Direct Determination of Safrole and Isosafrole from Soft Drinks by Gas Chromatographic Method

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2008-3-70-006

Department of Pharmacy

East West University

19th June, 2012

A thesis paper submitted to the Department of Pharmacy in conformity with the requirements for the Degree of Bachelor of Pharmacy

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This Gas Chromatography analysis study is done in the Advance Research Laboratory of East West University

Authorization by Head of the Department

This is to endorse that the dissertation entitled "Direct Determination of Safrole and Isosafrole from Soft Drinks by Gas Chromatographic Method*"* is a research work done by Maruf Kamal under the supervision of Kh. Tanvir Ahmed, Lecturer, Department of Pharmacy, East West University.

Dr. Sufia Islam

Chairperson and Associate professor

Department of Pharmacy

East West University

Certificate by the Supervisor

This is to certify that the dissertation entitled "Direct Determination of Safrole and Isosafrole from Soft Drinks by Gas Chromatographic Method*"* is a research work done by Maruf Kamal, in partial fulfillment of the requirement for the Degree of Bachelor of Pharmacy. It is a record of original research work carried out by me during fall 2011 to spring 2012 under the supervision and guidance of me.

Kh. Tanvir Ahmed

Lecturer, Department of Pharmacy

East West University

Affirmation by the Candidate

I, Maruf Kamal hereby declare that this dissertation entitled "Direct Determination of Safrole and Isosafrole from Soft Drinks by Gas Chromatographic Method*"* submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for award of the degree of bachelor of pharmacy. It is a record of original research work carried out by me during fall 2011 to spring 2012 under the supervision and guidance of Kh. Tanvir Ahmed, Lecturer, Department of Pharmacy, East West University.

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This thesis paper is dedicated to my parents------

Late Kamal Uddin

and

Sheela Kamal.

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Abstract

A high concentration (0.5%) of safrole and isosafrole has been shown to increase the occurrence rate of malignant tumors in mice. The major toxicity of safrole and isosafrole come from their carcinogenic nature after oxidation. For this reason, a simple and rapid method was developed to determine the safrole and isosafrole in soft drink using a semi-polar column Rtx 625 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 0.25 μg/mL. Seven commercial soft drinks were analyzed by the current method, and results indicated that these seven commercial soft drink samples contained safrole but isosafrole cannot be identified. One reason for this, isosafrole may be used after derivitization.

Key words: Soft drink, Safrole, Isosafrole, Direct injection, Gas Chromatography, Quantitative analysis

Chapter 1: Introduction

1.1 Introduction

Safrole is a natural constituent of a number of spices such as nutmeg, mace, cinnamon, anise, black pepper and sweet basil. The most important dietary sources are nutmeg, mace and their essential oils. Safrole is also present in cola drinks. 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 ontheir respective food consumption data and on concentration levels documented or assumed. (Borchert *et al*., 2012)

Isosafrole was evaluated by the International Agency for Research on Cancer (IARC) in 1975. 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. 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)

1.2 Safrole

Safrole or 4-allyl-l, 2-methylenedioxybenzene is a plant constituent that is a hepatotoxin for animals and man. It is a major (70 to 80%) component of oil of sassafras and certain oils from the Heterotropa genus, a minor component (1 to 4%) of some other essential oils, and a very minor (ca. 0.1%) component of oils of nutmeg, mace, and cinnamon leaf. 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. In that year the use of safrole as a food additive was banned in the United States after a study by the United States Food and Drug Administration

indicated that safrole was a weak hepatocarcinogen in the rat. In the early 1960's showed that administration of 0.04 to1.0% of safrole in the diet of male and female rats for 150 days to 2 years produced hepatic adenomas and hepatic carcinomas. Further confirmatory data were published shortlythereafter. Subsequently, it was noted that hepatomas were also induced in mice by the long-term administration of safrole beginning at the 7th day of life. Likewise, Epstein et al. (11 have reported that 58% ofmale Swiss mice killed 1 year after 4 s.c. injections of safrole (total dose of 6.6 mg) in infancy had hepatomas as compared to a 0%incidence in female mice treated with the same dose of safrole and a 6% incidence in control male mice. Thus, under appropriate conditions safrole can act as a moderately potent hepatocarcinogen. (Borchert, Wislocki and Miller, 2012)

1.2.1 Chemical characterization

Safrole (chemical formula: $C_{10}H_{10}O_2$) is a colorless or slightly yellow oily liquid. 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. Safrole was once widely used as a food additive in root beer, sassafras tea, and other common goods. However, the FDA barred the use of safrole after it was shown to be mildly carcinogenic. Today, safrole is used for making soap and perfumes, aroma therapeutic agents, and the insecticide heliotropin (piperonalbutoxide). 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)

Name: Safrole

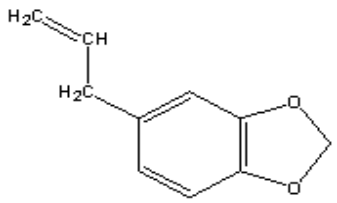
Synonyms: 1-allyl-3,4-methylene dioxy benzene; 5-allyl-1,3-benzodioxole;4-allyl-1, 2-methylene dioxy benzene

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

CAS No: 94-59-7

M.W.: 162.2

Structure:



1.2.2 Flavor concentration

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)

1.2.3 Intake estimate

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)

1.2.4 Biochemical aspects

1.2.4.1 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 therat. 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)

1.2.4.2 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 and guinea-pig. These safrole metabolites are Mannich bases which are believed to be formed by oxidation of the allyl group to yield a vinyl ketone 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. (IPCS INCHEM, 2012)

1.2.4.3 Hazard identification/characterization

Extensive metabolism occurs in animals (rat, mouse, and guinea-pig). The following metabolic transformations occur in all the investigated species, but the relative extent of each modification varies across species and strains:

- allylic hydroxylation to 1'-hydroxysafrole (HOS) and isomerization to 3'-hydroxysafrole excreted in conjugated form

- oxidation and cleavage of the methylene di-oxy moiety, leading to 4-allyl catechol, easily oxidized to 4-allyl-o-quinone
- epoxidation of the allylic side chain or, in small extent, of the aromatic ring
- gamma oxidation of the allylic side chain leading to a carboxylic acid (piperonylicacid) further conjugated with glycine (piperonylglycine).

The main metabolic pathways in all animal species are a) and b). Both these pathways as well as epoxidation form reactive metabolites able to react with macromolecules. However epoxide formation is low, whereas HOS and 4-allyl catechol represent the main metabolites. HOS can be conjugated by sulphate by 3'-phosphoadenosine-5-phosphosulfate (PAPS) or acetate; both esters can be easily split producing, in the case of sulphate, anelectrophilic carbonium ion. Small doses of safrole radio labeled with 14C in the 1'-position of the allylic side chain were absorbed rapidly and excreted almost completely via the urinein 24 hours in both man (0.165 mg or 1.655 mg) and rat (0.63 mg/kg). In rats treated with a higher dose (750 mg/kg) only 25% safrole was excreted in 24 hours. 1, 2-Dihydroxy-4-allylbenzene was the main urinary metabolite in both species; HOS was detected in the urine of rat and not in the urine of man. This does not exclude the formation of small amounts of HOS and subsequent binding to macromolecules (e.g. DNA) in the liver. (EUROPEAN COMMISSION, 2001)

1.2.4.4 Toxicity

"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 tea spoonful 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 hydroxylation systems, a property that could lead to toxicity problems if drugs metabolized by these enzymes are administered together with sassafras teas. (EUROPEAN COMMISSION, 2001)

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 off spring exposed to safrole in utero; none of the other experimental and control animals developed these tumors. Only male off spring 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)

1.2.4.5 Acute toxicity

The oral LD50 was reported to be 1950 mg/kg bw for rats and 2350 mg/kg bw for mice.

