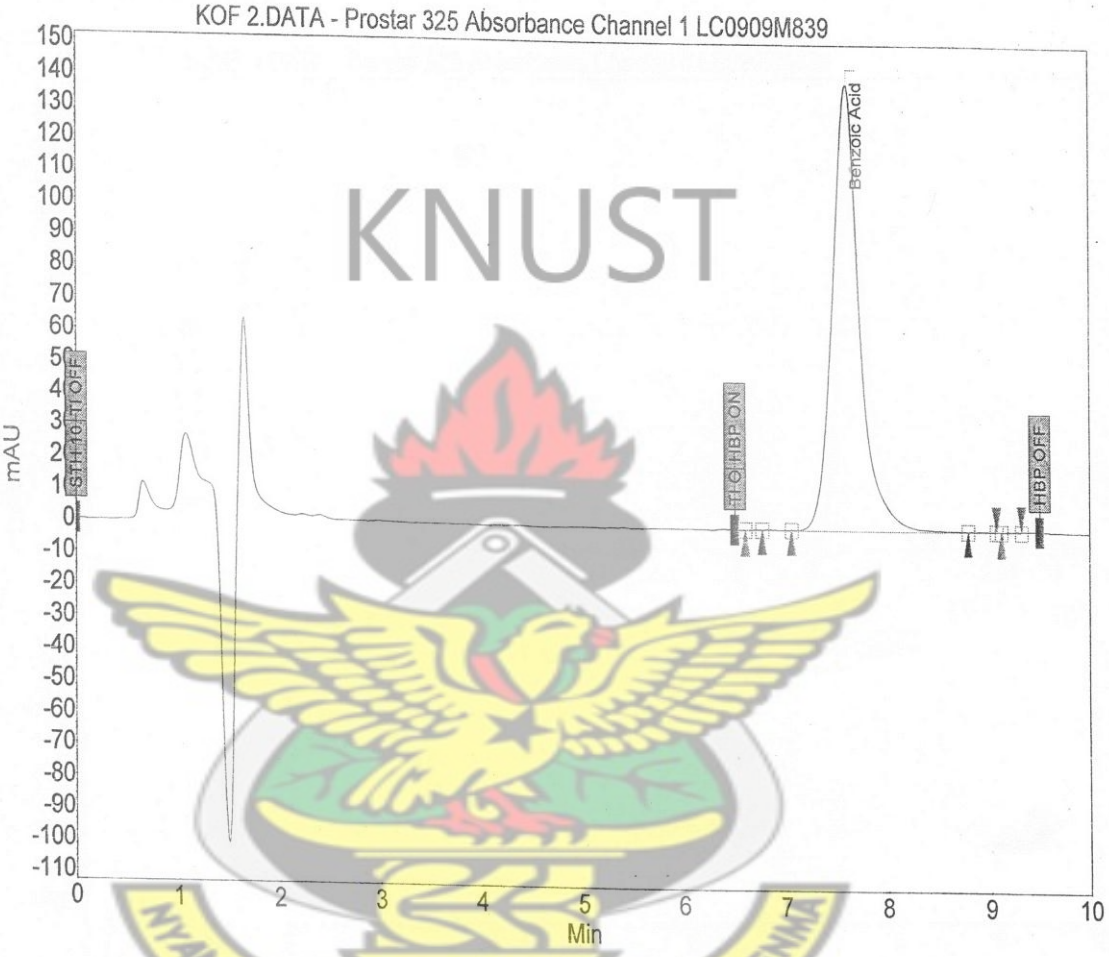
Peak results :

Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
2	Benzoic Acid	7.61	6.11	133.8	41.6	99.660
Total			6.11	134.8	41.7	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 12/07/2012 15:10:36
Processed : 16/08/2012 13:05:18
Printed : 16/08/2012 13:08:32



Peak results :

Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
3	Benzoic Acid	7.61	6.43	139.7	43.7	99.467
Total			6.43	141.1	43.9	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

