Determination of Safrole and Isosafrole from soft drinks using Gas Chromatogram

A thesis submitted to the department of pharmacy, East West University in the partial fulfillment of the requirements for the Degree of Bachelor of Pharmacy

Submitted by:
SHIFA- AT- ARA
I.D: 2008-3-70-094

JULY, 2012

Under the Guidance of
Mr. Md. Amran Howlader
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This Gas Chromatography analysis study was done in the Advance Research Lab of East West University

Thesis Certificate

This is to certify that the thesis entitled “Determination of Safrole and Isosafrole from soft drinks using Gas Chromatogram Method” submitted to the Department of Pharmacy, East West University in the partial fulfillment of the requirements for the Degree of Bachelor of Pharmacy, is the outcome of the investigations performed by Shifa-At-Ara, ID: 2008-3-70-094 under the supervision of Mr. Md. Amran Howlader, Senior Lecturer, Department of Pharmacy, East West University and that no part of thesis has been submitted for any other degree.

My honorable Chairperson, Dr. Sufia Islam always supports me and facilitates me all the time with the required tools and support.

Dr. Sufia Islam
Chairperson
Department of Pharmacy
East West University

Thesis Certificate

This is to certify that the thesis entitled “Determination of Safrole and Isosafrole from soft drinks using Gas Chromatogram Method” submitted to the Department of Pharmacy, East West University in the partial fulfillment of the requirements for the Degree of Bachelor of Pharmacy, is the outcome of the investigations performed by Shifa-At-Ara, (ID: 2008-3-70-094) under the supervision of Mr. Md. Amran Howlader, Senior Lecturer, Department of Pharmacy, East West University and that no part of thesis has been submitted for any other degree. We further certify that all the sources of information availed of this connection are duly acknowledged.

Mr. Md. Amran Howlader
Supervisor
Senior Lecturer
Department of Pharmacy
East West University

DEDICATION

This paper is dedicated to my parents and my only brother.

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LIST OF ABBREVIATIONS

AOAC: Association of Official Agricultural Chemists

HPLC: High Performance Liquid Chromatography

MS: Mass Spectroscopy

DHB: 1, 4-dihydroxybenzene

GC: Gas Chromatography

FID: Flame Ionization Detector

MDMA: 3, 4-methylenedioxy-*N*-methylamphetamine

MDA: 3, 4-Methylenedioxyamphetamine

MDE: Methylenedioxyethylamphetamine

HCl: Hydrochloric Acid

SCEs: Sister Chromatid Exchanges

UDS: Induced Unscheduled DNA Synthesis

CAS: Chemical Abstracts Service

FAO: Food and Agriculture Organization

WHO: World Health Organization

JECFA: Joint FAO/WHO Expert Committee on Food Additives

CEFS: Council of Europe Committee of Experts on Flavoring Substances

$LD_{50:}$ Median Lethal Dose

ISO: International Organization for Standardization

LC: Liquid Chromatographic

LOD: Limits of Detection

LOQ: Limits of Quantitation

SFE: Supercritical Fluid Extraction

WCOT: Wall-Coated Open Tubular

SCOT: Support-Coated Open Tubular

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July, 2012 **Shifa- At- Ara**

Author

ABSTRACT

The aim and objective of this study is to determine the presence and concentration of safrole and isosafrole in different commercially available soft drinks to ensure the good health and safety of our life. Gas chromatogram technique was used to determine the presence of those compounds and measure their concentration. For this reason, a simple and rapid method was developed to determine the safrole and isosafrole in soft drink using a semi-polar column (RT_X - 625) with direct injection gas chromatography. Direct quantitative analysis of safrole and isosafrole in soft drinks was carried out to determine their presence and concentration. The water soluble compound 1, 4-dihydroxybenzene (DHB) was used as the internal standard. Seven commercial soft drinks were analyzed by the current method, and results indicated that these seven commercial soft drink samples contained safrole, 0.39-.55 µg/ml safrole was detected, and most of them contained high level of safrole. Isosafrole was unable to detected using this method probably for its presence as derivatives.

Key words: **Soft drink, Safrole, Isosafrole, Direct injection, Gas Chromatography, Quantitative analyses, DHB**

CHAPTER - 1

Introduction

1.1. Introduction

Safrole also known as shikimol, is a phenylpropene. Safrole is one of the natural components of refined oils in more than fifty kinds of vegetables. Many of them can be made into seasonings and are one of the major components of essential oils, such as sassafras, camphor, nutmeg, black pepper, sweet basil, mace, cinnamon, anise and piper betle flower (Choong and Lin, 2000). The most important dietary sources are nutmeg, mace and their essential oils. Safrole is a precursor chemical of ring-substituted amphetamine-type substances such as MDMA or Ecstasy, MDA, MDE. Safrole and isosafrole (S-isomer of safrole) were once used extensively as a seasoning in soft drinks (Khlaeo-om, Identification of safrole). For example, Sarsaparilla and Coke have used sassafras oil as seasoning which contained nearly 85% safrole and isosafrole (Choong and Lin, 2000). Intake estimates of flavoring substances are generally very poor because of the lack of data on the concentrations of these chemicals naturally occurring or voluntarily added in foodstuffs. Within the Council of Europe, UK and France provided calculations based on their respective food consumption data and on concentration levels documented or assumed (Borchert *et al*., 2012).

Since safrole and isosafrole are carcinogens, adding sassafras oil in soft drinks has been prohibited in the US since 1970, while it was defined as a kind of food additive and treated as a special element of seasonings in the Republic of China. However, it can be used only in soft drinks with the use limit below 1μg/mL, according to food additive regulations. Safrole and isosafrole were once used extents have a connection with inductive liver tumors. A high concentration (0.5%) of safrole and isosafrole has been shown to increase the occurrence rate of malignant tumors in mice Safrole or 4-allyl-l; 2-methylenedioxybenzene is a plant constituent that is a hepatotoxin for animals and man.

The major toxicity of safrole and isosafrole come from their carcinogenic nature after oxidation. Safrole is oxidized into1-hydroxysafrole by many mammals and whose derivatives including isosafrole and dihydrosafrole, which are all both carcinogenic (Choong and Lin, 2000).

1.2 Safrol

1.2.1 Physical and chemical properties of safrole

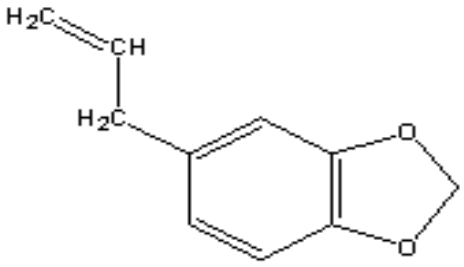
Safrole (chemical formula: $C_{10}H_{10}O_2$), a naturally occurring substance, is a derivative of the aromatic phenol ether 1,3-benzodioxole (HSDB, 2009). It exists at room temperature as a colorless or pale-yellow oil with an odor of sassafras. It is practically insoluble in water, insoluble in glycerine, slightly soluble in propylene glycol, soluble in alcohol, and miscible with chloroform and ether. Safrole is a colorless or slightly yellow oily liquid (Report on Carcinogens,2011). It is typically extracted from sassafras plants in the form of sassafras oil, or synthesized from other related methylenedioxy compounds. It is the principle component of brown camphor oil, and is found in small amounts in a wide variety of plants. It has a characteristic "candy-shop" aroma.

Synonyms: 1-allyl-3, 4-methylene dioxy benzene; 5-allyl-1, 3-benzodioxole; 4-allyl-1, 2-methylene dioxy benzene.

CAS Name: 1, 3-benzodioxole, 5(2-propenyl).

CAS No: 94-59-7

Table 1: **Physical and chemical properties of safrole are listed in the following table**

Property	Information	Structure
Molecular weight	162.2	
Density	1.1 g/cm^3 at 20°C	
Melting point	11.2°C	
Boiling point	234.5°C	
Log K_{ow}	3.45	
Water solubility	0.121 g/L at 25°C	
Vapor pressure	0.0618 mm Hg at 25°C	

(HSDB 2009, ChemIDplus 2009)

1.2.2. Synthesis of safrole

Safrole is produced by distillation of oils rich in safrole (such as sassafras, nutmeg, mace, cinnamon, anise, black pepper and sweet basil). The standard synthetic procedure for the production of MDMA from safrole is via isomerization in the presence of a strong base to isosafrole. This is then oxidized to 3, 4-methylenedioxy phenyl-2-propanone. Finally a reductive amination was done with either methylamine to make MDMA or ethylamine. It is nearly impossible to obtain large quantities of safrole and/or sassafras oil without arousing the suspicion of law enforcement, as Safrole is currently a List I chemical (The Chemistry Encyclopedia, 2005).

1.2.3. Sources of Safrole (Natural sources -different species)

- Lauraceae family (*Sassafras albidum, Cinnamomum camphora, Ocoteacymbarumand Ocoteapretiosa*).
- Piperaceae family (*Piper hispidinervium*) (Khlaeo-om, Identification of safrole).

1.2.4. The concentration of safrole as Flavoring agent:

The estimate by the "Observatoire des Consommations Alimentaires" (1998) was based on maximum limits for flavorings substances in industrially prepared foods and therefore on the amount of safrole potentially added to foods. The information on the quantity of foods to which safrole can be added was provided by industry. It is assumed that a consumer consumes randomly both industrially prepared and home-made foodstuffs. The exposure assessment was based on a selection of 28 food categories identified by industry to which safrole can be added. For these food categories, a concentration of 0.5 mg safrole/kg was assumed for food in general, a concentration of 2 mg/kg for food containing cinnamon and of 5 mg/kg for food containing nutmeg. For beverages, canned fish and chewing gum, the following concentrations were applied as specified by the Council of Europe (2001): beverages 5 mg/kg, canned fish 20 mg/kg and chewing-gum 10 mg/kg. Finally, the following correction factors were applied for the percentage of industrially prepared products. For beverages 4% of the market share will contain 5

mg/kg; for canned fish 30% of market share will contain 20 mg/kg and for chewing-gum 2% of market share will contain 10 mg/kg (IPCS INCHEM, 2012).

1.2.5. Intake estimate of safrole

Using the above assumptions the estimated average intake (for consumers only) amounts to 0.3 mg/day and the 97.5th percentile to 0.5 mg/day. It can be noted that in a previous evaluation a rough figure for estimating the intake of safrole was assumed to be 1 mg/person/day from food and spices and 1 mg/person/day from essential oils (IPCS INCHEM, 2012).

1.2.6. Pharmacological aspects of safrole

1.2.5.1. In case of absorption, distribution and excretion

Safrole was absorbed from the gastrointestinal tract by passive diffusion, with the absorption kinetics apparently dependent on its lipid solubility as determined in an in situ perfusion method in the rat. In this same procedure, safrole, at a level of 2 mg/ml of perfusion medium, reduced the absorption of glucose and methionine but not butyric acid (IPCS INCHEM, 2012).

1.2.6.2. In case of Biotransformation

Basic ninhydrin-positive substances were excreted in the urine of male rats treated with safrole or isosafrole in doses of 75-300 mg/kg i.p. These substances were not seen when dihydrosafrole was administered in similar doses. These substances readily decomposed tocarbonyl-containing compounds. The substances were not identified in this study, but in a later study the safrole metabolites were identified as tertiary amino propiophenones, 3-N, N-dimethylamino-1-(3,4-methylenedioxyphenyl)-1-propanone, 3-piperidyl-1-(3,4-methylenedioxyphenyl)-1-propanone, 3-pyrrolidinyl-1-(3,4-methylenedioxyphenyl)-1-propanone (III).I and III are excreted by the rat and II by both the rat andguinea-pig. These safrole metabolites are Mannich bases which are believed to be formed by oxidation of the allyl group to yield a vinylketone which condenses with an available amine. I, II and III were competitive inhibitors of rat liver mitochondrial monoamine oxidase with benzylamine-HCl as a substrate. Metabolite III inhibited rat liver, kidney,

and brain monoamineoxidase with tyramine HCl or serotonin as substrates (PCS INCHEM, 2012).

1.2.7. Toxicity of safrole

"As a result of research conducted in the early 1960's, safrole was recognized as a carcinogenic agent in rats and mice." "Several naturally occurring aromatic ethers, of which safrole [1-allyl-3,4-(methylenedioxy)-benzene] is one example, are hepatocarcinogens." "Both benign and malignant tumors have developed in laboratory animals, depending on the dose of safrole administered. Safrole is a chemical carcinogen, which can induce DNA modification. “No one really knows just how harmful it is to human beings, but it has been estimated that one cup of strong sassafras tea could contain as much as 200 mg [milligrams] of safrole, more than four times the minimal amount believed hazardous to humans if consumed on a regular basis."
"In concentrations of 1% of the diet, safrole is toxic, producing weight loss, testicular atrophy, and bone marrow depletion. It also induces hepatomas (liver cancer). "It is estimated that a few drops of sassafras oil are sufficient to kill a toddler and as little as one teaspoonful has proved fatal in an adult. Symptoms of poisoning are described as vomiting, stupor, and collapse. High doses may cause spasms followed by paralysis. Large amount of the oil are reported to be psychoactive with the hallucinogenic effects lasting for several days."
Recent studies have shown that even safrole-free sassafras produced tumors in two-thirds of the animals treated with it. Apparently, other constituents in addition to safrole are responsible for part of the root bark's carcinogenic activity. A 72-year-old woman drank sassafras tea up to 10 cups a day and developed diaphoresis and hot flashes. When the woman stopped drinking the tea, the diaphoresis and hot flashes promptly resolved. In preliminary pharmacological experiments that certain aqueous and alcoholic extracts prepared from sassafras root bark are capable of eliciting a variety of pharmacological responses in mice, including ataxia, ptosis, hypersensitivity to touch, central nervous system depression and hypothermia. Safrole is also a potent inhibitor of certain liver microsomal hydroxyl ting systems, a property that could lead to toxicity problems if

drugs metabolized by these enzymes are administered together with sassafras teas (European Commission, 2001).