1.2.4.6 Short-term toxicity

Groups of young adult Osborne-Mendel rats of both sexes received safrole by oral intubation at doses of 250, 500 and 750 mg/kg bw/day up to 105 days.At doses of 750 mg/kg/d for 19 days 9/10 animals died; with 500 mg/kg/d only 1/10animals died after 46 days; with 250 mg/kg/d no animal died within 34 days and the following effects were observed: liver hypertrophy and focal necrosis plus slight fibrosis, fatty infiltration (steatosis), bile duct proliferation, adrenal enlargement with fatty infiltration. (EUROPEAN COMMISSION, 2001)

1.2.4.7 Long-term and Chronic toxicity

In a study Groups of 25 male and 25 female Osborne-Mendel rats were fed 0, 100, 500, 1000 and 5000 mg/kg (0, 0.01, 0.05, 0.1 and 0.5 %) safrole in the diet for two years. The levels correspond approximately to average daily intakes of 0, 5, 25, 50, or 100 mg/kg bw/day. Changes in the liver including benign and malignant tumors (hepatic cell adenoma, hepatocholangioma, hepatic cell carcinoma and hepatocholangiocarcinoma) were observed. Reduced body weight gain was reported in both sexes at the highest dose; mildanemia and leukocytosis were also reported. The liver injury was rated as very slight at 100 mg/kg, slight at 500 mg/kg, slight to moderate at 1000 mg/kg, and moderate to severe at 5000 mg/kg. Tumor incidence was significantly increased at 5000 mg/kg rats with malignant tumors, 5 with benign tumors versus 2 and 1, respectively, in the controls. Tumor incidence in the other groups was: 8 benign on 1000 mg/kg, 2 malignant and 1 benign on 500 mg/kg, and 1 benign on 100 mg/kg. Groups of 25 male and 25 female Osborne-Mendel rats were fed 0, 100, 500, 1000 and 5000 mg/kg (0, 0.01, 0.05, 0.1 and 0.5 %) safrole in the diet for two years. The levels correspond approximately to average daily intakes of 0, 5, 25, 50, or 100 mg/kg bw/day. Changes in the liver including benign and malignant tumors (hepatic cell adenoma, hepatocholangioma, hepatic cell carcinoma and hepatocholangiocarcinoma) were observed. Reduced body weight gain was reported in both sexes at the highest dose; mild anemia and leukocytosis were also reported. The liver injury was rated as very slight at 100 mg/kg, slight at 500 mg/kg, slight to moderate at 1000 mg/kg, and moderate to severe at 5000 mg/kg. Tumor incidence was significantly increased at 5000 mg/kg (14 rats with malignant tumors, 5 with benign tumors versus 2 and 1, respectively,

in the controls. Tumor incidence in the other groups was: 8 benign on 1000 mg/kg, 2 malignant and 1 benign on 500 mg/kg, and 1 benign on 100 mg/kg. (EUROPEAN COMMISSION, 2001)

1.2.4.7.1 Rats

Weanling Osborne-Mendel rats were fed safrole in the diet at levels of 0 (35M & 35F), 1000 (10M & 10F), 2500 (10M & 10F), 5000 (25M & 25F) and 10000 mg/kg (10M &10F) (0, 0.1, 0.25 and 1%) for two years. Growth was depressed in both sexes at the two highest doses and in the females at 1000 mg/kg. At 10000 mg/kg no rats survived beyond 62 weeks. These animals showed testicular atrophy, stomach atrophy and changes in the liver including tumor formation. The livers were enlarged, irregularly nodular, with single and multiple tumor masses. Microscopically, there was hepatic cell enlargement which resulted in nodule formation. The nodules tended to progress in: cystic necrosis, cirrhosis and hyperplasia leading to tumors. The liver damage was slight at 1000 mg/kg and lacked tumors and cirrhosis, moderate at 2500 but lacked cirrhosis and severe at 5000 mg/kg, where there was a statistically significant increase of malignant livertumors. There was mild hyperplasia of the thyroid at 5000 mg/kg and an increase of chronic nephritis at lower doses. (EUROPEAN COMMISSION, 2001)

1.2.4.7.2 Dogs

Two males and two female dogs were given orally safrole at 5 and 20 mg/kg bw for six years. Liver changes, and no tumors were observed at both doses. At the higher dose there was liver enlargement with a nodular surface. At the lower dose the liver changes were focal necrosis, bile-duct proliferation, fatty metamorphosis, and hepatic cell atrophy and leucocytes infiltration. (EUROPEAN COMMISSION, 2001)

1.2.4.8 Carcinogenicity

1.2.4.8.1 Mice

(C57BL/6 x C3Hanf)F1 or (C57BL/6 x AKR)F 1 hybrid mice of 7 days of age (18 M 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 or 5000 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. The results of a large multipart study carried out by Miller *et al.* (1983) are summarized as follows:

- Pre weanling CD-1 mice (groups of about 100 animals of both sexes) were administered 400 mg/kg of safrole ten times, twice weekly by gavage. The study wasterminated at 14 months. The mice bearing hepatoma were 30/49 (61%) versus 14/59(24%) in controls in males, and 7/48 (13%) in females versus 1/47 (2%) in controls.The lung adenomas in males were 3/49 versus 0/59 in controls and in females 3/48 versus 2/47 in controls.
- Pre weanling male CD-1 mice (groups of about 50 animals) received i.p. injections of safrole (total dose: 81 mg/kg bw) and of its metabolite 1'-hydroxysafrole (total dose: 45 mg/kg bw) distributed by four weekly injections in the first three weeks of life.The experiment was terminated at 12 months. The mice bearing hepatoma were 33/48 (67%) versus 11/42 (26%) in the controls. The lung adenomas were 7/48 (14%) versus 1/42 (2%) in controls. For 1'-hydroxysafrole the mice bearing hepatoma were 31/46 (65%) versus 11/42 (26%) in controls. The lung adenomas were 5/46 (10%) versus 1/42 (2%) in controls.
- Groups of 50 CD-1 female mice, approximately 8 weeks old, were maintained for 12 months on grain diets containing 2300 or 4600 mg/kg of safrole. According to the authors the dietary levels correspond to an average daily intake of 150-300 and 300-600 mg/kg bw. The experiment was terminated at 19 months. The mice bearing hepatomas were at the low dose 23/34 (68%) and at the high dose 27/39 (69%) versus 0/39 in controls.
- Groups of 40 CD-1 female mice were treated topically four days/week for 6 weeks with safrole-2', 3'-oxide and 1'-hydroxysafrole-2', 3'-oxide. 1 week afterthe last dose croton oil was applied. The experiment was terminated at 40 weeks. Skin papillomas were 14/40 (35%) in mice treated with safrole-2', 3'-oxide and 33/40(82%) in mice treated with 1'-hydroxysafrole-2', 3'-oxide, versus 3/40 (7%) papillomasin acetone controls.

- Groups of 25 female A/J mice were administered by i.p. injections safrole (total dose 3900 mg/kg bw), 1'-hydroxysafrole (total dose 2000 mg/kg bw) and 1'-hydroxysafrole-2', 3'-oxide at two doses (total doses 2100 mg/kg and 4200 mg/kg bw).

The injections were given twice weekly for 12 weeks. The experiment was terminated at 8 months. Lung adenomas were found in 1/19 (5%) mice treated with safrole, in 2/21 (10%) mice treated with 1'-hydroxysafrole, and in 5/18 (28%) and 9/20 (45%) respectively at the low and high doses in mice treated with 1'-hydroxysafrole-2', 3'- oxide. In uninjected controls the lung adenomas were 1/25 (4%). Safrole was administered by gavage to pregnant B6C3F1 mice starting at the 12th day of gestation by 4 doses (total dose 500 mg/kg bw) and to lactating mice from the delivery day (total dose 1500 mg/kg bw). The off spring groups were of 70-100 animals for each sex.

The experiment was terminated at 92 weeks. The mice bearing hepatoma in trans placentally treated mice were 2/63 (3.2%) in males and zero in females. In lactation treated mice the animals bearing hepatoma were 28/85 (34%) in males and 2/80 (2.5%) in females. In controls the hepatomas were 3/100 (3%) in males and absent in females. In the combined trans placental female groups 14/199 (7%) kidney epithelial tumors were found. These tumors were absent in treated males and in both male and female controls. (EUROPEAN COMMISSION, 2001)

1.2.4.8.2 Rats

In the same large study by Miller *et al.* (1983) groups of 20 male Fischer rats were administered by s.c. injection 1'-hydroxysafrole, safrole-2',3'-oxide and 1'-hydroxysafrole- 2',3'-oxide (total doses about 1000, 1100 and 1200 mg/kg bw) twice weekly for 10 weeks. The experiment was terminated at 24 months. Rats treated with 1'-hydroxysafrole developed 11/20 (55%) hepatic carcinomas, whereas no hepatic carcinomas occurred in rats injected with safrole-2', 3'-oxide or 1'- hydroxysafrole-2', 3'-oxide. No tumor was seen in controls injected with trioctanoin only. Whereas 1'-hydroxysafrole did not induce sarcomas at the injection site, one sarcoma was induced by safrole-2', 3'-oxide and four by 1'-hydroxysafrole-2', 3'-oxide. Groups of 15 male CD rats were fed with 5000 mg/kg safrole for 18 months. One group was administered phenobarbital in drinking water (0, 1%). The experiment was terminated at 22 months. The rats bearing

hepatomas were 3/15 (20%) in safrole treated rats, and 12/15 (80%) in rats treated with safrole and phenobarbital. In the controls no hepatomas were found. (EUROPEAN COMMISSION, 2001)