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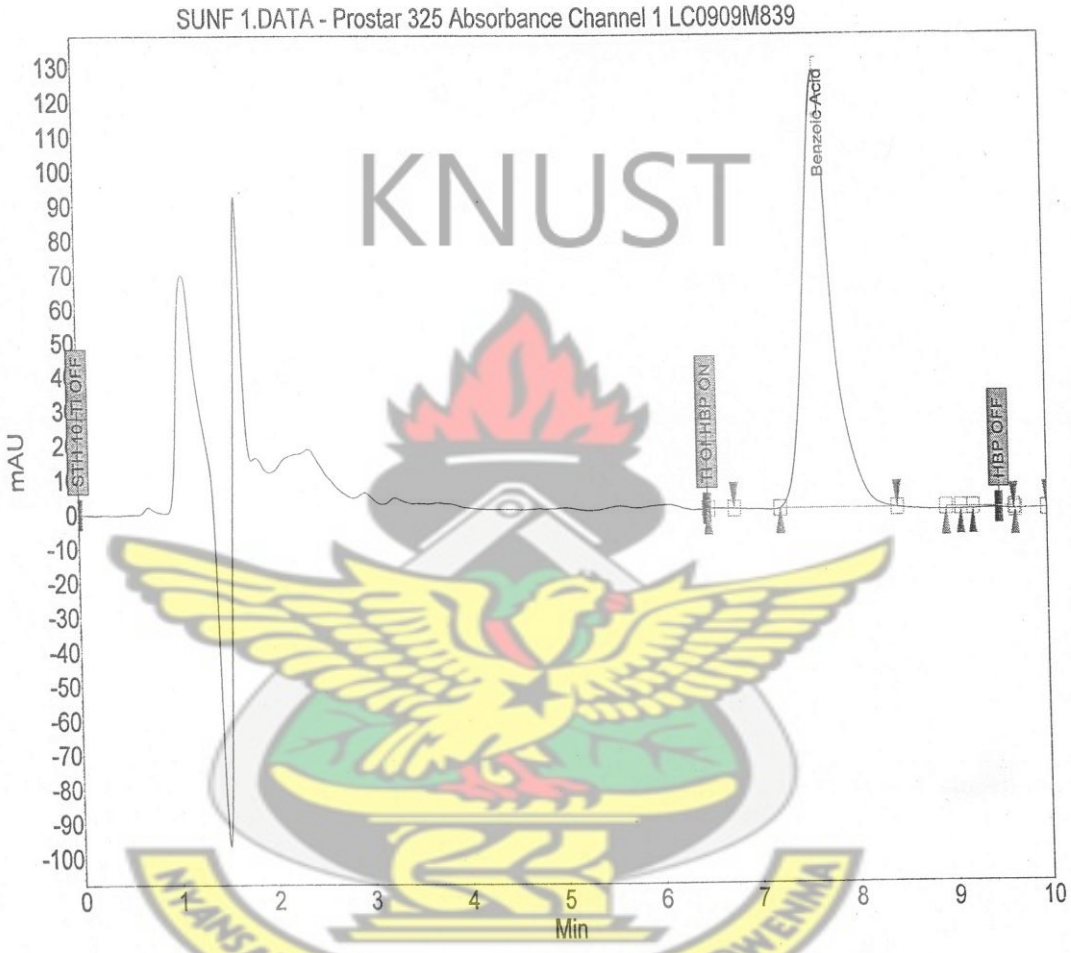
Processed : 16/08/2012 13:05:39
Printed : 16/08/2012 13:08:16



Peak results :