To ensure safe and effective drug therapy, it would seem appropriate for physicians to evaluate their patients in terms of extemporaneous herb tea usage and to discourage these practices whenever feasible." Carcinogenicity of safrole following trans placental exposure of the mouse fetus and exposure of the neonatal mouse via the mother's milk was investigated in mice by intra gastric administration of the agent to pregnant and lactating females. Renal epithelial tumors were observed in 7% of female offspring exposed to safrole in utero; none of the other experimental and control animals developed these tumors. Only male offspring nursed during the pre weaning period by mothers treated with safrole developed hepatocellular tumors. In contrast, direct administration of safrole, beginning at the time of weaning and continuing for the duration of the experiment, led to a significantly high incidence of hepatocellular tumors in females, but not in males. Eighty-six percent of the liver tumors observed in females were hepatocellular carcinomas with a high rate of pulmonary metastasis. The data suggest that safrole or its metabolites came into contact with fetuses by crossing the placenta and with infants through its excretion in milk to exert the perinatal carcinogenicity (European Commission, 2001).

1.2.7.1. Carcinogenicity

(C57BL/6 x C3Hanf)F1 or (C57BL/6 x AKR)F 1 hybrid mice of 7 days of age (18M and 18 F per group) were administered safrole by stomach tube for 21 days (total dose: 464 mg/kg bw), followed by dietary administration (1112 mg/kg) for 82 weeks (total dose: 1265 mg/kg bw). Liver-cell tumors were found in 11/17 (65%) males and 16/16 (100%) females and 3/17 (18%) males and 16/17 (94%) females of the two strains, respectively, versus 8/79 (11%) males and 0/87 (0%) female and 7/90 (8%) male and 1/82 (1%) female controls, respectively. Groups of 35-40 male CD-1 mice were fed for 13 months with a diet containing 4000 or5000 mg safrole per kg. The study was terminated at 16 months. Hepatocellular carcinomas were found in 23/87 (26%) surviving animals versus 7/70 (10%) in the controls (Miller *et al.*1983).

1.2.7.2. Genotoxicity

Safrole was generally negative or weakly positive in the *Salmonella* reverse mutation assay (Ames test). 1-hydroxysafrole was directly mutagenic for strain TA100; its mutagenicity was increased by supplementation with NADPH-fortified rat liver microsomes and cytosol. Other possible metabolites such as 1-acetoxysafrole, safrole-2,3-oxide, 1-acetoxysafrole and 1-oxosafrole are directly mutagenic in strain TA100 and also, except 1-acetoxysafrole, in strain TA1535. Safrole was positive in *Escherichia coli* and *Saccharomyces cerevisiae* and in a cell transformation assay. Safrole was positive in various *in vitro* mammalian cell genotoxicity assays such as chromosomal aberrations; genemutations and sister chromatid exchanges (SCEs). It induced unscheduled DNA synthesis (UDS) in cultured rat hepatocytes. It induced DNA damage (single-strandbreaks) in cultured rat hepatocytes (European Commission, 2001). Safrole was positive in the *in vivo* i.p. host-mediated assay with *S. typhimuriums* trainTA1535 or *S. cerevisiae.* Safrole was found negative in a bone-marrow micronucleus assay, in an *in vivo* rat liver UDS and in a mouse dominant lethal assay. More recently it has been shown that safrole is able to induce chromosome aberrations, SCEs and DNA adducts in hepatocytes of F344rats exposed *in vivo*. Five repeated doses of 125 and 250 mg/kg bw induced dose-dependent increase of aberrant cells in the liver, with a maximum frequency of 13.4 % compared with the control value of 1.8%. A dose-dependent induction of SCEs in the liver was observed after a single dose of safrole at doses of 10-500 mg/kg bw. Safrole produces liver tumors in mice and rats by oral administration; safrole also produces liver and lung tumors in male infant mice following its subcutaneous injection. The carcinogenic potency appears to be relatively low and dependent on the metabolism. Mice appear to be more susceptible than rats to the carcinogenic effect of safrole. Safrole is metabolically activated through the formation of intermediates able to directly react with DNA. Safrole is genotoxic in various *in vitro* mammalian cell systems causing induction of gene mutations, chromosomal aberrations, UDS and SCE. Several metabolites of safrole are directly mutagenic in *Salmonella*. *In vivo*, safrole was able to induce chromosome aberrations, SCE and DNA adducts in the liver of rats. Safrole has been demonstrated to be genotoxic and carcinogenic. Therefore

the existence of a threshold cannot be assumed and a safe exposure limit could not be established. Consequently, reductions in the exposure and restrictions in the use levels are indicated (European Commission, 2001).

1.3. Physical and chemical properties of isosafrole

Isosafrole is a propenylbenzene derivative.

Synonyms: 5-(1-Propenyl)-1, 3-benzodioxole; 1, 2-(methylenedioxy)-4-(1-propenyl) benzene

CAS no.: 120-58-1

Structure:

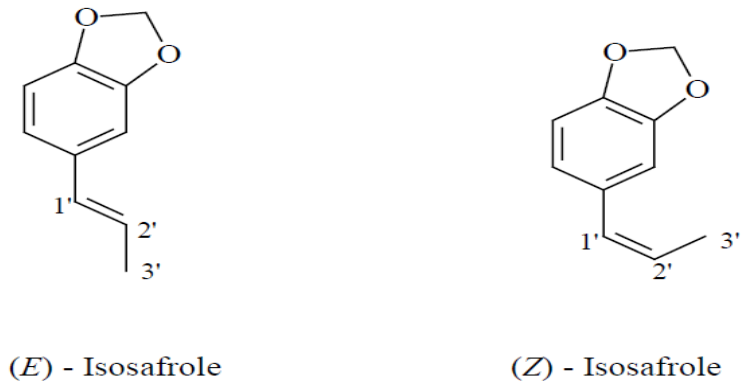


***Figure 1*: Isosafrole**

Isosafrole exists as a trans-(E-) isomer (beta-isosafrole) (CAS no: 4043-71-4) and as a cis-(Z) isomer (alfa-isosafrole), (CAS no: 17627-76-8) (IPCS INCHEM, 2012).

1.3.1. Intake estimate of Isosafrole

According to CEFS (1999) natural source materials for flavorings used by the flavor industry do not contain isosafrole. Former information on occurrence of isosafrole in ylang-ylang oil and products of sassafras could not be confirmed. On the other hand, small amounts of isosafrole were found in some samples of nutmeg oils (range < 0.1 - 3.4%) and oleoresins (range < 0.1 - 2.7%). In a study by Adam and Postel (1992) isosafrole was found in trace amounts (0.01 and 0.03 mg/kg) in two out of 24 alcoholic beverages. According to available data isosafrole only occurs sporadically and then

together with safrole but at much lower concentrations than safrole, roughly an order of magnitude lower. It has therefore been speculated, whether isosafrole occurs as an artifact, formed due to heating during the analytical process and/or during the preparation of source material extracts, essential oils, processed foods and others, containing safrole. This supposition is backed up by the fact that isosafrole can be produced by isomerisation of safrole under hot alkaline conditions and hence the possibility that traces of isosafrole may be formed even under neutral conditions. A rough intake estimate for isosafrole could be based on the estimated average safrole intake (for consumers only), assuming that the intake of isosafrole is one tenth of the safrole intake. Using this assumption, the estimated average per capita intake of isosafrole amounts to 0.03 mg/day and the 97.5% percentile to 0.05 mg/day (IPCS INCHEM, 2012).

1.3.2. Pharmacological aspects of Isosafrole

1.3.2.1. Absorption, Distribution and Excretion

In an in vitro study with epithelial cells from adult rat liver, the major metabolite of isosafrole was 1', 2'-dihydro- 1', 2'-dihydroxyisosafrole with lesser amounts of 1', 2'-epoxyisosafrole and 1'- hydroxysafrole. The metabolites were identified by gas chromatography-mass spectrometry, but no quantitative data are given. At 1 mmol (162 mg) isosafrole ("Cis-trans mixture")/kg bw given to three male albino rats by stomach tube, metabolite excretion accounted for 89 % of the dose in urine, in 72 hours. Demethylenation leading mainly to 1, 2-dihydroxy-4-(1'-propenyl) benzene was the most prominent reaction (92% of the urinary metabolites were demethylenated) but also allylic hydroxylation and epoxide-diol pathway took place. Allylic hydroxylation took place at the 3'- position, but this was a minor pathway in the present rat study. Only 1.3% of the dose was recovered as 3'-hydroxyisosafrole and, contrary to the above in vitro study, no 1'-hydroxysafrole was detected. However, the authors did not exclude that some formation of this may occur when very large doses of isosafrole are administered. This is supported by the study by Peele and Oswald (1978) who did in fact demonstrate excretion of traces of 1'- hydroxysafrole in the urine of rats to which they had

administered 3'-hydroxyisosafrole. It has also been shown that 1'-hydroxysafrole may undergo chemical rearrangement to 3'-hydroxyisosafrole and that this equilibrium between the two isomers strongly favors 3'- hydroxyl isosafrole. Isosafrole is an efficient inducer of some of the liver cytochrome P-450's, and is a weak liver carcinogen in rats and mice. Liver DNA adduct formation (32P post labeling) is low. Allgenotoxicity tests are negative. Overall, these data provide support for a non- genotoxic mechanism of hepatocarcinogenicity associated with hepatic enzyme induction, but the available data from carcinogenicity studies in mouse and rat do not allow establishing a clear NOEL (IPCS INCHEM, 2012).

1.3.3. Toxicity of Isosafrole

Isosafrole was evaluated by the International Agency for Research on Cancer (IARC) in 1975. It was concluded that isosafrole is carcinogenic in mice and rats, producing liver tumors following its oral administration. Based on this monograph, IARC in 1987 concluded for isosafrole that there were no adequate human carcinogenicity data but limited evidence of animal carcinogenicity, and isosafrole was classified in group 3, "not classifiable as to its carcinogenicity for humans". In 1981 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) also concluded that isosafrole is carcinogenic in rats and mice and no ADI was allocated. In 1999, the Council of Europe Committee of Experts on Flavoring Substances (CEFS) was informed by the International Organization of Flavor Industry that isosafrole does not occur in any natural source material for flavorings used by flavor industry and therefore the CEFS deleted isosafrole from the list of active principles. In 2002 CEFS was informed that isosafrole does occur in relevant source materials for flavorings and CEFS decided to reconsider isosafrole at its next meeting. Isosafrole is listed in the Directive 88/388/EEC on flavorings in Annex II with maximum limits for isosafrole (and safrole) of 1 mg/kg in foodstuffs and beverages with exceptions of 2 mg/kg and 5mg/kg in alcoholic beverages with not more than and with more than 25% volume of alcohol, respectively, and 15 mg/kg in foodstuffs containing mace and nutmeg (IPCS INCHEM, 2012).

In two strains of mice (C57BL/6 x C3H/Anf) F1and (C57BL/6 x AKR) F1 (group size 18 males and 18 females) isosafrole (vehicle: water) was given by stomach tube from 7 days of age until 28 days of age at 215 mg/kg bw, then subsequently fed ad libitum at 517 mg/kg diet for up to 82 weeks. The study included control groups of up to 18 males and up to 18 females per strain. Liver cell tumors occurred in 5/18 males and 1/16 females and in 2/17 males and 0/16 females, pulmonary tumors in 3/18 males and 1/16 females and in 0/17 males and 0/16 females, and lymphomas in 1/18 males and 0/16 females, and in 1/17 males and 0/16 females; of the two strains, respectively. The difference from in 1/17 males and 0/16 females; of the two strains, respectively. The difference from controls was only statistically significant for the liver tumors (P=0.05) in (C57BL/6 x C3H/Anf) F1mice (males and females combined). No hepatocarcinogenic activity was found in male B6C3F1 mice given a single i.p. injection (solvent: trioctanoin) of isosafrole (52 % cis-/48 % trans-isomer) to a group of 29 animals or transisosafrole(90 % trans-/10 % cis-isomer) to a group of 32 animals, at 12 days of age (dose: 0.75 mmol/kg bw equal to 122 mg/kg bw) and killed at 10 months. Thirty-two animals only given the solvent, served as a control group (IPCS INCHEM, 2012).

Isosafrole (19,7% cis/78.2% trans isomer) did not induce gene mutations in *Salmonella typhi* muriumstrains TA 98, TA 100, TA 1535, TA 1537, TA 1538 or in Escherichia coli WP 2 uvr with or without S9. It was negative in a Bacillus subtilis DNA repair test without S9.In contrast to safrole, estragole and methy leugenol, it did not induce UDS in cultured rat hepatocytes (IPCS INCHEM, 2012).