1.2.4.9 Genotoxicity

1.2.4.9.1 In vitro

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; gene mutations and sister chromatid exchanges (SCEs). Itinduced unscheduled DNA synthesis (UDS) in cultured rat hepatocytes. It induced DNA damage (single-strandbreaks) in cultured rat hepatocytes. (EUROPEAN COMMISSION, 2001)

1.2.4.9.2 In vivo

Safrole was positive in the *in vivo* i.p. host-mediated assay with *S. typhimurium*s train TA1535 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 withthe 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 chromosom aberrations, SCE and DNA adducts in the liver of rats. (EUROPEAN COMMISSION, 2001)

1.2.4.9.3 DNA adduct formation

DNA adducts (two major and two minor) were detected in rat liver DNA after single doses of safrole at 1 or 100 mg/kg bw. These results suggest that the cytogenetic effects may result from covalent DNA modification in the rat liver. Previous studies have shown that two major DNA adducts are formed in N2 position of guanine after administration of safrole or 1'-hydroxysafrole to mice; minor reaction occurs with N6 of adenine. The covalent binding index (CBI) value was about 30 for safrole, consistent with its weak hepatocarcinogenic activity. However, it has been shown that DNA adduct formation in mouse liver was linear over a dose span of 10000-fold down to the lowest dose range, i.e. 1-10 g (6-60 nmol) safrole/mouse, with no indication of a threshold; moreover the persistence of DNA adducts was rather long (up to140 days in the liver of treated mice) and independent from their levels. For the high dose (10 mg/mouse), a slight deviation from linearity was observed (hepatotoxic effect with regenerative hyperplasia in liver tissue). (EUROPEAN COMMISSION, 2001)

In another study safrole-DNA adducts, and to a greater extent, myristicine-DNA adducts were identified in livers of mice given cola beverages instead of drinking water. Inhibition of both DNA adduct formation and carcinogenicity of 1'-hydroxysafrole was shown in the liver of mice deficient in the synthesis of PAPS or treated with pentachlorophenol, a strong inhibitor of sulfotransferase and PAPS formation. In rodents, trans placental passage of the reactive metabolites of safrole was demonstrated by the presence of liver DNA adducts in the fetuses of mothers dosed with safrole. The effects of pregnancy on the covalent binding of several carcinogens to DNA were investigated in ICR mice. Pregnancy lowered the binding of the ultimate carcinogenic metabolite of B (a) P and increased 2.3 and 2.5 fold the binding of safrole and 1'-hydroxysafrole to liver and kidney DNA. (EUROPEAN COMMISSION, 2001)

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 Isosafrole

Isosafrole 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 2002 CEFS was informed that isosafrole does occur in relevant source materials for flavorings and CEFS decided to reconsider isosafrole at its next meeting. (IPCS INCHEM, 2012)

1.3.1 Exposure assessment

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 isomerization 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. By 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 Chemical characterization

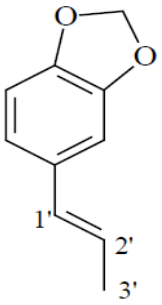
Isosafrole is a propenylbenzene derivative.

Name: Isosafrole

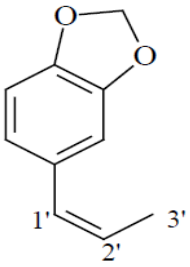
Synonyms: 5-(1'-Propenyl)-1, 3-benzodioxole; 1, 2-(methylenedioxy)-4-(1'-propenyl) benzene

CAS no.: 120-58-1

Structure:



(*E*) - Isosafrole



(*Z*) - 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)

1.3.3 Hazard identification / characterization

1.3.3.1 Absorption, distribution, metabolism 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 livercarcinogen in rats and mice. Liver DNA adduct formation (32P post labeling) is low. All genotoxicity 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)

1.3.3.2 Acute toxicity

LD 50 oral (mice): 2.47 g/kg bw. LD 50 oral (rats): 1.34 g/kg bw.

1.3.3.3 Sub-acute/sub chronic toxicity

1.3.3.3.1 Rat

Rats (Osborne-Mendel strain, 10 males and 10 females) given 10000 mg isosafrole/kg in diet showed growth retardation in both sexes. No rats survived beyond 11 weeks of treatment. The

livers were enlarged and microscopically slight hepatic cell hypertrophy, which was usually focal and resulted in the formation of nodules, was shown Daily doses of 460 mg isosafrole/kg bw given by stomach tube for four days to rats (3 males and 3 females) produced severe liver lesions consisting of discoloration, enlargement, and loss of normal texture. No histopathology was performed. Two of the rats died during the test period. Apparently, no control group was included in the study (Taylor et al., 1964). Oral intubation to young Osborne- Mendel rats of both sexes of 500 mg isosafrole/kg bw/day for 41 days resulted in mortality ratios of 8/10 rats and of 250 mg isosafrole/kg bw/day for 34 days of 2/10 rats. All ten rats of the control group survived. The following effects were observed: liver hypertrophy and slight focal necrosis and fibrosis, slight degree of focal fatty metamorphosis and bile duct proliferation. (IPCS INCHEM, 2012)

1.3.3.4 Chronic toxicity/Carcinogenicity

1.3.3.4.1 Mouse

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 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)

1.3.3.4.2 Rat

Isosafrole was given in the diet for two years to Osborne-Mendel rats at 0, 1,000, 2,500 or 5,000 mg isosafrole/kg (control group: 35 males and 35 females). At the two lowest dose levels (10 males and 10 females per group) there was slight growth retardation in females. Microscopically, slight hepatic cell hypertrophy but no primary hepatic tumors were reported. At 2,500 mg/kg in the diet there was also slight hyperplasia in the thyroid. At the highest dose, 5,000 mg/kg in the diet (25 males and 25 females) growth retardation was reported in both sexes. The liver was enlarged and microscopically slight hepatic cell hypertrophy, which was usually focal and resulted in the formation of nodules, was shown. Five rats had primary hepatic tumors (two adenomas and three carcinomas). Slight hyperplasia in the thyroid and an increase in the incidence of chronic nephritis were demonstrated. An increased number of interstitial cell tumorswas found in testes. The study is poorly reported, and it does not allow a clear NOEL to be established. No local tumors were observed in 18 male rats given a total of 20 (twice weekly) s.c. injections, each of 3 mg isosafrole (trioctanoin solution) per rat. The rats were examined after surviving 18 months. (IPCS INCHEM, 2012)

1.3.3.5 Genotoxicity

1.3.3.5.1 In vitro

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)

1.3.3.5.2 DNA adduct formation

DNA adduct formation using 32P post labeling analysis was studied in livers from adult female CD1 mice, isolated 24 hours after i.p. administration of different alkenyl benzenes, including isosafrole (2 or 10 mg/mouse). After administration of isosafrole, only low binding to the mouse liver DNA was demonstrated, with the two major DNA adducts formed in the N2-position of

guanine. The low DNA binding could also be expressed by the covalent binding index (CBI) value of about 1 for isosafrole. For comparison the CBI values for safrole, estragole and methyl eugenol were all about 30. (IPCS INCHEM, 2012)

1.4 Other studies

Isosafrole is a known inducer of some of the liver enzymes of the cytochrome P-450 group in rodents, especially CYP1A2. Finally, it cannot be completely excluded that high exposure to isosafrole may give rise to some isomerization of 3'-hydroxyisosafrole to 1'-hydroxysafrole, the anticipated proximate carcinogen of safrole. (IPCS INCHEM, 2012)

1.4.1 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 asignificant effect on ethanol sleeping time. In case of acute toxicity,

Table: 1 Pharmacological study in case of acute toxicity

	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 DNAin 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)

Chapter 2-Study Hypothesis

2.1 Hypothesis

This thesis is done because in some previous studies we have found that there was use of safrole and isosafrole (e.g. Sarsaparilla, Coca-Cola, Pepsi, Root Sar and Apple Cider) in different soft drinks as flavoring agent. Recently Food and Drug Administration prevents the use of safrole and isosafrole in soft drinks. So the hypothesis of this thesis is, there may be presence of safrole and isosafrole in the soft drinks as flavoring agent those are under analysis.

2.2 Aims and objective of the study

Safrole and isosafrole has been shown to increase the occurrence rate of malignant tumors in mice at a high concentration (0.5%). The major toxicity of safrole and isosafrole show carcinogenic toxicity after oxidation.

Safrole induced DNA damage (single-strand breaks) in cultured rat hepatocytes. 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 rats exposed *in vivo*.