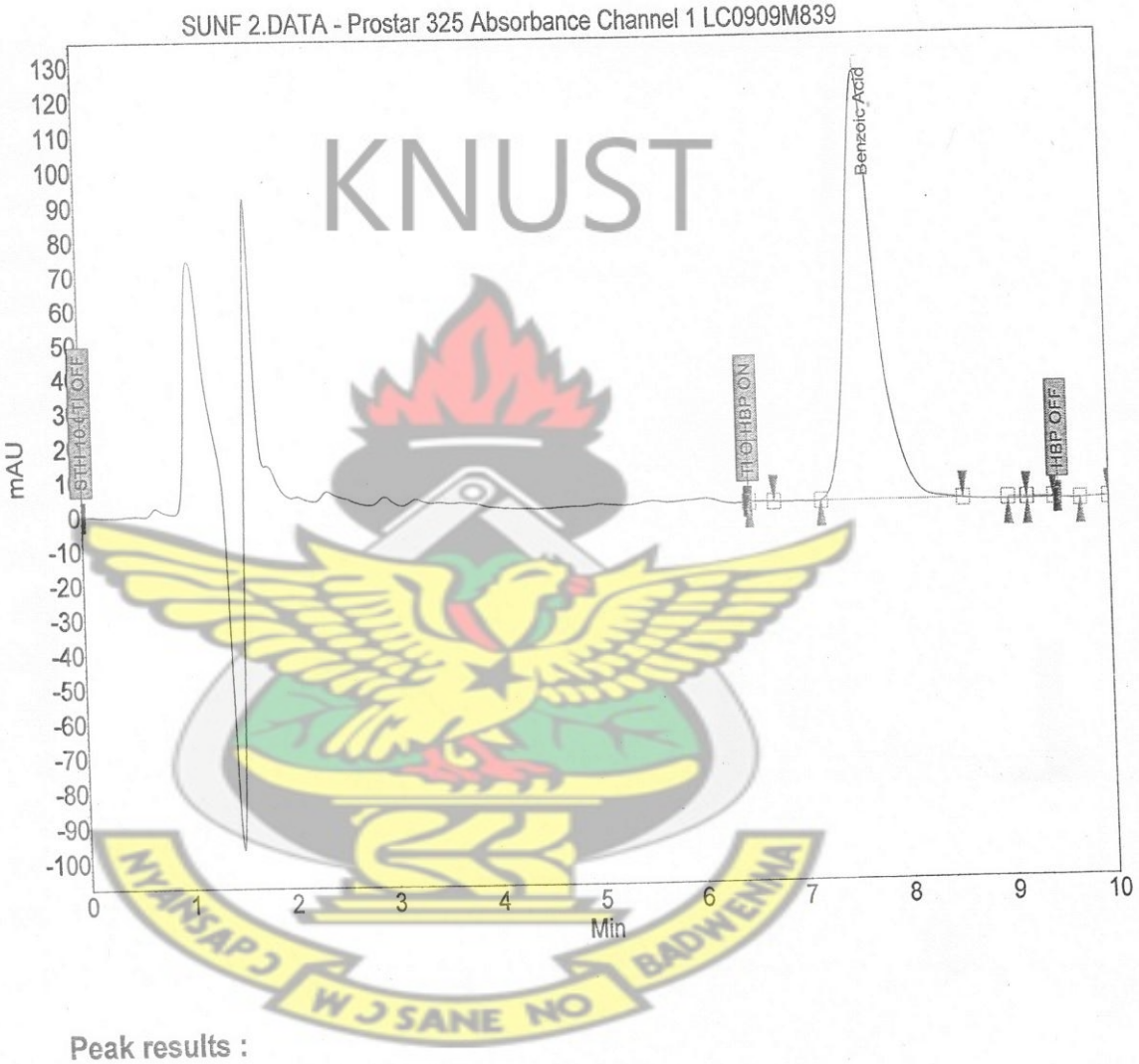
Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
2	Benzoic Acid	7.66	6.18	126.6	42.1	98.873
Total			6.18	129.1	42.5	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 12/07/2012 19:08:58
Processed : 16/08/2012 13:05:42
Printed : 16/08/2012 13:08:11

SUNF 2.DATA - Prostar 325 Absorbance Channel 1 LC0909M839



Peak results :

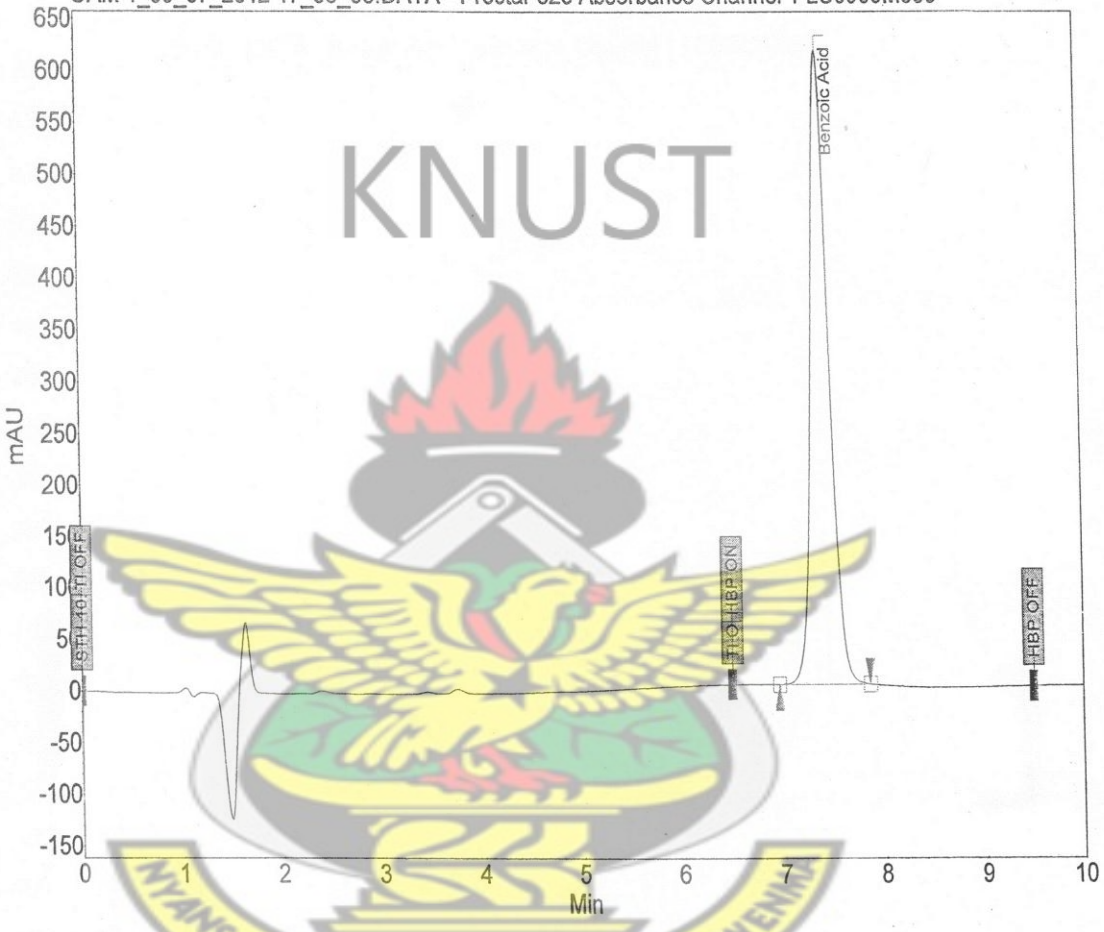
Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
2	Benzoic Acid	7.64	6.21	123.9	42.2	99.400
Total			6.21	125.4	42.5	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : Elvis Baidoo