1.4. Application of Safrole and Isosafrole

Safrole can be made into seasonings and one of the major components of essential oils, such as sassafras, camphor, nutmeg, black pepper, sweet basil, mace and cinnamon. Safrole and isosafrole were once used extensively as a seasoning in soft drinks. Safrole is thus a natural ingredient of sassafras tea, and it was used as a flavoring component in soft drinks such as root beer in the United States until 1960.

Safrole and isosafrole were used:

- As flavoring agent in beverages and foods.

◙ Perfume and Soap

◙ Oil of sassafras, which contains safrole, was formerly used to flavor some soft drinks, such as root beer.

◙ Safrole has been used as a flavoring agent in drugs and in the manufacture of heliotropin, perfumes, soaps, and piperonyl butoxide (a compound used in a variety of insecticides to enhance the pesticidal properties of other active ingredients).

◙ It has also been used as a preservative in mucilage and library paste and as a flotation frother.

◙ Safrole has also been used in the illicit production of the drug 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), and the U.S. Drug Enforcement Administration has designated safrole a List I Chemical.

However, this use or any other addition of safrole or oil of sassafras to food was banned in the United States in 1960 (Report on Carcinogens, 2011).

CHAPTER - 2

Literature Review

2.1. Literature Review

The methods for safrole and isosafrole analysis include GC; HPLC etc are most commonly used. Among them, AOAC is one of the methods to determine safrole and isosafrole in soft drinks. The principle of AOAC is to distill safrole and isosafrole with steam, extract with an organic solvent (such as $CHC1_3$), and then analyze with gas chromatography (GC). Sample pretreatment in AOAC is complicated and time-consuming, and safrole is likely to disappear causing low recovery. Therefore, it is not a good method for routine analysis. In addition, it is necessary to use $CHCl_3$ in the process of extraction, a toxic solvent that can be used only within certain limits and is not available easily. Therefore, it is urgent and important to develop a new simple and rapid analysis method for quantitative determination of safrole and isosafrole (Choong and Lin, 2000).

2.2. Direct Method for determination of safrole in soft drinks

A simple and rapid method was developed to determine the safrole and isosafrole in soft drink using a mega pore semi-polar column (CP-SIL 8CB, 30 m × 0.53 mm, 1.5 μm) with direct injection gas chromatography. Direct quantitative analysis of safrole and isosafrole in soft drinks was carried out without any sample pretreatment procedure. The water soluble compound 1,4-dihydroxybenzene (DHB) was used as the internal standard. The detection limit for safrole and isosafrole was 0.25μg/mL. A recovery study was performed using one of the soft drinks by spiking 1mL with safrole and isosafrole at 5.0 and 10.0μg, respectively. The recovery was found in the range of 98-108% with coefficients of variation being less than 8.7%. Twenty-five commercial soft drinks were analyzed by the current method, and results indicated that 20 out of 25 soft drink samples contained safrole and isosafrole, and the amount of safrole were 3-5 fold over the regulated amount, 1μg/mL (Choong and Lin, 2000).

2.3. Some pharmacological study

Safrole at a dose of 20 mg/kg bw i.p. approximately doubled the sleeping time of mice treated with sodium pentobarbital. Isosafrole in the same dose was slightly less active.

Neither substance had a significant effect on ethanol sleeping time. In case of acute toxicity,

Table 2: **Pharmacological study of safrole, Dihydrosafrole and isosafrole**

	Animal	**LD_{50} Route**	**(mg/kg bw)**
Safrole	Mouse	Oral	2 350
	Rat	Oral	1 950
Dihydrosafrole	Mouse	Oral	3 700
		Oral	4 300
	Rat	Oral	2 260
Isosafrole	Mouse	Oral	2 470
	Rat	Oral	1 340

(IPCS INCHEM, 2012)

Isosafrole is a weak rodent hepatocarcinogen; the carcinogenicity is probably mediated by a nongenotoxic mechanism. Isosafrole metabolites may give rise to only very low binding to liver DNA in mice. It cannot be excluded that high exposure to isosafrole may give rise to isomerization of 3'-hydroxy-isosafrole to 1'-hydroxysafrole, the proximate carcinogen metabolite of safrole. However, generally the exposure to isosafrole is estimated to be very low. A clear NOEL could not be demonstrated for hepatic effects in the long-term studies. Therefore, the Committee could not establish a TDI. The Committee notes that isosafrole occurs together with safrole, but at much lower concentrations. Any measure to restrict exposure to safrole in food would also cover isosafrole (European Commission, 2003).

2.4. Determination of safrole by Gas chromatographic method on packed columns

ISO 7355 (1985) (Now withdrawn) analysize "Oils of sassafras and nutmeg, Determination of safrole and cis- and trans-isosafrole content by Gas chromatographic method on packed columns". A small quantity of the oils is analyzed by gas chromatography under specified conditions on a packed column. The safrole and cis- and

trans-isosafrole contents are determined by using the internal standard method. As stationary phase are used polydimethylsiloxane, or polyethylene glycol 20000. Operating temperatures: Oven: isotherm about 100 °C when using polydimethylsiloxane; isotherm about 125 °C when using polyethylene glycol 20000. Injection system and detector: about 170 °C.

2.5. Determination of safrole in sassafras-derived herbal products Liquid chromatographic method

Carlson M. & Thompson R.D. (1997) "Liquid chromatographic determination of safrole in sassafras-derived herbal products". A liquid chromatographic (LC) method was developed for determining safrole in herbal products derived from sassafras (Sassafras albidum), as well as related compounds such as isosafrole and dihydrosafrole. The procedure involves solvent extraction and isolation of analyte by reversed phase LC with UV detection at 235 nm. Safrole is resolved from related compounds and other sample constituents including thymol, a component of thyme. A linear concentration range of 0.003-0.200 mg/mL was obtained for safrole, isosafrole, and dihydrosafrole. Limits of detection (LOD) and quantitation (LOQ) were 0.0015 and 0.0051µg/mL for safrole, 0.0018 and 0.006µg/mL for isosafrole, and 0.0038 and 0.0125 µg/mL for dihydrosafrole, respectively. Intraday relative standard deviations (RSDs) for safrole (n=5) from various samples ranged from 1.30 to 5.39% at analyte levels of 0.01-1.5%. Safrole contents of 26 samples including root bark powder, leaves, oils, tea concentrate, herbal extract tinctures, and herbal powder capsules ranged from <LOD for most leaf samples to 92.4% for an oil. Recoveries of safrole from fortified samples ranged from 83.6% for an oil to 117.2% for a tincture preparation. Safrole contents of 0.09-4.6mg/cup were found for brewed teas prepared from sassafras root bark powders and tinctures.

2.6. Determination of safrole and related allylbenzenes in sassafras teas by GC and MS with SFE

Heikes D.L. (1994) "SFE with GC and MS determination of safrole and related

allylbenzenes in sassafras teas". Safrole (4-allyl-1,2-methylenedioxybenzene), a natural plant component of the aromatic oil of sassafras root bark, possesses carcinogenic and mutagenic activity. Legal restrictions have been placed on safrole as a food additive. However, sassafras teas continue to be accessible from health food establishments in the United States. Supercritical fluid extraction (SFE) with gas chromatographic-mass spectrometric (GC-MS) determination is utilized in the formulation of a rapid, accurate, and specific method for the determination of safrole and related allylbenzenes in unbrewed sassafras teas. Samples are extracted in a static-dynamic mode with CO2 at 690 bar and 80 degrees C with methanol as an extractor-added modifier. Levels of safrole exceeding 10,000 mg/kg (1.0%) are commonly encountered. Lesser amounts of other allylbenzenes, including eugenol and 4-allyl-1,2-dimethoxybenzene, are also reported. Recoveries of safrole and related compounds from previously extracted tea samples fortified at 100 and 1000 mg/kg ranged from 96 to 101%.

2.7. Identification of a human liver cytochrome P-450

The rat 3-methylcholanthrene-inducible family of liver cytochromes P-450 contains two proteins (P-450c and P-450d) that are immunochemically related, possess 68% total sequence homology, and are induced by a number of toxic or carcinogenic compounds. To determine whether equivalent isozymes of hepatic cytochrome P-450 are expressed in humans, as they are in several mammalian species, we performed immunoblot analyses on microsomes prepared from 14 human liver specimens and found that each one contained a 52.5-kDa protein (termed HLd) that reacted with antibodies specific for rat P-450d. In addition, one specimen contained a 54-kDa protein (termed HLc) that reacted with antibodies specific for rat P-450c. HLd was purified through the use of immune affinity chromatography and was found to be 56% homologous to rat P-450d and 61% homologous to the equivalent isozymes in the rabbit (P-450 LM4) through their first 18 NH2-terminal amino acids. Finally, levels of immuno reactive HLd varied more than 10-fold among these patients but were unrelated to the patients' drug treatments, smoking habits, or amount of immuno reactive HLp, a human liver cytochrome P-450 related to the glucocorticoid-inducible family of rat cytochromes P-450. We conclude that, in man,

there is a cytochrome P-450 family composed of two isozymes (HLc and HLd) that are immunochemically and structurally related to the 3-methylcholanthrene-inducible family observed in several other species **(**Wrighton et al. 1986).

CHAPTER - 3

Statement of Purpose

3.1. Statement of Purpose

Safrole is one of the natural components of refined oils. This thesis is done because in some previous studies or from literature review we have found that there was use of safrole and isosafrole (e.g. Sarsaparilla, Coca-Cola, 7up, Root Sar and Apple Cider) in different soft drinks as flavoring agent. Since safrole and isosafrole are carcinogens, adding sassafras oil in soft drinks has been prohibited in many countries during 1970s. Due to this reason we want to do the study for quantitative determination of safrole and isosafrole in soft drinks. The purpose of this study is to ensure and determine that our soft drinks still contain safrole and isosafrole or not. However, it can be used only in soft drinks with the use limit below 1μg/mL, according to food additive regulations. For quantitative determination gas chromatography method is the best method. To do the analysis study we choose gas chromatography method. For quantitative determination gas chromatography method is the best method. There are many reasons to choose the method. GC machine is very robust, method is simple and we can easily determine the quantitaty of safrole and isosafrole in soft drinks by determining the peck area by calculations by retention time, need not be washed frequently the column. Clean up could be done only after finishing analysis of more than 100 samples. In addition, it is very simple to clean the glass liner. Simply remove and soak in hydrochloric acid solution for 10 minutes, then remove the glass wool and rinse with water, replace with new glass wool and place back into the injector for use after drying it out. By adopting this direct injection gas chromatography, our research has developed a rapid method for analyzing liquid foods, such as soft drinks. Based on the above reasons, our research selected soft drinks as a liquid sample without any pretreatment procedure, and direct injected into GC after adding an appropriate internal standard solution in coordination with proper columns and gas chromatographic conditions used. Thus, we developed a simple and rapid analysis method for quantitative determination of safrole and isosafrole in soft drinks.

CHAPTER - 4

Methodology

4. 1. Materials and Method

Gas chromatography is a popular and widely used chromatographic separation and analysis technique based on the difference in the distribution of species between two non-miscible phases in which the mobile phase is a carrier gas moving through or passing the stationary phase contained in a column. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different retention time(characteristic time takes for a particular analyte to pass through the system from the column inlet to the detector under set conditions).GC helps in identifying a compound. In our research study, we selected soft drinks as a liquid sample without any pretreatment procedure, and direct injected into GC after adding an appropriate internal standard solution in coordination with proper columns and gas chromatographic conditions used. It is a simple and rapid analysis method due to this reason we selected GC for quantitative determination of safrole and isosafrole in soft drinks (Wikipedia, 2012).

4.1.1. The apparatus consist of Gas Chromatograph

Gas chromatograph is equipment by which gas chromatographic separations and analysis can be done or a chemical analysis instrument for separating chemicals in a complex sample.

Gas chromatograph consists of:

- **Injector/ Sample port**
- **Carrier gas:**
- **Chromatographic column contained in an oven**
- **Detector**
- **Recorder**

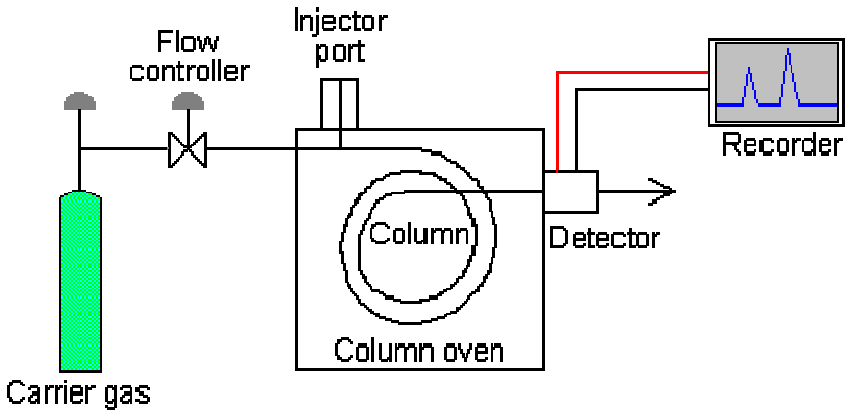


Figure 2: **Schematic diagram of a gas chromatograph**

4.1.2. Carrier Gas

The carrier gas flows through the column at a controlled rate of pressure and then through the detector. It must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependant upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities. Helium is the most common carrier gas used .Helium has a larger range of flow rates that are comparable to hydrogen in efficiency, with the added advantage that helium is non-flammable and works with a greater number of detectors. Therefore we used helium as carrier gas in our study (Wikipedia, 2012).