On the other hand, Isosafrole is carcinogenic in mice and rats, producing liver tumors following its oral administration. IARC in 1987 concluded for isosafrole that there were no adequate human carcinogenicity data but limited evidence of animal carcinogenicity.

So, the aim and objective of this experiment is to determine the concentration of safrole and iso safrole in soft drinks those are carcinogenic to ensure the good health and safety of our life.

2.3 Significance of the study

Safrole (4-allyl-1, 2-methylene dioxybenzene) is one of the 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 and piper betel flower. Safrole and isosafrole were once used extend 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. The major toxicity of safrole and isosafrole come from their carcinogenic nature after oxidation. Safrole is oxidized into 1-hydroxysafrole by many mammals and whose derivatives including isosafrole and dihydrosafrole, which are all both carcinogenic. Safrole and isosafrole were once used extensively as a seasoning in soft drinks. 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 1 μg/mL, according to food additive regulations. 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. (CHOONG and LIN, 2000)

Chapter 3: Methodology

3.1Materials and Method

3.1.1 Materials

Seven various commercial soft drinks, including Pepsi, Speed, Mojo, Tiger, Virgin, Black horse, and Mirinda, were purchased from convenience stores between January and March 2012. Safrole, its isomer isosafrole and standards 1, 4-dihydroxybenzene (DHB), Methanol, each have a purity of over 98%. Among them, isosafrole consisted of cis-isosafrole and trans-isosafrole.

3.1.2 Chromatographic condition

Column: Rtx 625 (30m x 0.25mm x 0.25um, polysiloxane, mid polar polysiloxane)

Oven temperature:

Time	0-3 min	3-18 min	I8-21 min
Temperature	120° C	120° C-150° C	150° C- 300°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: 300 ml/min

Detector temperature: 290°C

3.1.3 Standard

Safrole (4-allyl-1, 2-methylene dioxybenzene) and isosafrole [1, 2-(methylenedioxy)-4-(1'-propenyl) benzene]

3.1.4 Internal standard

1, 4-dihydroxy benzene (DHB)

3.2 Preparation of standard and internal standard solution

3.2.1 Preparation of standard

- Safrole (0.112% w/v) was prepared. 0.112gm/ml safrole was taken into 100 ml volumetric flask. Then methanol was added up to the volume 100 ml.
- Isosafrole (0.112% w/v) was prepared. 0.112gm/ml isosafrole was taken into 100 ml volumetric flask. Then methanol was added up to the volume 100 ml.

3.2.2 Preparation of Internal standard solution

- 1, 4-dihydroxy benzene (0.1% w/v) was prepared. 0.1gm dihydroxy benzene was taken into 100 ml volumetric flask. Then methanol was added up to the volume 100 ml.

3.3 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 then 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.

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

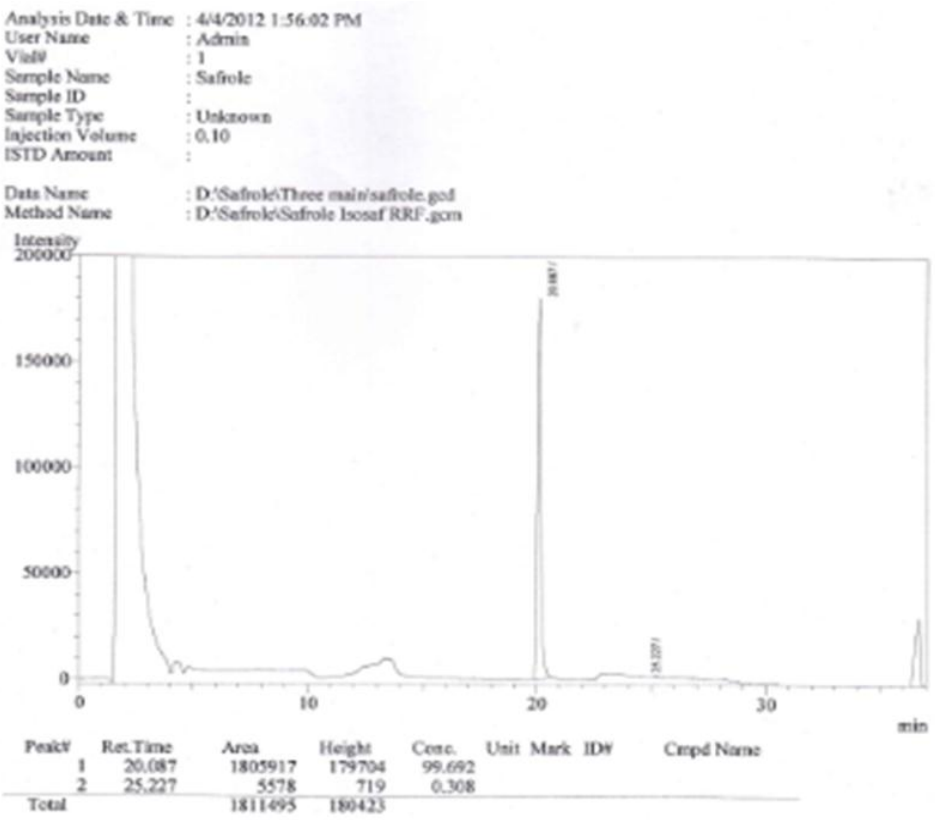


Figure 1: Chromatogram of standard safrole

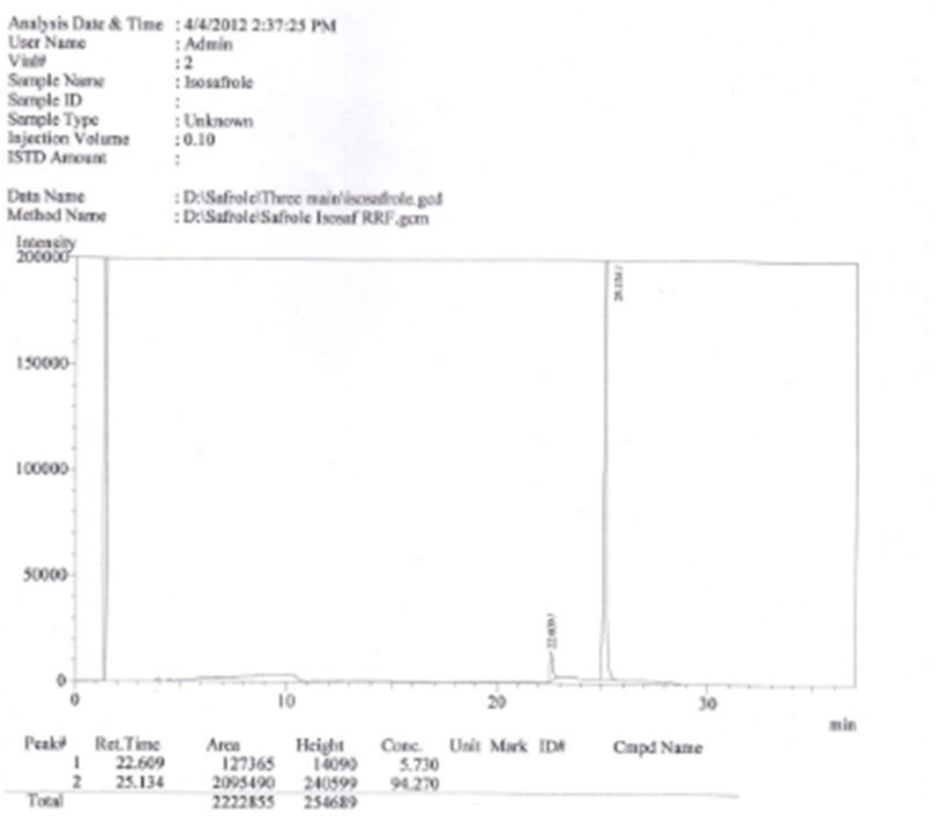
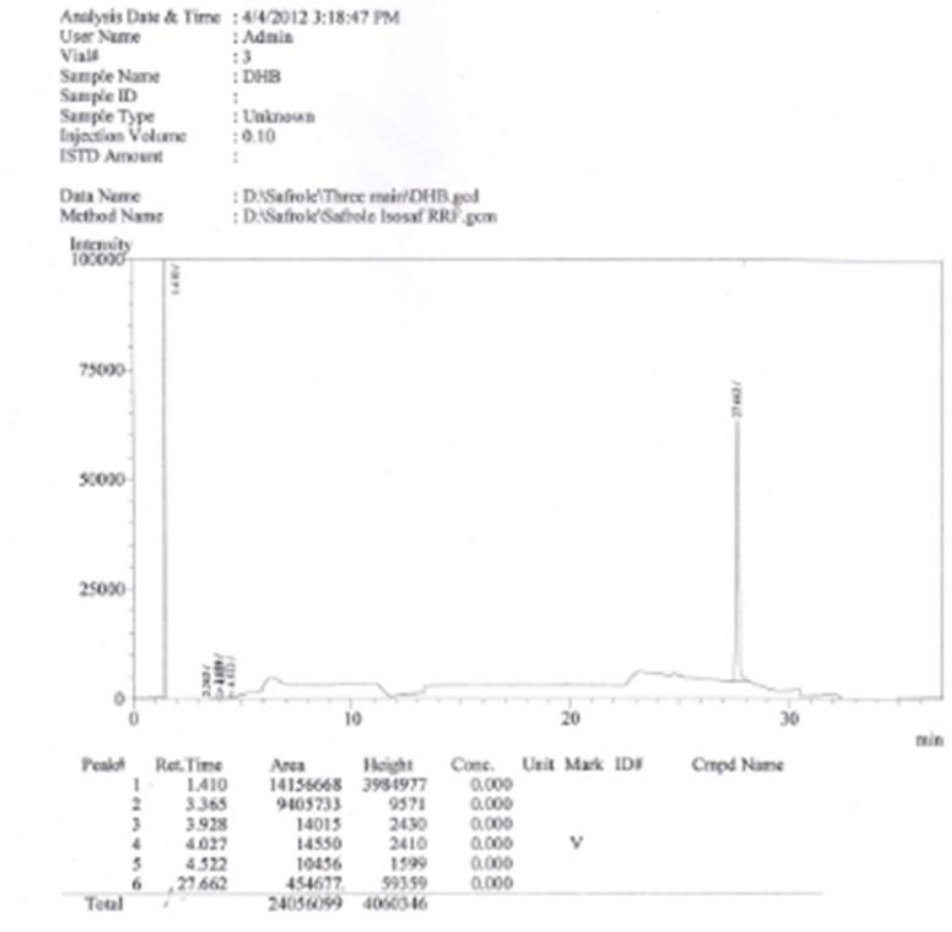