Acquired : 09/07/2012 17:53:58
Processed : 16/08/2012 13:04:52
Printed : 16/08/2012 13:09:24

OAM 1_09_07_2012 17_53_36.DATA - Prostar 325 Absorbance Channel 1 LC0909M839



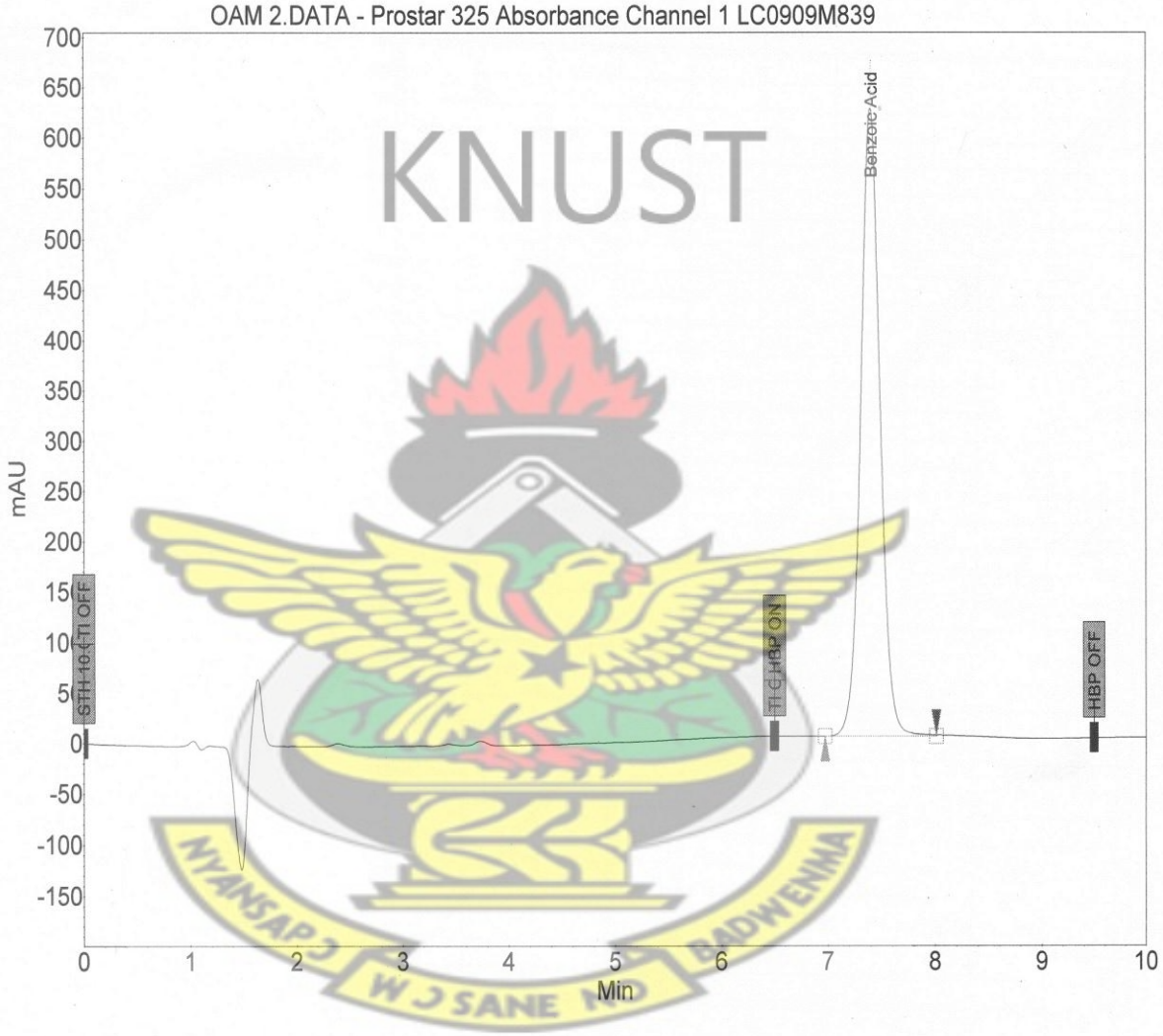
Peak results :

Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	Benzoic Acid	7.41	18.83	612.6	128.0	100.000
Total			18.83	612.6	128.0	100.000

Kwame Nkrumah University of Science & Technology

System : KNUST HPLC
Method : Kusi Benzoic Acid
Work by: Kusi James
User : ELvis Baidoo

Acquired : 09/07/2012 18:05:20
Processed : 16/08/2012 12:22:57
Printed : 16/08/2012 12:33:48



Index	Name	Time [Min]	Quantity [ppm]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	Benzoic Acid	7.40	19.10	622.4	129.9	100.000
Total			19.10	622.4	129.9	100.000

APPENDIX D Names of Coding

SAMPLE AND CODE

Herbal Prepared Products

SAMPLE	CODE
Elephant Herbal Bitters	EHM
Adutwumwaa Bitters	ADB
Alafia Bitters	AB
Yaakson Mixture	YM
Onyame Ama Mixture	OAM
Ebetoda Bitters	EB
Living Bitters tonic	LBT
Top Fever Syrup	TFS
Kingdom Garlic Bitters	KGB
Diagellates Elixir	DE
Tete Wobika Garlic Bitters	TWGB
Fralena Tafoos Mixture	FTM
Adutwumwaa Tonic	ADT
Adom W&G Mixture	AWGM
Pomaah Herbal Mixture	PHB
Taabea Herbal Mixture	THM
Navina Bitters	NB
Kwasu Ooko Aduro	KOA
Spanish Garlic Bitters	SGB
Adom Koo Mixture	AKM
Time Herbal Mixture	TIHM
Angel Herbal Mixture	AHM
Agbevo Tonic	AT
Madam Catherine Tonic	MCT
Fralena Koo Bitters	FKB

FRUIT JUICES

SAMPLE	CODE
Kalyppo pineapple	KP
Pinea juice	Pe
Fruity	FTY
Kalyppo fruitmix	KFM
5 star strawberry juice	5SSJ
Sunfruits	SUNF
New age pineapple	NAP
Pina juice	PIJ
Maslenda pineapple juice	MPJ
Lotti fruity	LFTY
5 star apple juice	5SAJ
Tampico	TAM
Fandango	FDG
Kalyppo guava	KG
Kalyppo apple	KA
Kalyppo orange	KO

SOFT DRINKS

SAMPLE	CODE
Club orange	CO
Alvaro pineapple	ALP
Africa cola	AFC
B-cool	B-C
Master cai pineapple	MCP
Tasty orange flavour	TOF
Decco pineapple flavour	DPF
Vika cola	VC
Sprite	SP
Anabe fun orange	AFO
Fanta	FTA
Klassy orange flavour	KOF
Club soda	CS
Decco cola	DC
Anabe fun cola	ANFC
Orange vika	OV
Hita orange flavour	HOF
Royal orange flavour	ROF
Kalah cool	KAC
Africa fun orange	AFFO
Fresh orange flavour	FOF
Hita cola flavour	HICF
Vanda cool	VANC
Klassy cola	KC
Alvaro passion fruit	ALPF
Coca cola	CC
U-fresh coke	UFC
Sil mixed fruit	SMF
Africa fun limonade	AFFL
Club muscatella	CM
Master cai cocktail	MCC
Alvaro pear	ALPE
Malta guinness	MG
Fresh cola flavour	FCF