4.1.3. Columns

There are two general types of column, *packed* and *capillary* (also known as *open tubular*). Packed columns contain a finely divided, inert, solid support material (commonly based on *diatomaceous earth*) coated with liquid stationary phase. Most packed columns are 1.5 - 10m in length and have an internal diameter of 2 - 4mm. Capillary columns have an internal diameter of a few tenths of a millimeter. They can be one of two types; *wall-coated open tubular* (WCOT) or *support-coated open tubular* (SCOT). Wall-coated columns consist of a capillary tube whose walls are coated with liquid stationary phase. In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, onto which the stationary phase has been adsorbed. SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns. In 1979, a new type of WCOT column was devised - the *Fused Silica Open Tubular*

(FSOT) column. These have much thinner walls than the glass capillary columns, and are given strength by the polyimide coating. These columns are flexible and can be wound into coils. They have the advantages of physical strength, flexibility and low reactivity (Wikipedia, 2012).

4.1.4. Column Temperature

For precise work, column temperature must be controlled to within tenths of a degree. The optimum column temperature is dependant upon the boiling point of the sample. As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes. Minimal temperatures give good resolution, but increase elution times. If a sample has a wide boiling range, then temperature programming can be useful. The column temperature is increased (either continuously or in steps) as separation proceeds (British Pharmacopoeia, 2010).

4.1.5. Detectors

There are many detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A *non-selective* detector responds to all compounds except the carrier gas, a *selective detector* responds to a range of compounds with a common physical or chemical property and a *specific detector* responds to a single chemical compound. Detectors can also be grouped into *concentration dependant detectors* and *mass flow dependant detectors*. The signal from a concentration dependant detector is related to the concentration of solute in the detector, and does not usually destroy the sample. Dilution of with make-up gas will lower the detectors response. Mass flow dependant detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependant detector is unaffected by make-up gas **(**Introduction of Gas chromatography).

4.1.5.1. Flame Ionization Detector

In our lab we have gas chromatograph installed with flame ionization detector. Therefore we had to use flame ionization detector for our analysis purpose. In flame ionization detector (FID) the effluent from the column is mixed with hydrogen and air, and ignited. Organic compounds burning in the flame produce ions and electrons which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The current resulting from the pyrolysis of any organic compounds is measured. FIDs are mass sensitive rather than concentration sensitive; this gives the advantage that changes in mobile phase flow rate do not affect the detector's response. The FID is a useful general detector for the analysis of organic compounds; it has high sensitivity, a large linear response range, and low noise. It is also robust and easy to use, but unfortunately, it destroys the sample **(**Introduction of Gas chromatography).

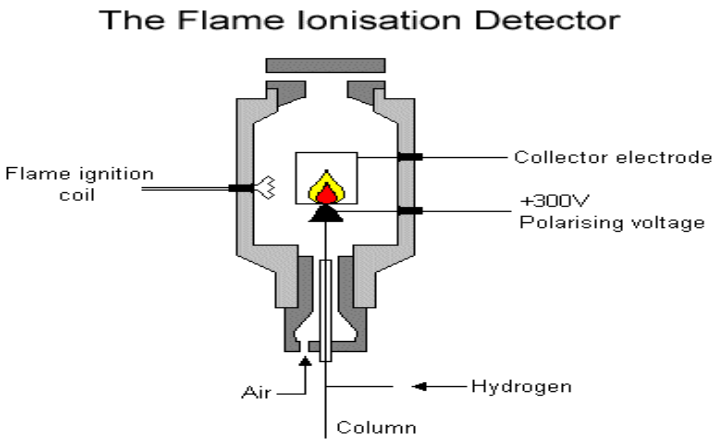


***Figure 3*: Diagram of a Flame Ionization Detector**

4.2. Method

The method for gas chromatography is the collection of conditions in which the GC operates for a given analysis. Method development is the process of determining what conditions are adequate or ideal for the analysis required. Conditions which can be varied to accommodate a required analysis include inlet temperature, detector temperature, to accommodate a required analysis include inlet temperature, detector temperature, column

temperature and temperature program, carrier gas and carrier gas flow rates, the column's stationary phase, diameter and length, inlet type and flow rates, sample size and injection technique. Depending on the detectors installed on the GC, there may be a number of detector conditions that can be varied. Since we had flame ionization detector, we had to set detector conditions according to it required. We developed a simple and rapid analysis method for quantitative determination of safrole and isosafrole in soft drinks. Our research selected soft drinks as a liquid sample without any pretreatment procedure, and direct injected into GC after adding an appropriate internal standard solution in coordination with proper columns and gas chromatographic conditions used.

4.2.1. Apparatus

***Table 3*: Apparatus**

● Gas chromatograph	● Analytical Balance
● Volumetric Flasks (50 and 100ml)	● Reagents
● Conical Flasks	● Fume Hood
● Burette	● Distillatory Machine (Rotary Evaporator)
● Micropipette	● Vacuum Pump
● Pipettes	● Measuring Cylinder
● Test Tubes	● Foil Paper
● Test Holder	● Measuring Cylinder
● Sonicator	● Vials

4.2.2. Reagent /Materials

Fourteen various commercial soft drinks, including CocaCola, Pepsi, 7 up, Speed etc were purchased from convenience stores between January and March 2012. Safrole, its

isomer isosafrole and standards 1,4-dihydroxybenzene (DHB), each have a purity of over 98%. Among them, isosafrole consisted of cis-isosafrole and trans-isosafrole.

- Soft drinks (Sample)
- Safrole and isosafrole (Standards)
- 1,4-dihydroxybenzene /DHB (Internal Standard)
- Methanol

4.3. Chromatographic Conditions

Column: RT_X - 625

Column temperature: 120° C

Oven temperature:

Table 4: **Oven Temperature**

Time	0-3 min	3-18 min	I8-30 min
Temperature	120° C	120° C-150° C	150° C- 290°C
Rate	0°C/min	2°C/min	50°C/min

Injection volume: 0.1µL,

Injection mode: Direct injection mode

Injector temperature: 240°C

Mobile phase: Helium (He), Mobile phase flow rate: 5ml/min

Detector: FID; Hydrogen gas flow rate: 30ml/min; Air flow rate: 300ml/min

Detector temperature: 290°C

Standard

Safrole and isosafrole

Internal standard

1, 4-dihydroxy benzene

4.4. Preparation of standard and internal standard solution:

4.4.1. Preparation of standard

- Safrole (0.1% w/v) was prepared. 50µl safrole was taken into 50ml volumetric flask. Then methanol was added up to the volume 50 ml.

● Isosafrole (0.1% w/v) was prepared. 50µl isosafrole was taken into 50ml volumetric flask. Then methanol was added up to the volume 50ml.

4.4.2. Internal standard solution

● 1, 4-dihydroxy benzene (0.1% w/v) was prepared. 0.05gm dihydroxy benzene was taken into 50ml volumetric flask. Then methanol was added up to the volume 50ml.

4.5. Preparation of sample

● Samples were taken in conical flask at first to remove carbon dioxide by sonication.

● After completing sonication 2 ml of samples was taken into 7 ml vials and then 100 µl of 0.1 % DHB (dihydroxy benzene) i.e. equal to the 100 µg of DHB in methanol.

● And after mixing, the mixtures were transferred into GC for analysis.

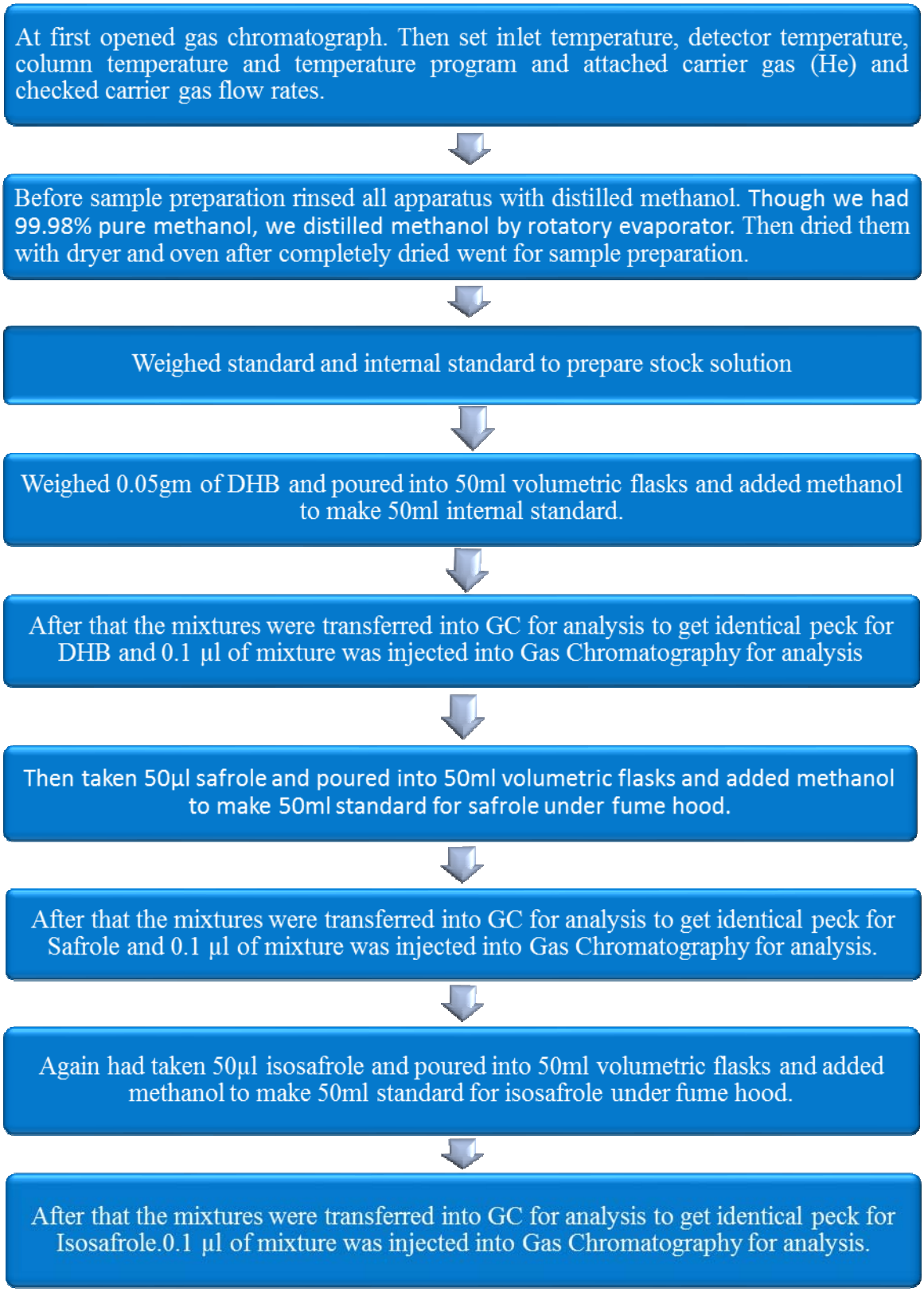
● 0.1 µl of mixture was injected into Gas Chromatography for analysis.