Figure 2: Chromatogram of standard Isosafrole



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 3: Chromatogram of internal standard 1, 4-dihydroxy benzene (DHB)

From the chromatograms, we can see that for the standard safrole we have got a peak with the retention time 20.087 min and the GC peak area is 1805917. For the standard isosafrole we have got a peak with the retention time 25.134 min and the GC peak area is 2095190. For the internal standard 1, 4-dihydroxy benzene (DHB) we have got a peak with the retention time 27.662 min and the GC peak area is 454677.

Chapter 4-Calculation

4.1 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, 4-dihydroxybenzene (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:

$RRF = (A_s/W_s) \div (A_{is}/W_{is})$..... (1)

A_s = GC peak area of safrole or isosafrole

A_{is} = GC peak area of DHB

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

W_{is} = weight (µg) of DHB

For safrole,

Density of safrole, is 1.12 gm/ml= 1.12 mg/µl. Then it was diluted 1000 times to make 1.12 mg/ml to prepare the stock solution. This conc. is used in the analysis.

In the ratio 2: 1 :: Safrole: 1, 4-dihydroxy benzene (DHB), the weight of safrole will be,

3 ml= 3000 µl contain 2× 1.12 mg of safrole

Therefore, 0.1 µl contain (2.24× 0.1)/ 3000 mg of safrole

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

= 0.07467 µg of safrole

= 0.075 µg of safrole

The weight of 1, 4-dihy droxy benzene will be,

3 ml= 3000 µl contain 1 mg of 1, 4-dihydroxy benzene (DHB)

Therefore, 0.1 µl contain (1× 0.1)/ 3000 mg of DHB

$= 3.3\times10^{-5}$ mg of DHB

= 0.033 µg of DHB

The RRF value will be,

$RRF = (A_s/W_s) \div (A_{is}/W_{is})$

A_s = GC peak area of safrole = 1179058

A_{is} = GC peak area of DHB = 187543

W_s = weight (µg) of safrole = 0.075 µg

W_{is} = weight (µg) of DHB = 0.033 µg

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

RRF = 2.77

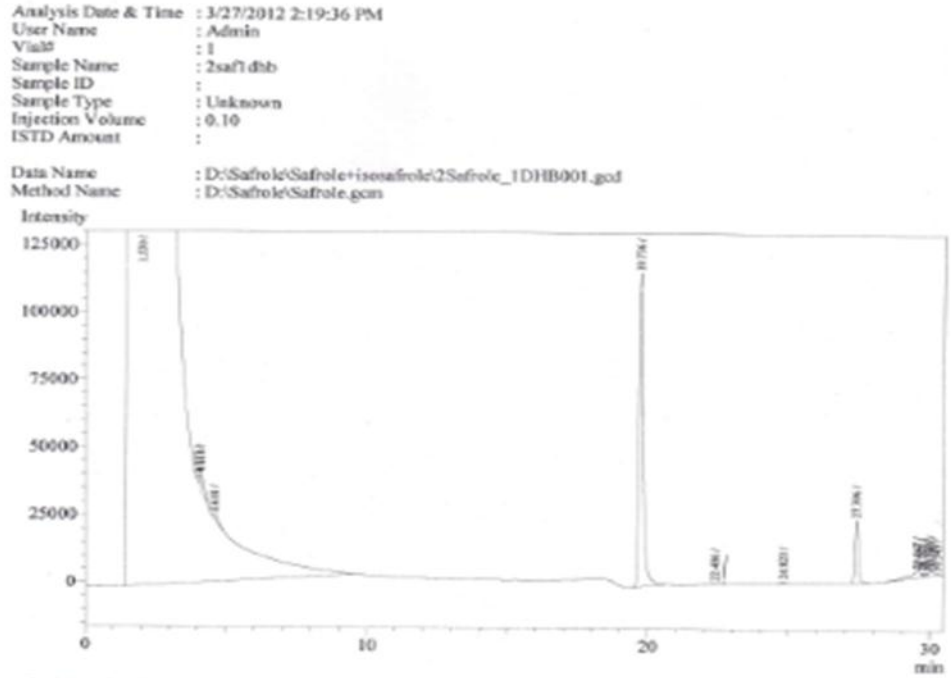
In the ratio 1: 1 :: Safrole: 1, 4-dihydroxy benzene(DHB), the weight of safrole will be,

2 ml= 2000 µl contain 1× 1.12 mg of safrole

Therefore, 0.1 µl contain (1.12× 0.1)/ 2000 mg of safrole

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

= 0.056 µg of safrole



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

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

2 ml= 2000 µl contain 1 mg of 1, 4-dihydroxy benzene (DHB)

Therefore, 0.1 µl contain (1× 0.1)/ 2000 mg of DHB

$= 5\times 10^{-5}$ mg of DHB

= 0.05 µg of DHB

RRF value will be,

$RRF = (A_s/W_s) \div (A_{is}/W_{is})$

A_s = GC peak area of safrole = 870221

A_{is} = GC peak area of DHB = 259680

W_s = weight (µg) of safrole =0.056 µg

W_{is} = weight (µg) of DHB = 0.05 µg

RRF = (870221/0.056) ÷ (259680/0.05)

RRF = 2.99

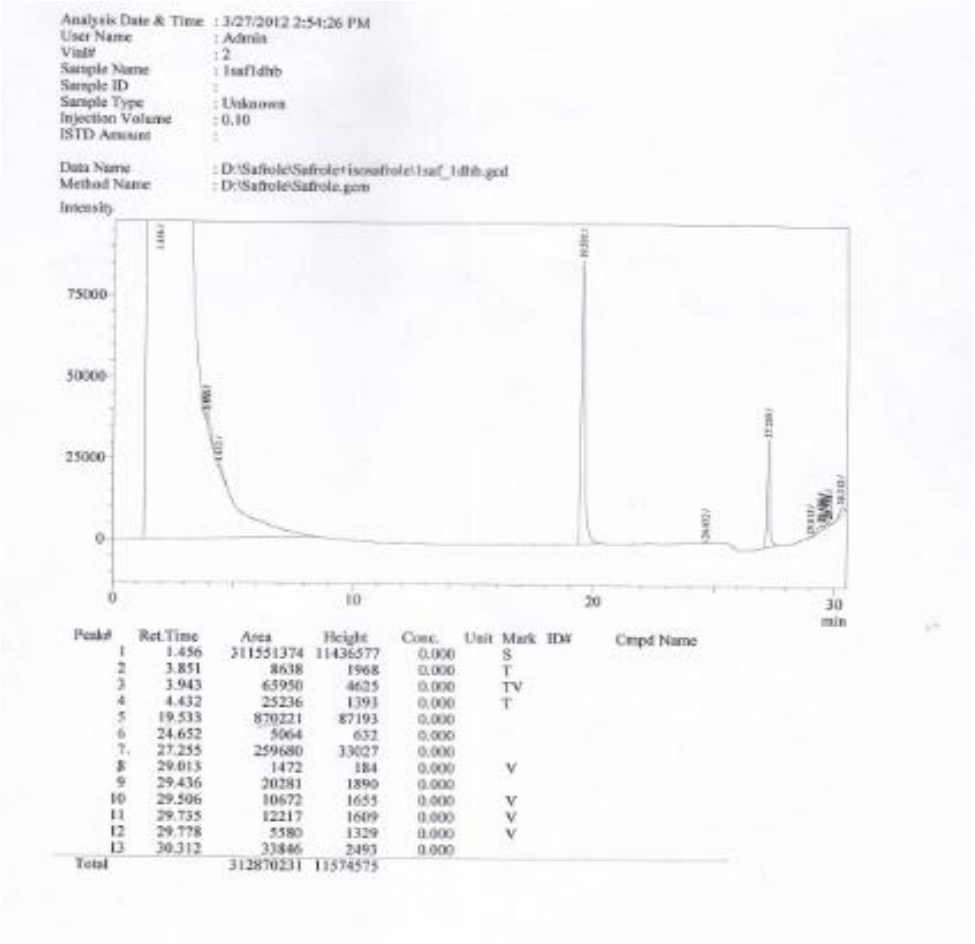
In the ratio 1: 2 :: Safrole: 1, 4-dihydroxy benzene (DHB), the weight of safrole will be,

3 ml= 3000 µl contain 1× 1.12 mg of safrole

Therefore, 0.1 µl contain (1.12× 0.1)/ 3000 mg of safrole

$= 3.373 \times 10^{-5}$ mg of safrole

= 0.0373 µg of safrole



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	1.456	211551374	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 5: Chromatogram of safrole and 1, 4-dihydroxy benzene in (1:1) ratio

The weight of 1, 4-dihy droxy benzene will be,

3 ml= 3000 µl contain 2×1 mg of 1, 4-dihydroxy benzene (DHB)

Therefore, 0.1 µl contain (2× 0.1)/ 3000 mg of DHB

$= 6.67\times10^{-5}$ mg of DHB

= 0.067 µg of DHB

The RRF value will be,

$RRF = (A_s/W_s) \div (A_{is}/W_{is})$

A_s = GC peak area of safrole = 418676

A_{is} = GC peak area of DHB = 239785

W_s = weight (µg) of safrole = 0.0373 µg

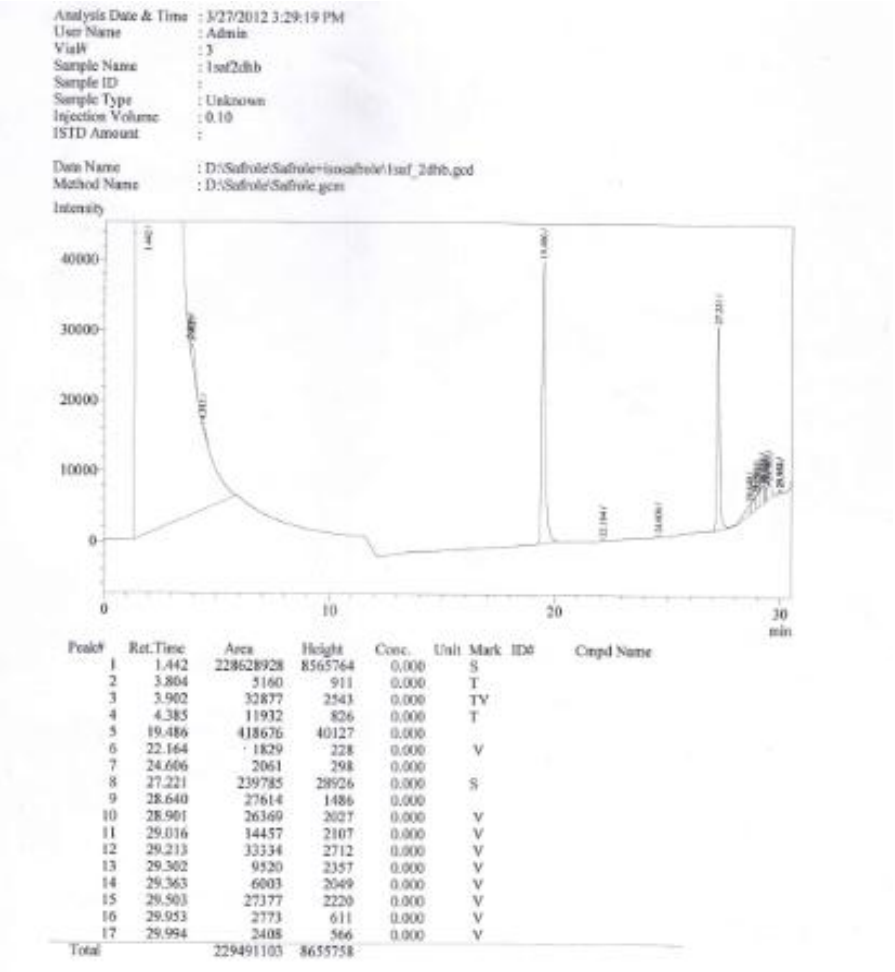
W_{is} = weight (µg) of DHB = 0.067 µg

RRF = (418676/0.037) ÷ (239785/0.067),

RRF = 3.16

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

= 2.97



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

4.2 Determination of Safrole

For every sample,

In the vial, 2ml of sonicated soft drink and 100 µl of 1, 4-dihydroxy benzene (DHB) was taken for analysis so,

2ml sample + 100 µl DHB

Total volume (2ml + 100 µl) = 2000 µl + 100 µl = 2100 µl

So, 2100 µl of preparation contains 100 µg DHB

Therefore, 0.1 µl of preparation contains = (100×0.1)/2100 = 4.76×10^{-3} µg DHB

4.2.1 Speed

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

Here, A_s = GC peak area of safrole = 1836

A_{is} = GC peak area of DHB = 261841

W_s = weight (µg) of safrole

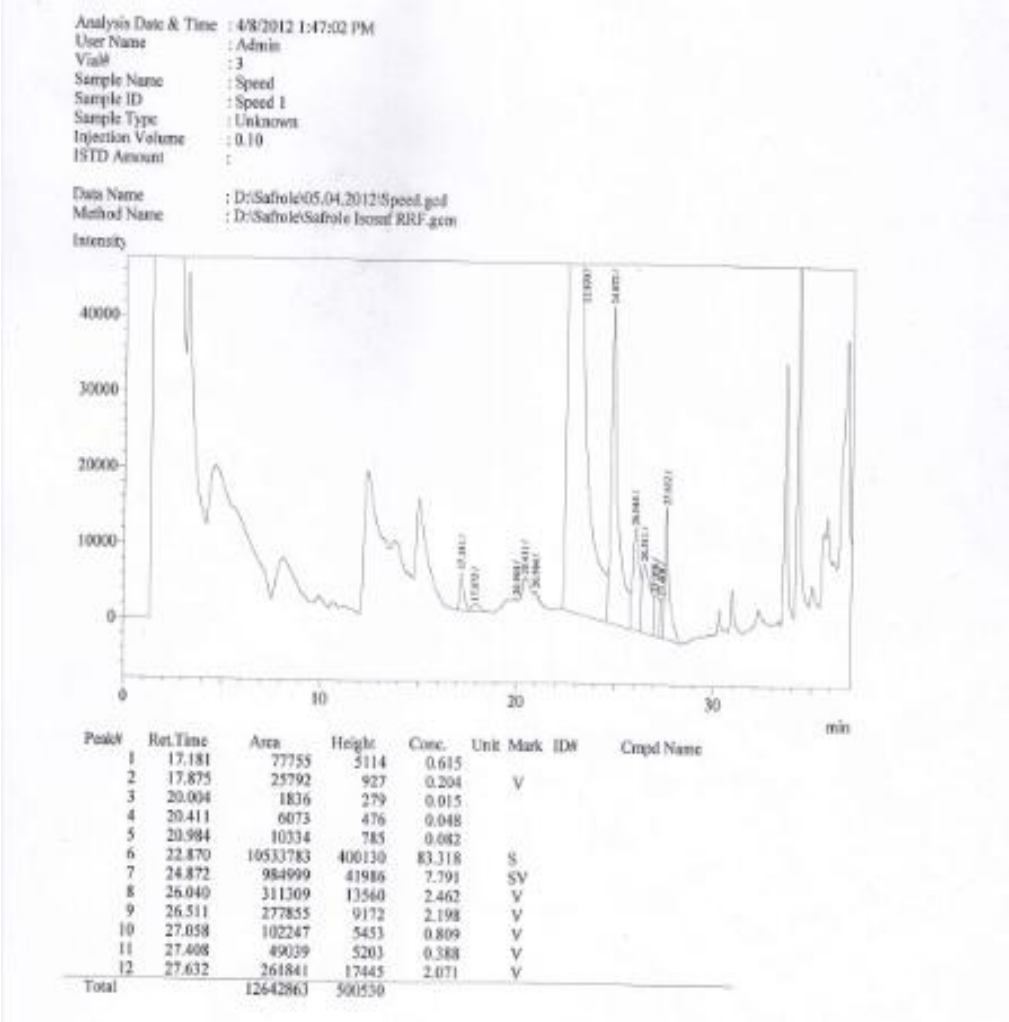
W_{is} = weight (µg) of DHB = 4.76×10^{-3} µg