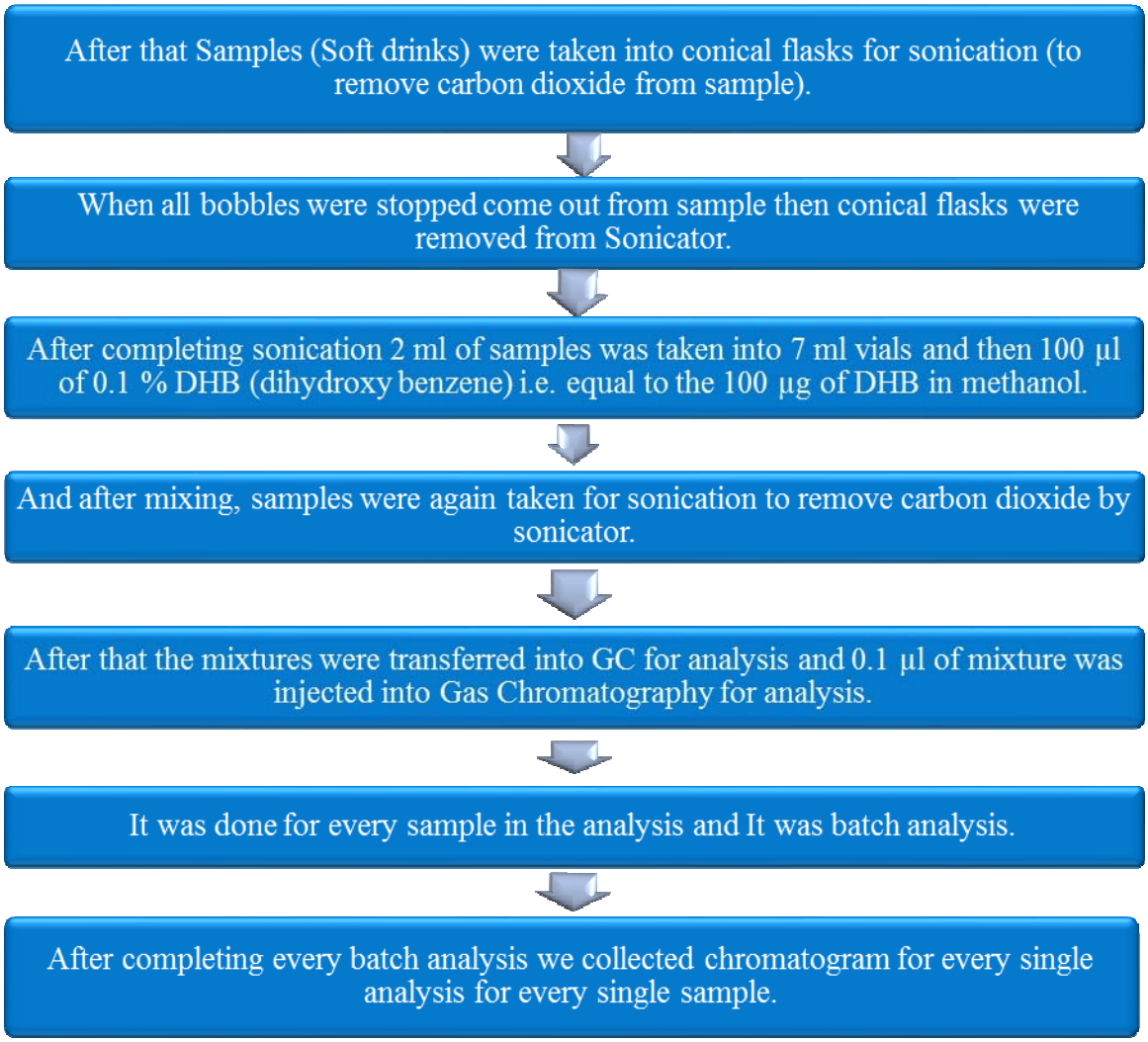
● It was done for every sample in the analysis.

4.6. Procedure

We used simple and rapid direct Gas chromatographic method. Gas chromatograph is equipment by which gas chromatographic separations and analysis can be done or a chemical analysis instrument for separating chemicals in a complex sample. During GC operation we had to set inlet temperature, column temperature, detector temperature and then started for further process.

The procedure is given in a flow chart to the next page.


At first opened gas chromatograph. Then set inlet temperature, detector temperature, column temperature and temperature program and attached carrier gas (He) and checked carrier gas flow rates.
Before sample preparation rinsed all apparatus with distilled methanol. Though we had 99.98% pure methanol, we distilled methanol by rotatory evaporator. Then dried them with dryer and oven after completely dried went for sample preparation.
Weighed standard and internal standard to prepare stock solution
Weighed 0.05gm of DHB and poured into 50ml volumetric flasks and added methanol to make 50ml internal standard.
After that the mixtures were transferred into GC for analysis to get identical peck for DHB and 0.1 µl of mixture was injected into Gas Chromatography for analysis
Then taken 50µl safrole and poured into 50ml volumetric flasks and added methanol to make 50ml standard for safrole under fume hood.
After that the mixtures were transferred into GC for analysis to get identical peck for Safrole and 0.1 µl of mixture was injected into Gas Chromatography for analysis.
Again had taken 50µl isosafrole and poured into 50ml volumetric flasks and added methanol to make 50ml standard for isosafrole under fume hood.
After that the mixtures were transferred into GC for analysis to get identical peck for Isosafrole.0.1 µl of mixture was injected into Gas Chromatography for analysis.


After that Samples (Soft drinks) were taken into conical flasks for sonication (to remove carbon dioxide from sample).
When all bobbles were stopped come out from sample then conical flasks were removed from Sonicator.
After completing sonication 2 ml of samples was taken into 7 ml vials and then 100 µl of 0.1 % DHB (dihydroxy benzene) i.e. equal to the 100 µg of DHB in methanol.
And after mixing, samples were again taken for sonication to remove carbon dioxide by sonicator.
After that the mixtures were transferred into GC for analysis and 0.1 µl of mixture was injected into Gas Chromatography for analysis.
It was done for every sample in the analysis and It was batch analysis.
After completing every batch analysis we collected chromatogram for every single analysis for every single sample.

CHAPTER - 5

Calculation

5.1. Determination of the Relative Response Factor (RRF) of Safrole and Isosafrole to 1, 4-Dihydroxybenzene (DHB)

0.1% (w/v) of safrole and isosafrole were mixed with 0.1% (w/v) of internal standard 1,4dihydroxybenzene (DHB) in methanol solution in various ratios: safrole or isosafrole / DHB = 2/1, 1/l, 1/2. The relative response factor of safrole and isosafrole to DHB was calculated according to their peak area ratio and concentration ratio in a gas chromatographic device:

$$RRF = \frac{A_s}{W_s} / \frac{A_{is}}{W_{is}}$$

As = GC peak area of safrole or isosafrole

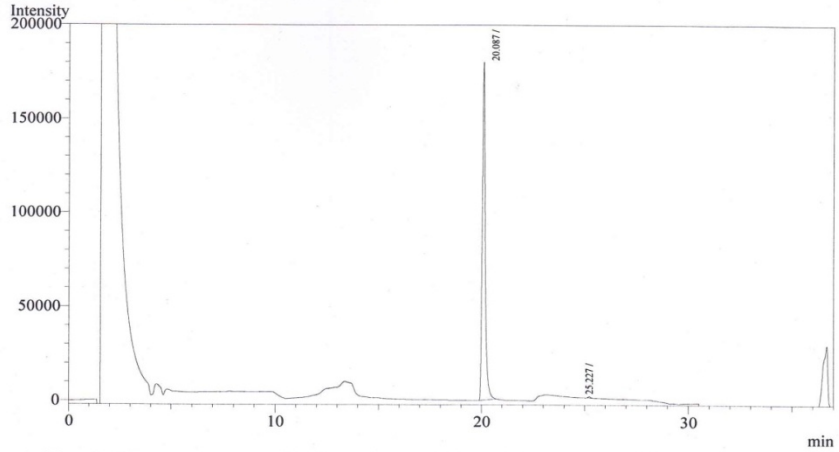
Ais = GC peak area of DHB

Ws = weight (μg) of safrole or isosafrole (cis- or trans-)

Wis = weight (μg) of DHB

Analysis Date & Time : 4/4/2012 1:56:02 PM
User Name : Admin
Vial# : 1
Sample Name : Safrole
Sample ID :
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\Three main\safrole.gcd
Method Name : D:\Safrole\Safrole Isosaf RRF.gcm

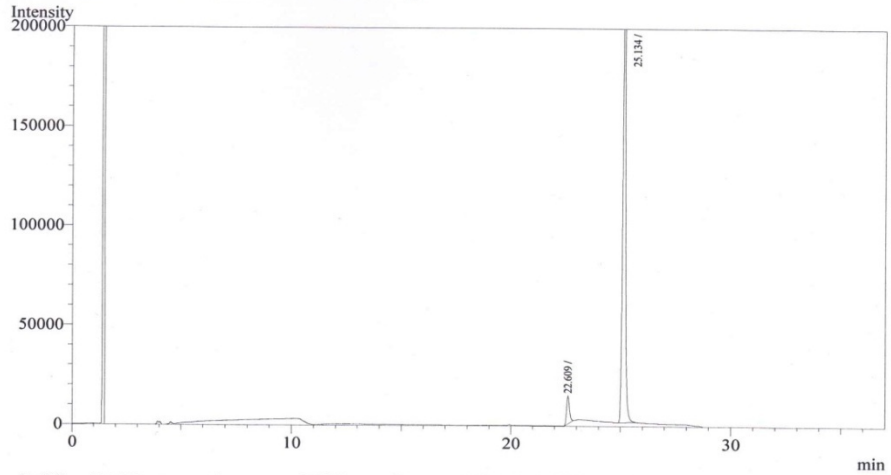


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	20.087	1805917	179704	99.692				
2	25.227	5578	719	0.308				
Total		1811495	180423					

Figure 4: **Chromatogram of standard safrole**

Analysis Date & Time : 4/4/2012 2:37:25 PM
User Name : Admin
Vial# : 2
Sample Name : Isosafrole
Sample ID :
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\Three main\isosafrole.gcd
Method Name : D:\Safrole\Safrole Isosaf RRF.gcm

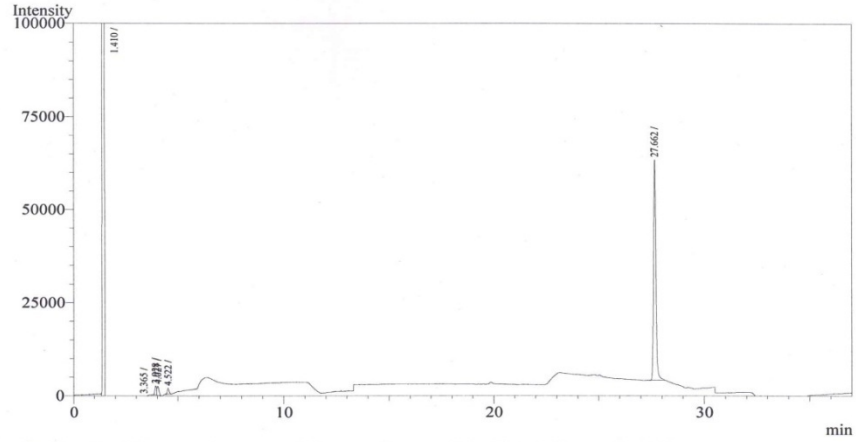


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	22.609	127365	14090	5.730				
2	25.134	2095490	240599	94.270				
Total		2222855	254689					

Figure 5: **Chromatogram of standard Isosafrole**

Analysis Date & Time : 4/4/2012 3:18:47 PM
User Name : Admin
Vial# : 3
Sample Name : DHB
Sample ID :
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\Three main\DHB.gcd
Method Name : D:\Safrole\Safrole Isosaf RRF.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	1.410	14156668	3984977	0.000				
2	3.365	9405733	9571	0.000				
3	3.928	14015	2430	0.000				
4	4.027	14550	2410	0.000		V		
5	4.522	10456	1599	0.000				
6	27.662	454677	59359	0.000				
Total		24056099	4060346					

Figure 6: **Chromatogram of internal standard 1, 4dihydroxy benzene**

For safrole, In the ratio 2:1:: Safrole: 1, 4-dihydroxy benzene (DHB),

Total volume = 3ml

Amount of safrole = (2×1.12) mg [Density of safrole=1.12 gm/ml=1.12mg/µl]

= 2.24 mg

The weight of safrole will be,

3 ml contain 2.24mg of safrole

3000 µl contain (2.24/ 3000) mg/µl of safrole

.1µl contain (2.24×.1 / 3000) mg/µl of safrole

$= 7.467 \times 10^{-5}$ mg/µl of safrole

=0.07467 µg/µl

The weight of 1, 4- dihydroxy benzene will be,

Ws = 1/3 × Density of DHB × Injection volume

=0.33 × 1µg/µl × 0.1µl

=0.033 µg/µl

The RRF value will be,

$$RRF = \frac{A_s}{W_s} / \frac{A_{is}}{W_{is}}$$

As = GC peak area of safrole = 1179058

Ais = GC peak area of DHB = 187543

Ws = weight (µg) of safrole = 0.07467 µg/µl

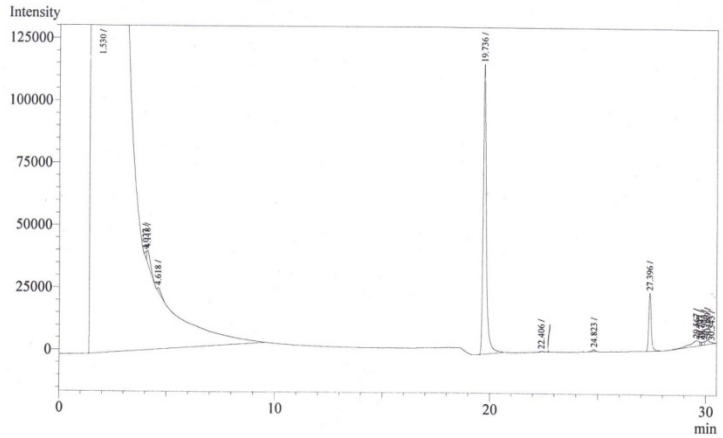
Wis = weight (µg) of DHB = 0.033 µg/µl

RRF = (1179058/0.075) ÷ (187543/0.033)

RRF = 2.77

Analysis Date & Time : 3/27/2012 2:19:36 PM
User Name : Admin
Vial# : 1
Sample Name : 2saf1dhb
Sample ID :
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\Safrole+isosafrole\2Safrole_1DHB001.gcd
Method Name : D:\Safrole\Safrole.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	1.530	313884606	11361392	0.000		S		
2	4.027	11356	2579	0.000		T		
3	4.118	72706	5240	0.000		TV		
4	4.618	20065	1541	0.000		T		
5	19.736	1179058	116108	0.000		S		
6	22.406	3267	295	0.000				
7	24.823	8282	816	0.000				
8	27.396	187543	23359	0.000		S		
9	29.567	66089	2244	0.000				
10	29.727	5412	1557	0.000		V		
11	29.792	3382	1363	0.000		V		
12	29.923	10176	1350	0.000		V		
13	30.089	27448	1991	0.000		V		
14	30.343	2130	435	0.000		V		
Total		315481520	11520270					