RRF = 2.97

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

$W_s = (6073/261841) \times (4.76 \times 10^{-3}$ µg /2.97)

$= 3.71 \times 10^{-5}$ µg



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	17.181	77755	5114	0.615				
2	17.875	25792	927	0.204		V		
3	20.004	1836	279	0.015				
4	20.411	6073	476	0.048				
5	20.984	10334	785	0.082				
6	22.870	10533783	400130	83.318		S		
7	24.872	984999	41986	7.791		SV		
8	26.040	311309	13560	2.462		V		
9	26.511	277855	9172	2.198		V		
10	27.058	102247	5453	0.809		V		
11	27.408	49039	5203	0.388		V		
12	27.632	261841	17445	2.071		V		
Total		12642863	500530					

Figure 7: Chromatogram of Speed

0.1 µl contains 3.71×10^{-5} µg of safrole

1 µl contains 37.71×10^{-5} µg of safrole

Therefore, 1000 µl or 1 ml contains $37.71\times 10^{-5} \times 1000$ µg of safrole

= 0.38µg of safrole

So, 250 ml contains 250× 0.38 µg of safrole

= 95 µg of safrole

For Speed, Content of safrole, 95 µg/250 ml

4.2.2 Pepsi

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

Here, A_s = GC peak area of safrole = 21296

A_{is} = GC peak area of DHB = 57562

W_s = weight (µg) of safrole

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

RRF = 2.97

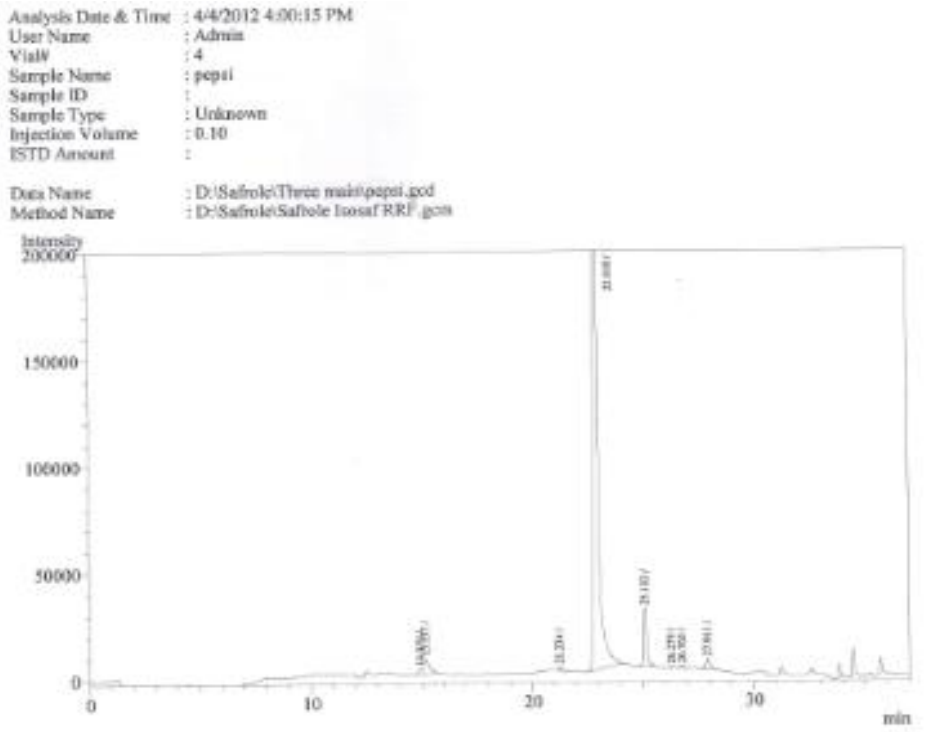
$W_s = (A_s/A_{is}) \times (W_{is}/RRF)$

$W_s = (21296/57562) \times (4.76 \times 10^{-3}$ µg /2.97)

$= 5.93\times 10^{-4}$ µg

0.1 µl contains 5.93×10^{-4} µg of safrole

1 µl contains 59.3×10^{-4} µg of safrole



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	14.929	30090	2846	0.652				
2	15.157	134974	7177	2.925		V		
3	21.234	21296	1868	0.461				
4	23.010	4091081	320518	88.651		S		
5	25.102	261869	27295	5.675				
6	26.279	3049	474	0.066				
7	26.763	14902	1168	0.323				
8	27.911	57562	4833	1.247				
Total		4614823	366179					

Figure 8: Chromatogram of Pepsi

Therefore, 1000 µl or 1 ml contains $59.3\times 10^{-4} \times 1000$ µg of safrole

$= 5.93$ µg of safrole

So, 250 ml contains 250×5.93 µg of safrole

$= 1482.5$ µg of safrole

For Pepsi, Content of safrole, 1482.5 µg/250 ml

4.2.3 Black Horse

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

Here, A_s = GC peak area of safrole = 83562

A_{is} = GC peak area of DHB = 312403

W_s = weight (µg) of safrole

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

RRF = 2.97

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

$W_s = (83562/312403) \times (4.76 \times 10^{-3}$ µg $/2.97)$

$= 4.29 \times 10^{-4}$ µg

0.1 µl contains 4.29×10^{-4} µg of safrole

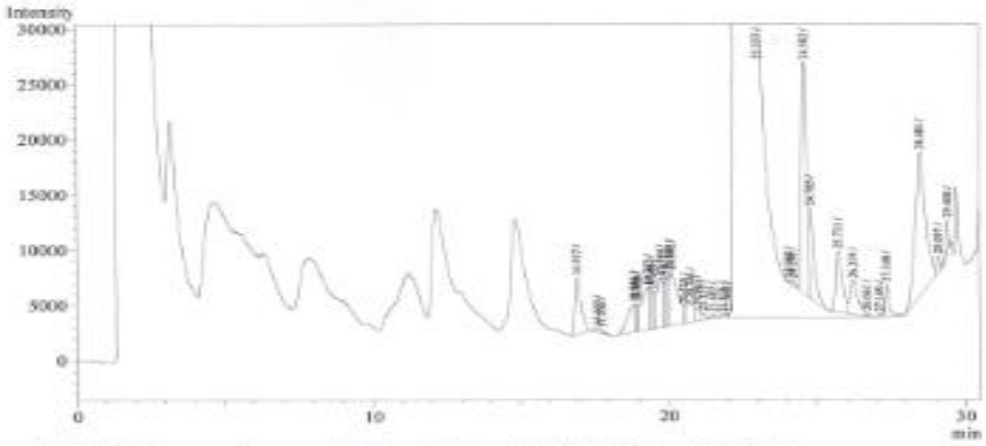
1 µl contains 42.9×10^{-4} µg of safrole

Therefore, 1000 µl or 1 ml contains $42.9\times 10^{-4} \times 1000$ µg of safrole

$= 4.29$ µg of safrole

Analysis Date & Time : 4/5/2012 4:54:45 PM
User Name : Admin
Vial# : 3
Sample Name : Blackhorse
Sample ID : BH1
Sample Type : Unknown
Injection Volume : 0.10
ISTD Amount :