Figure 7: **Chromatogram of safrole and 1, 4-dihydroxy benzene in (2:1) ratio**

In the ratio 1:1 :: Safrole: 1, 4-dihydroxy benzene(DHB),

The weight of safrole will be,

Total volume = 2ml

Amount of safrole = (1×1.12) mg [Density of safrole=1.12 gm/ml=1.12mg/µl]

= 1.12 mg

The weight of safrole will be,

2 ml contain 1.12mg of safrole

2000 µl contain (1.12/ 3000) mg/µl of safrole

.1µl contain (1.12×.1 / 2000) mg/µl of safrole

$= 5.6 \times 10^{-5}$ mg/µl of safrole

=0.056 µg/µl

The weight of 1, 4- dihydroxy benzene will be,

Ws = 1/2 × Density of DHB × Injection volume

=0.5 × 1µg/µl × 0.1µl

=0.05 µg/µl

The RRF value will be,

$$RRF = \frac{A_s}{W_s} / \frac{A_{is}}{W_{is}}$$

As = GC peak area of safrole = 870221

Ais = GC peak area of DHB = 259680

Ws = weight (µg) of safrole = 0.056 µg/µl

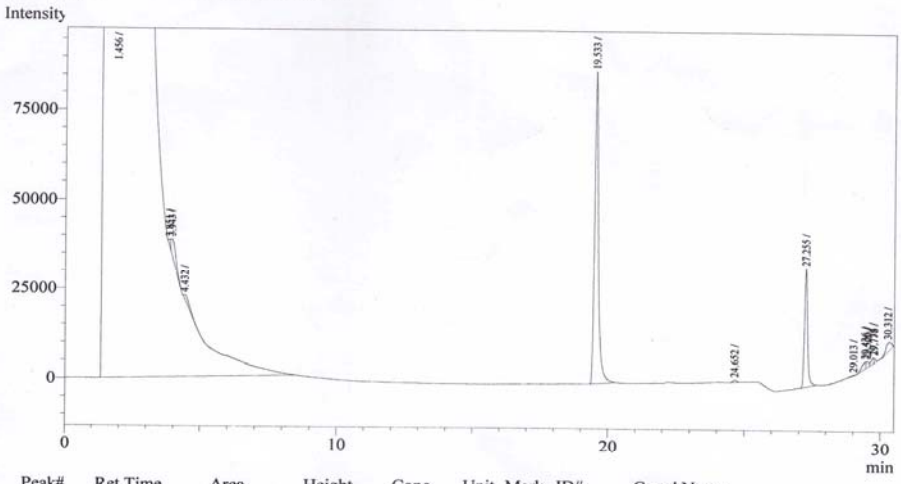
Wis = weight (µg) of DHB = 0.05 µg/µl

RRF = (870221/0.056) ÷ (259680/0.05)

RRF = 2.99

Analysis Date & Time : 3/27/2012 2:54:26 PM
User Name : Admin
Vial# : 2
Sample Name : 1saf1dhb
Sample ID :
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\Safrole+isosafrole\1saf_1dhb.gcd
Method Name : D:\Safrole\Safrole.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	1.456	311551374	11436577	0.000		S		
2	3.851	8638	1968	0.000		T		
3	3.943	65950	4625	0.000		TV		
4	4.432	25236	1393	0.000		T		
5	19.533	870221	87193	0.000				
6	24.652	5064	632	0.000				
7	27.255	259680	33027	0.000				
8	29.013	1472	184	0.000		V		
9	29.436	20281	1890	0.000				
10	29.506	10672	1655	0.000		V		
11	29.735	12217	1609	0.000		V		
12	29.778	5580	1329	0.000		V		
13	30.312	33846	2493	0.000				
Total		312870231	11574575					

Figure 8: **Chromatogram of safrole and 1, 4-dihydroxy benzene in (1:1) ratio**

In the ratio 1: 2 :: Safrole: 1, 4-dihydroxy benzene(DHB),

The weight of safrole will be,

Total volume = 3ml

Amount of safrole = (1×1.12) mg [Density of safrole=1.12 gm/ml=1.12mg/µl]

= 1.12 mg

The weight of safrole will be,

3 ml contain 1.12mg safrole

3000 µl contain (1.12/ 3000)mg/µl safrole

.1µl contain (1.12×.1 / 3000)mg/µl safrole

$= 3.73 \times 10^{-5}$ mg/µl safrole

=0.037 µg/µl

The weight of 1, 4- dihydroxy benzene will be,

Ws = 2/3 × Density of DHB × Injection volume

=0.67 × 1µg/µl × 0.1µl

=0.067 µg/µl

The RRF value will be,

$$RRF = \frac{A_s}{W_s} / \frac{A_{is}}{W_{is}}$$

As = GC peak area of safrole = 418676

Ais = GC peak area of DHB = 239785

Ws = weight (µg) of safrole = .037 µg/µl

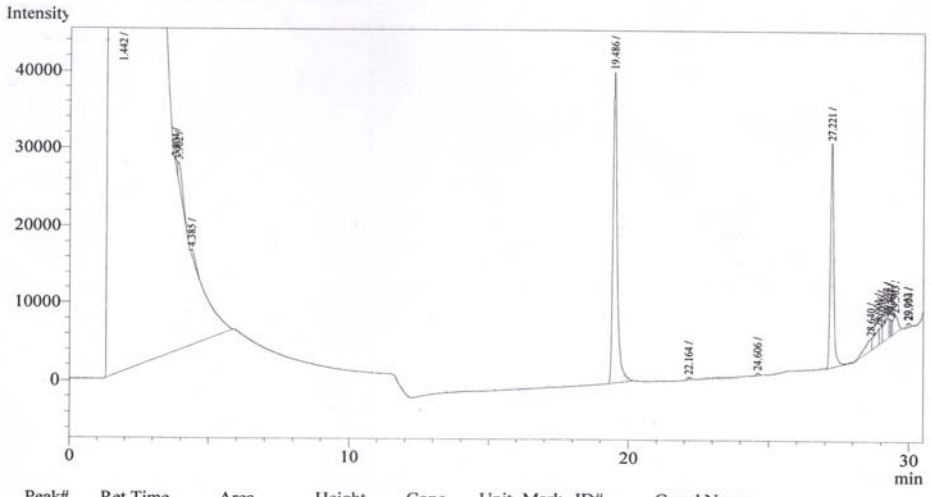
Wis = weight (µg) of DHB = 0.067 µg/µl

RRF = (418676/.037) ÷ (239785/0.067)

RRF = 3.16

Analysis Date & Time : 3/27/2012 3:29:19 PM
User Name : Admin
Vial# : 3
Sample Name : 1saf2dhb
Sample ID :
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\Safrole+isosafrole\1saf_2dhb.gcd
Method Name : D:\Safrole\Safrole.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	1.442	228628928	8565764	0.000		S		
2	3.804	5160	911	0.000		T		
3	3.902	32877	2543	0.000		TV		
4	4.385	11932	826	0.000		T		
5	19.486	418676	40127	0.000				
6	22.164	1829	228	0.000		V		
7	24.606	2061	298	0.000				
8	27.221	239785	28926	0.000		S		
9	28.640	27614	1486	0.000				
10	28.901	26369	2027	0.000		V		
11	29.016	14457	2107	0.000		V		
12	29.213	33334	2712	0.000		V		
13	29.302	9520	2357	0.000		V		
14	29.363	6003	2049	0.000		V		
15	29.503	27377	2220	0.000		V		
16	29.953	2773	611	0.000		V		
17	29.994	2408	566	0.000		V		
Total		229491103	8655758					

Figure 9: **Chromatogram of safrole and 1, 4-dihydroxy benzene in (1:2) ratio**

The average RRF value = (2.77 + 2.99 + 3.16)/3

= 2.97

Content of safrole and isosafrole (μg/ml), Ws = (As/Ais) × (Wis/RRF)

5.2. Determination of Safrole

For every sample,

In the vial, 1ml of sonicated soft drink and 50 μl of 1, 4-dihydroxy benzene (DHB) was taken for analysis so,

1ml sample + 50 μl DHB

Total, (1ml + 50 μl) = 1000 μl + 50 μl = 1050 μl

So, 1050 μl of preparation contains 50 μg DHB

Therefore, 0.1 μl of preparation contains = (50×0.1)/1050 = 4.76×10^{-3} μg DHB

5.2.1. For 7up, Content of safrole (μg/ml), Ws = (As/Ais) × (Wis/RRF)

Here, As = GC peak area of safrole = 153601

Ais = GC peak area of DHB = 697423

Ws = weight (μg) of safrole

Wis = weight (μg) of DHB = 4.76×10^{-3} μg

RRF = 2.97

Ws = (As/Ais) × (Wis/RRF)

Ws = (153601/697423) × (4.76×10^{-3} μg /2.97)

= 3.53×10^{-4} μg

0.1μl contain 3.53×10^{-4} μg safrole

1 μl contain 35.3×10^{-4} μg safrole

1ml contain $35.3 \times 10^{-4} \times 1000$μg safrole

= 35.3μg/ml

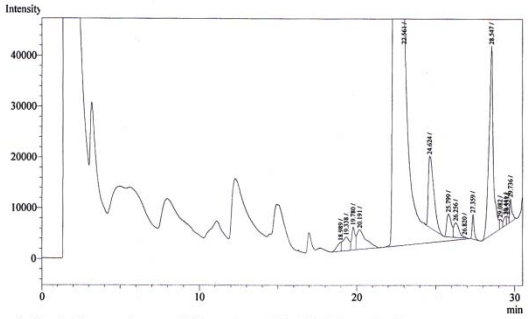
250ml contain $35.3 \times 10^{-4} \times 1000 \times 250$μg/ml safrole

= 882.5 μg/250ml safrole

For 7up, Content of safrole, Ws = 882.5μg / 250ml

Analysis Date & Time : 4/5/2012 4:19:55 PM
User Name : Admin
Vial# : 2
Sample Name : Sevenup
Sample ID : 7Up1
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\05.04.2012\Sevenup.gcd
Method Name : D:\Safrole\Safrole.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	18.989	31041	1717	0.343		V		
2	19.338	78145	2667	0.864		V		
3	19.780	64721	4509	0.716		V		
4	20.191	153601	3843	1.699		V		
5	22.561	7374930	211052	81.566		SV		
6	24.624	341150	14037	3.773		TV		
7	25.799	90323	4513	0.999		T		
8	26.256	65465	2898	0.724		TV		
9	26.820	2020	303	0.022		TV		
10	27.359	42966	4833	0.475				
11	28.547	697423	37140	7.713				
12	29.082	19629	1915	0.217		V		
13	29.441	21429	1732	0.237		V		
14	29.537	14537	1625	0.161		V		
15	29.736	44265	4560	0.490		V		
Total		9041645	297344					

Figure 10: **Chromatogram of 7up**

5.2.2. For Cocacola

Content of safrole (µg/ml), Ws = (As/Ais) × (Wis/RRF)

Here, As = GC peak area of safrole = 17208

Ais = GC peak area of DHB = 707195

Ws = weight (µg) of safrole

Wis = weight (µg) of DHB = 4.76×10^{-3} µg

RRF = 2.97

Ws = (As/Ais) × (Wis/RRF)

Ws = (17208/707195) × (4.76×10^{-3} µg /2.97)

= 3.9×10^{-5}µg

0.1µl contain 3.9×10^{-5} µg safrole

1 µl contain 3.9×10^{-4} µg safrole

1ml contain $3.9 \times 10^{-4} \times 1000$µg safrole

= .39µg/ml

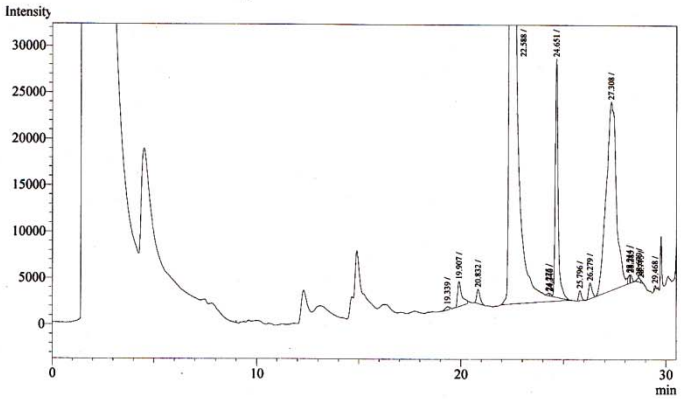
250ml contain $3.9 \times 10^{-4} \times 1000 \times 250$µg/ml safrole

= 97.5µg/250ml safrole

For CocaCola, Content of safrole, Ws = 97.5µg/250 ml

Analysis Date & Time : 4/5/2012 3:45:05 PM
User Name : Admin
Vial# : 1
Sample Name : Cocacola
Sample ID : Coca1
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\05.04.2012 New\Cocacola.gcd
Method Name : D:\Safrole\Safrole.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	19.339	4565	341	0.091				
2	19.907	38406	2679	0.769		V		
3	20.832	17208	1519	0.345				
4	22.588	3928012	310797	78.689		S		
5	24.275	1938	290	0.039		T		
6	24.346	1620	262	0.032		TV		
7	24.651	245985	25717	4.928		TV		
8	25.796	9438	1075	0.189				
9	26.279	19142	1713	0.383				
10	27.308	707195	20364	14.167		V		
11	28.214	5411	930	0.108		V		
12	28.285	5693	839	0.114		V		
13	28.679	4529	494	0.091				
14	28.775	1105	300	0.022		V		
15	29.468	1599	318	0.032				
Total		4991846	367638					

Figure 11: **Chromatogram of CocaCola**

5.2.3. For Sprite

Content of safrole (μg/ml), Ws = (As/Ais) × (Wis/RRF)

Here, As = GC peak area of safrole = 4129

Ais = GC peak area of DHB = 26773

Ws = weight (μg) of safrole

Wis = weight (μg) of DHB = 4.76×10^{-3} μg

RRF = 2.97

Ws = (As/Ais) × (Wis/RRF)

Ws = (4129/26773) × (4.76×10^{-3} μg /2.97)

= 2.5×10^{-4}μg

0.1μl contain 2.5×10^{-4}μg safrole

1 μl contain 2.5×10^{-3}μg safrole

1ml contain 2.5×10^{-3}μg ×1000μg safrole

= 2.5μg/ml

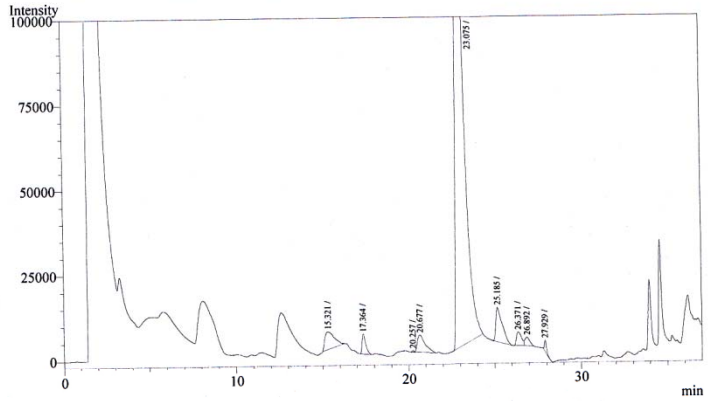
250ml contain 2.5×10^{-3} ×1000×250μg/ml safrole

= 625μg/250ml safrole

For Sprite, Content of safrole, Ws = 625μg/250 ml

Analysis Date & Time : 4/9/2012 1:15:38 PM
User Name : Admin
Vial# : 2
Sample Name : Sprite
Sample ID : S1
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\09.04.2012\Sprite.gcd
Method Name : D:\Safrole\Safrole Isosaf RRF.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	15.321	195594	5169	3.290				
2	17.364	85794	5836	1.443				
3	20.257	4129	510	0.069				
4	20.677	158787	5101	2.670		V		
5	23.075	5085257	156345	85.525				
6	25.185	260207	10165	4.376				
7	26.371	80132	4025	1.348				
8	26.892	49289	2535	0.829		V		
9	27.929	26773	3329	0.450				
Total		5945962	193015					

Figure 12: **Chromatogram of Sprite**

5.2.4. For RC Lemon

Content of safrole (µg/ml), Ws = (As/Ais) × (Wis/RRF)

Here, As = GC peak area of safrole = 9154

Ais = GC peak area of DHB = 2653

Ws = weight (µg) of safrole

Wis = weight (µg) of DHB = 4.76×10^{-3} µg

RRF = 2.97

Ws = (As/Ais) × (Wis/RRF)

Ws = (9154/2653) × (4.76×10^{-3} µg /2.97)

= 5.5×10^{-3}µg

0.1µl contain5.5×10^{-3}µg safrole

1 µl contain 0.055µg safrole

1ml contain 0.055µg ×1000µg safrole

= 55µg/ml

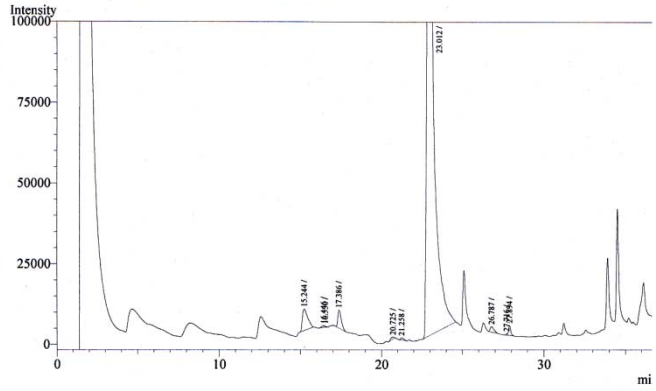
250ml contain 0.055 ×1000×250µg/ml safrole

= 13750µg/250ml safrole

For RC Lemon, Content of safrole, Ws = 13750µg/250 ml

Analysis Date & Time : 4/9/2012 3:19:48 PM
User Name : Admin
Vial# : 5
Sample Name : RC lemon
Sample ID : RL1
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\09.04.2012\RC lemon.gcd
Method Name : D:\Safrole\Safrole Isosaf RRF.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	15.244	152480	6679	2.476				
2	16.446	7199	538	0.117				
3	16.530	1109	365	0.018		V		
4	17.386	81793	5647	1.328				
5	20.725	9154	534	0.149				
6	21.258	8661	706	0.141				
7	23.012	5840441	271165	94.822				
8	26.787	27564	1700	0.448				
9	27.716	2653	424	0.043				
10	27.894	28307	3149	0.460		V		
Total		6159361	290907					

Figure 13: **Chromatogram of RC Lemon**

5.2.5. For Clemon

Content of safrole (μg/ml), Ws = (As/Ais) × (Wis/RRF)

Here, As = GC peak area of safrole = 185103

Ais = GC peak area of DHB = 86218

Ws = weight (μg) of safrole

Wis = weight (μg) of DHB = 4.76×10^{-3} μg

RRF = 2.97

Ws = (As/Ais) × (Wis/RRF)

Ws = (185103/86218) × (4.76×10^{-3} μg /2.97)

= 3.4×10^{-3}μg

0.1μl contain3.4×10^{-3}μg safrole

1 μl contain 0.034μg safrole

1ml contain 0.034μg ×1000μg safrole

= 34μg/ml

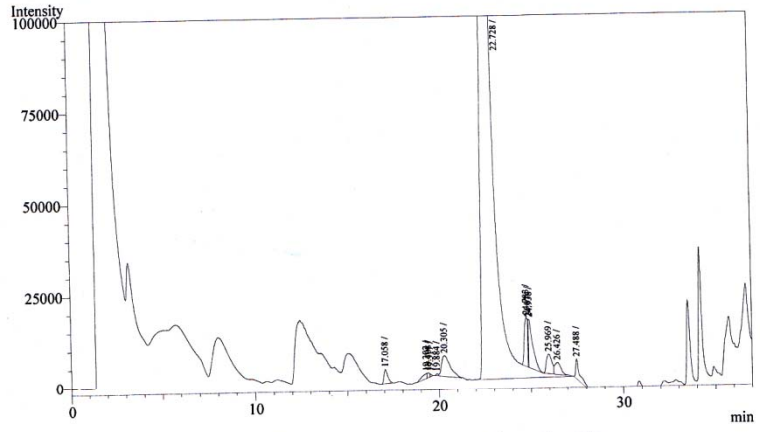
250ml contain 0.034 ×1000×250μg/ml safrole

= 8500μg/250ml safrole

For Clemon, Content of safrole, Ws = 8500μg/250 ml

Analysis Date & Time : 4/8/2012 3:09:46 PM
User Name : Admin
Vial# : 5
Sample Name : Clemon
Sample ID : Clemon 1
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\05.04.2012\Clemon.gcd
Method Name : D:\Safrole\Safrole Isosaf RRF.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	17.058	55310	3889	0.618				
2	19.302	28066	1378	0.314				
3	19.408	11599	1315	0.130		V		
4	19.517	4008	803	0.045		V		
5	19.884	3431	524	0.038				
6	20.305	185103	5680	2.068		V		
7	22.728	7994981	214840	89.334		SV		
8	24.783	155292	13542	1.735		T		
9	24.874	36589	13191	0.409		TV		
10	24.938	204550	13212	2.286		TV		
11	25.969	108032	5359	1.207		T		
12	26.426	76379	3296	0.853		TV		
13	27.488	86218	5502	0.963				
Total		8949558	282531					

Figure 14: **Chromatogram of Clemon**

5.2.6. For Dew

Content of safrole (µg/ml), Ws = (As/Ais) × (Wis/RRF)

Here, As = GC peak area of safrole = 47187

Ais = GC peak area of DHB = 37970

Ws = weight (µg) of safrole

Wis = weight (µg) of DHB = 4.76×10^{-3} µg

RRF = 2.97

Ws = (As/Ais) × (Wis/RRF)

Ws = (47187/37970) × (4.76×10^{-3} µg /2.97)

= 1.99×10^{-3}µg

0.1µl contain1.99×10^{-3}µg safrole

1 µl contain 0.0199µg safrole

1ml contain 0.0199µg ×1000µg safrole

= 19.9µg/ml

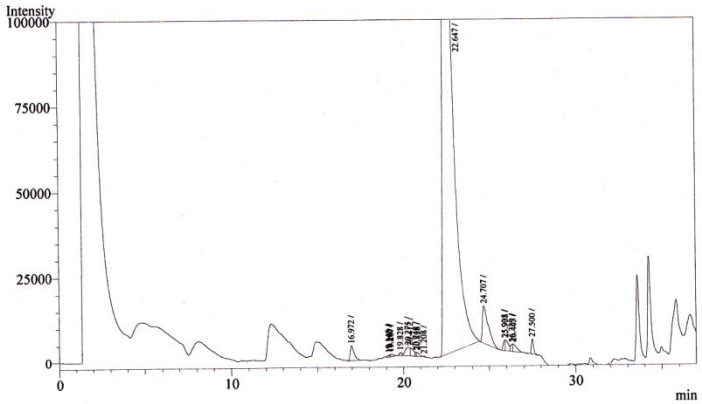
250ml contain 0.0199 ×1000×250µg/ml safrole

= 4975µg/250ml safrole

For Dew, Content of safrole, Ws = 4975µg/250 ml

Analysis Date & Time : 4/8/2012 3:51:12 PM
User Name : Admin
Vial# : 6
Sample Name : Dew
Sample ID : Dew
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

Data Name : D:\Safrole\05.04.2012\Dew.gcd
Method Name : D:\Safrole\Safrole Isosaf RRF.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	16.972	68259	4356	0.883				
2	19.148	4634	561	0.060		V		
3	19.203	2936	693	0.038		V		
4	19.267	7302	731	0.094		V		
5	19.828	9591	935	0.124				
6	20.275	47187	2528	0.610		V		
7	20.412	31670	2367	0.410		V		
8	20.748	5939	1159	0.077		V		
9	20.828	11837	1149	0.153		V		
10	21.208	1544	222	0.020				
11	22.647	7121526	201955	92.133				
12	24.707	263951	11101	3.415				
13	25.905	23242	3109	0.301				
14	25.938	40714	3188	0.527		V		
15	26.329	16766	2060	0.217		V		
16	26.403	34547	2219	0.447		V		
17	27.500	37970	4397	0.491				
Total		7729615	242730					

Figure 17: **Chromatogram of Dew**

5.2.7 Mirinda

Content of safrole (μg/ml), $W_s = (A_s/A_{is}) \times (W_{is}/RRF)$

Here, A_s = GC peak area of safrole = 5348

A_{is} = GC peak area of DHB = 26470

W_s = weight (μg) of safrole

W_{is} = weight (μg) of DHB = 4.76×10^{-3} μg

RRF = 2.97

$W_s = (A_s/A_{is}) \times (W_{is}/RRF) \times 1/V$

$W_s = (5348/26470) \times (4.76 \times 10^{-3}$ μg /2.97)

$= 3.24 \times 10^{-4}$ μg

0.1 μl contains 3.24×10^{-4} μg of safrole

1 μl contains 32.4×10^{-4} μg of safrole

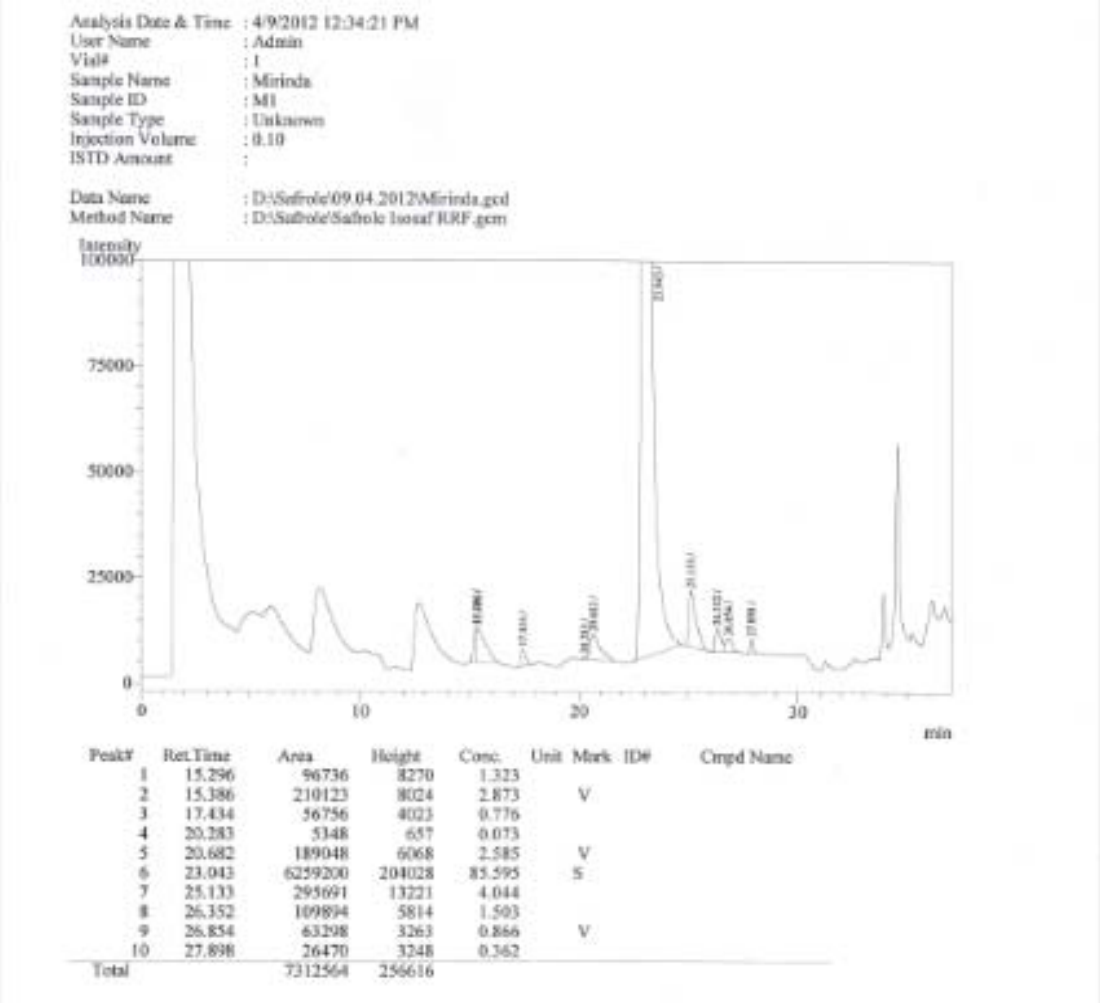
1 ml contains $32.4 \times 10^{-4} \times 1000$ μg of safrole

= 3.24 μg of safrole

So, 250 ml contains 250× 3.24 μg of safrole

=810μg /250ml of safrole

For Mirinda, Content of safrole, W_s = 810 μg/250 ml



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	15.296	96736	8270	1.323				
2	15.386	210123	8024	2.873		V		
3	17.434	56756	4023	0.776				
4	20.283	5348	657	0.073				
5	20.682	189048	6068	2.585		V		
6	23.043	6259200	204028	85.595		S		
7	25.133	295691	13221	4.044				
8	26.352	109894	5814	1.503				
9	26.854	63298	3263	0.866		V		
10	27.898	26470	3248	0.362				
Total		7312564	256616					

Figure 18: **Chromatogram of Mirinda**

CHAPTER - 6

Result and Discussion

6.1 Results

In the analysis we have found safrole in these soft drinks. **For 7up,** Content of safrole, Ws = 882.5µg / 250ml, **For Cocacalo,** Content of safrole, Ws = 97.5µg/250 ml, **For Sprite,** Content of safrole, Ws = 625µg/250 ml, **For RC Lemon,** Content of safrole, Ws = 13750µg/250 ml, **For Clemon,** Content of safrole, Ws = 8500µg/250 ml, **For Dew,** Content of safrole, Ws = 4975µg/250 ml, **For Mirinda**, Content of safrole, Ws = 810µg/250 ml which was confirmed in recovery test.

Table 5: **Content of Safrole**

Names (soft drinks)	Amount of Safrole (1µl contain)	Amount of Safrole (1ml contain)	Amount of Safrole (250ml contain)	Approved limit by FDA
7up	35.3×10^{-4} µg	3.53 µg	882.5 µg	Below 1µg/ml
Coca cola	3.9×10^{-4} µg	0.39 µg	97.5 µg	
Sprite	2.5×10^{-3} µg	2.5µg	625µg	
RC Lemon	0.055 µg	55 µg	13750 µg	
Clemon	0.034 µg	34 µg	8500 µg	
Dew	0.0199 µg	19.9 µg	4975 µg	
Mirinda	32.4×10^{-4} µg	3.24 µg	810 µg	

But we are not sure that is there presence of isosafrole in soft drinks or not because we got peak for isosafrole in the test of samples but it fails in the recovery test. One reason for this, isosafrole may be used after derivitization.

Table 6: **Content of Isosafrole**

Names (soft drinks)	Amount of Isosafrole	Approved limit by FDA
7up	Not found	Below 1µg/ml
Coca cola	Not found	
Sprite	Not found	
RC Lemon	Not found	
Clemon	Not found	
Dew	Not found	
Mirinda	Not found	

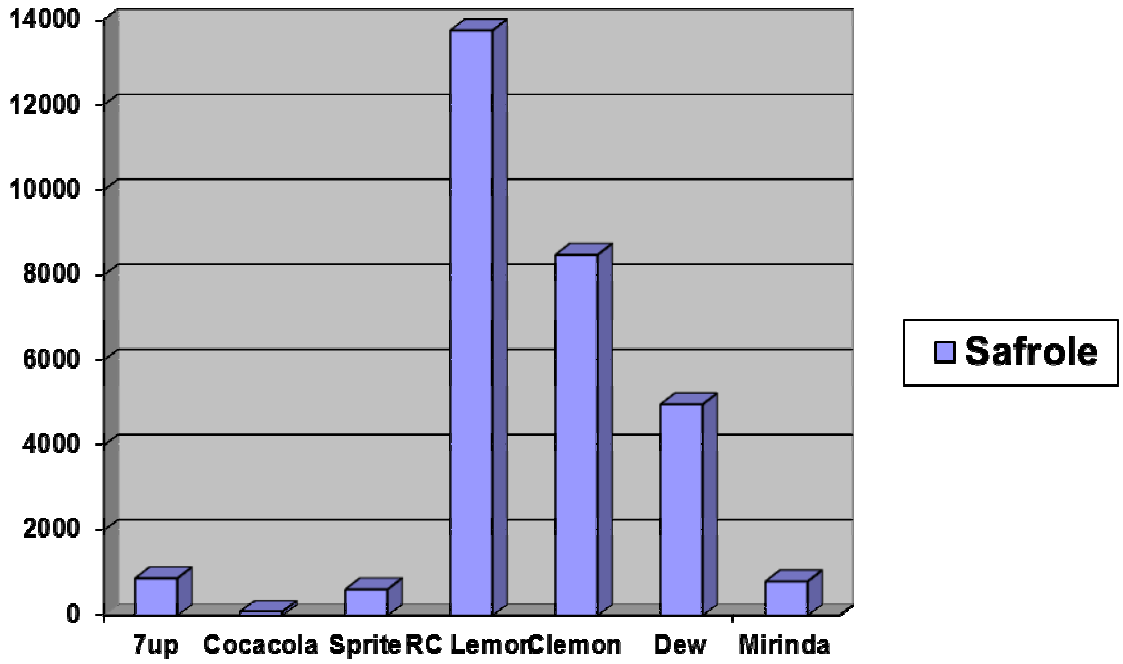


Figure 17: **Comparison of conc. of safrole between different soft drinks in (µg/250 ml)**

From the figure we can see that RC Lemon soft drink contains the maximum amount of safrole, then Clemon and then Dew and Cocacola contain the minimum amount of safrole.

6.2. Discussion

RT_X-625 column was used for trial with respect to the selection of the analytical column. Results demonstrated that the column was the most suitable one to be used for analysis of polarized safrole and isosafrole. The direct injection mode was adopted for sample analysis and the appropriate temperature raising program as described in Method. The retention times of safrole, isosafrole and DHB were 20.087, 25.134 and 27.662 min respectively according to the above analysis conditions. The GC chromatograms of soft drink samples and standards were showed in Figure (4-18), respectively. With respect to the selection of internal standard, a small amount of water-soluble standard of 1,4-dihydroxybenzene (DHB) were individually added into soft drink samples, including 7up, Cocacola, Sprite, RC Lemon, Clemon, Dew, Mirinda. According to the GC retention times of various standards with the above GC conditions used, a suitable internal standard was then chosen for determination analysis of safrole and isosafrole. Results showed that the retention times of safrole, isosafrole and DHB were 20.087, 25.134 and 27.662 min, respectively. By comparing the retention times of standards to various elements in soft drink samples as shown in Figure (7-13), the peaks of DHB and all elements in soft drink samples did not display any overlapping. Therefore, DHB was found to be a proper internal standard (IS) used for determination analysis of safrole and isosafrole.

CHAPTER - 7

Conclusion

7.1. Conclusion

In the United States, it was once widely used as a food additive in root beer, sassafras tea, and other common goods, but was banned by the Food and Drug Administration (FDA) after its carcinogenicity in rats was discovered (Choong and Lin, 2000). Today, safrole is also banned for use in soap and perfumes by the International Fragrance Association. According to a 1977 study of the metabolites of safrole in both rats and humans, two carcinogenic metabolites of safrole found in the urine of rats, 1-hydroxysafrole and 3-hydroxyisosafrole were not found in human urine. The Gas Chromatographic analysis developed a modest, rapid and accurate method for quantitative determination of safrole and isosafrole in seven commercial soft drinks purchased in the market. By using this method, various soft drink samples were added with a proper volume of water-soluble internal standard DHB and then 0.1 µl of mixture was injected directly into GC for analysis without any sample pretreatment procedure. From the method we determined that there may be present of safrole (0.39-55 µg/ml), we cannot confirm it because we could not do the validation test and recovery test but isosafrole cannot be determined. One reason for this, isosafrole may be used after derivitization. Results showed that all soft drinks contain safrole and or isosafrole, and most exceeded 1-5 times the regulated concentration of 1µg/mL.

CHAPTER - 8

Reference

Reference

○ Borchert, Wislocki and Miller, 2012, *The Metabolism of the Naturally Occurring Hepatocarcinogen,* Available at: http://cancerres.aacrjournals.org/content/33/3/575.full.pdf (Accessed: 3rd May, 2012).

○ Carlson M. & Thompson R.D, 1997, "*Liquid chromatographic determination of safrole in sassafras-derived herbal products*".

○ ChemIDplus, 2009, *National Library of Medicine*, Available at: http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp and select Registry Number and search on CAS number (Accessed: 9th September, 2009).

○ Choong Y M and Lin H J, 2000, *A Rapid and Simple Gas chromatographic Method for Direct Determination of safrole in soft drinks*, Journal of Food and Drug Analysis, vol. 9, No. 1, Pages: 27-32.

○ European Commission, (2001), Opinion of the Scientific Committee on Food on the safety of the presence of safrole (1-allyl-3,4-methylene dioxy benzene) in flavorings and other food ingredients with flavouring properties Available at: http://ec.europa.eu/food/fs/sc/scf/out116_en.pdf (Accessed: 3rd May, 2012).

○ European Commission, (2003), Opinion of the Scientific Committee on Food on Isosafrole Available at: http://ec.europa.eu/food/fs/sc/scf/out188_en.pdf (Accessed: 3rd May, 2012).

○ Gas chromatography, Available at: http://en.wikipedia.org/wiki/Gas_chromatography (Accessed: 2nd June, 2012).

○ Heikes D.L., 1994, "*SFE with GC and MS determination of safrole and related allylbenzenes in sassafras teas*".

○ HSDB, 2009, *Hazardous Substances Data Bank, National Library of Medicine* Available at: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB and search on CAS number (Accessed: 14th July, 2009).

○ Introduction of Gas chromatography, Available at: http://teaching.shu.ac.uk/hwb/chemistry/tutorials/chrom/gaschrm.htm (Accessed: 2nd June, 2012).

○ Khlaeo-om T, *Identification of safrole*, Bureau of Drug and Narcotic, Department of Medical Science, Nonthaburi.

○ Report on Carcinogens, 2011, Twelfth Edition, National Toxicology Program, Department of Health and Human Services, Page 374.

○ The Chemistry Encyclopedia, (2005) *Safrole* Available at: http://www.chemistrydaily.com/chemistry/Safrole (Accessed: 3rd May, 2012).

○ IPCS INCHEM, 2012 SAFROLE Available at: http://www.inchem.org/documents/jecfa/jecmono/v16je22.htm (Accessed: 3rd May, 2012).

○ ISO 7355, 1985, *Oils of sassafras and nutmeg , Determination of safrole and cis- and trans-isosafrole content*, Gas chromatographic method on packed columns.

○ Wrighton et al. 1986, Identification of a human liver cytochrome P-450 homologous to the major isosafrole-inducible cytochrome P-450 in the rat, 29(4):405-10.