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



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	16.917	75338	5189	0.753				
2	17.600	6117	401	0.061				
3	17.766	2115	290	0.021		V		
4	18.844	42460	2497	0.425				
5	18.896	12631	2565	0.126		V		
6	18.966	6767	2462	0.068		V		
7	19.322	60818	3836	0.608		V		
8	19.362	30341	3771	0.303		V		
9	19.493	10206	3361	0.102		V		
10	19.744	74653	4524	0.746		V		
11	19.871	12381	4032	0.124		V		
12	20.030	45996	4839	0.460		V		
13	20.092	83562	4877	0.836		V		
14	20.529	6055	1588	0.061		V		
15	20.746	34228	1994	0.342		V		
16	20.963	11737	1192	0.117		V		
17	21.159	5742	684	0.057		V		
18	21.485	3800	417	0.038		V		
19	21.826	2590	266	0.026				
20	22.008	1048	316	0.010		V		
21	22.557	8605394	360035	86.049		SV		
22	24.058	2571	341	0.026		T		
23	24.190	4729	469	0.047		TV		
24	24.582	263860	21023	2.638		TV		
25	24.785	79445	8173	0.794		TV		
26	25.721	85113	5595	0.851		TV		
27	26.219	55343	3039	0.553		TV		
28	26.661	1524	287	0.015		TV		
29	27.109	2675	275	0.027		V		
30	27.348	28770	2978	0.288		SV		
31	28.481	312403	13031	3.124				
32	29.097	12511	1515	0.125		V		
33	29.408	17607	2968	0.176				
Total		10000530	468830					

Figure 9: Chromatogram of Black Horse

So, 250 ml contains 250× 4.29 µg of safrole

= 1072.5 µg of safrole

For Black Horse, Content of safrole, 1072.5 µg/250 ml

4.2.4 Tiger

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

Here, A_s = GC peak area of safrole = 29549

A_{is} = GC peak area of DHB = 84095

W_s = weight (µg) of safrole

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

RRF = 2.97

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

RRF = 2.96×10^{-3}

$W_s = (As/Ais) \times (Wis/RRF) \times 1/V$

$W_s = (29549/84095) \times (4.76 \times 10^{-3}$ µg /2.97) × 1/1ml

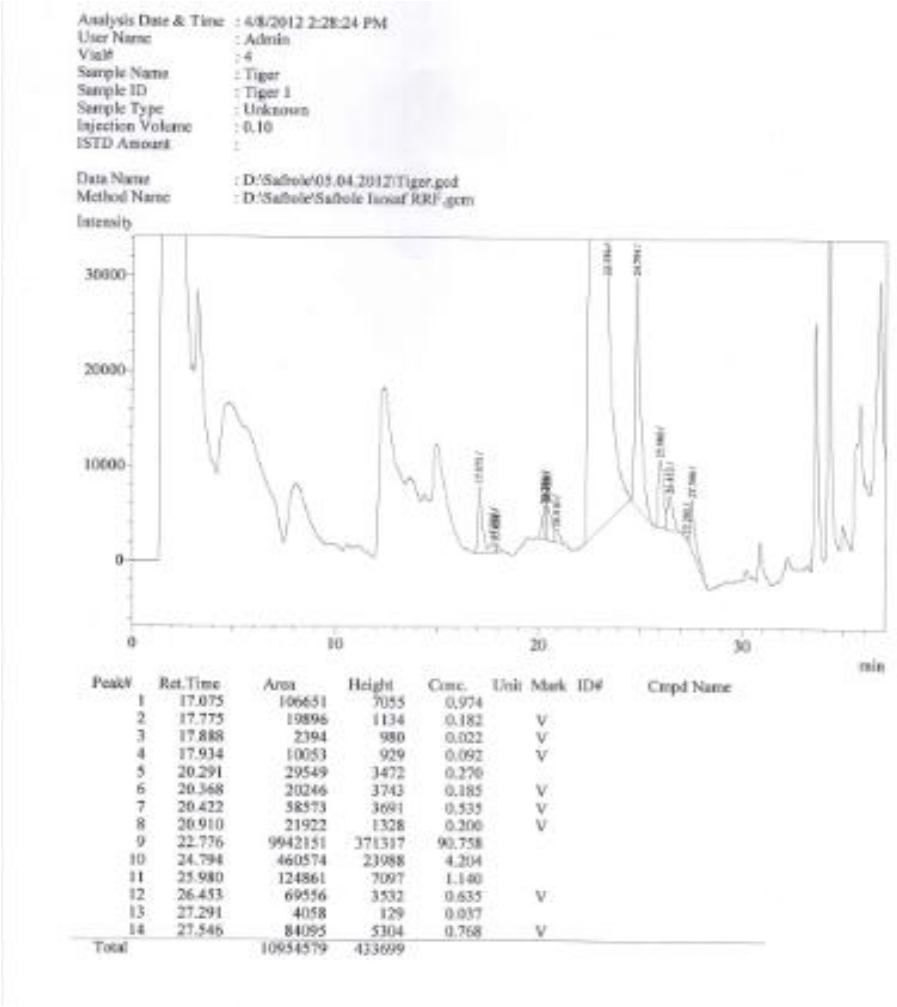
= 5.63×10^{-4} µg

0.1 µl contains 5.63×10^{-4} µg of safrole

1 µl contains 56.3×10^{-4} µg of safrole

Therefore, 1000 µl or 1 ml contains $56.3\times 10^{-4} \times 1000$ µg of safrole

= 5.63 µg of safrole



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	17.075	106651	7055	0.974				
2	17.775	19896	1134	0.182		V		
3	17.888	2394	980	0.022		V		
4	17.934	10053	929	0.092		V		
5	20.291	29549	3472	0.270				
6	20.368	20246	3743	0.185		V		
7	20.422	58573	3691	0.535		V		
8	20.910	21922	1328	0.200		V		
9	22.776	9942151	371317	90.758				
10	24.794	460574	23988	4.204				
11	25.980	124861	7097	1.140				
12	26.453	69556	3532	0.635		V		
13	27.291	4058	129	0.037				
14	27.546	84095	5304	0.768		V		
Total		10954579	433699					

Figure 10: Chromatogram of Tiger

So, 250 ml contains 250× 5.63 µg of safrole

= 1407.5 µg of safrole

For Tiger, Content of safrole, 1407.5 µg/250 ml

4.2.5 Mojo

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

Here, A_s = GC peak area of safrole = 19328

A_{is} = GC peak area of DHB = 134234

W_s = weight (µg) of safrole

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

RRF = 2.97

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

$W_s = (19328/134234) \times (4.76 \times 10^{-3}$ µg /2.97)

$= 2.31\times 10^{-4}$ µg

0.1 µl contains 2.31×10^{-4} µg of safrole

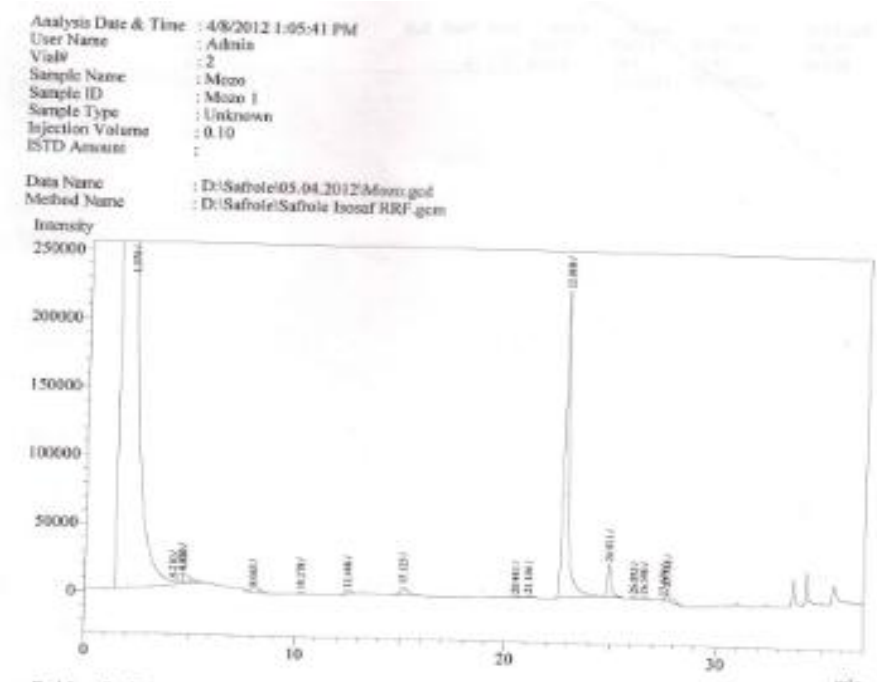
1 µl contains 23.1×10^{-4} µg of safrole

Therefore, 1000 µl or 1 ml contains $23.1\times 10^{-4} \times 1000$ µg of safrole

= 2.31 µg of safrole

So, 250 ml contains 250× 2.31 µg of safrole

= 577.5 µg of safrole



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	1.570	39114533	1410653	89.572		S		
2	4.210	1155	206	0.003		TV		
3	4.626	81114	6172	0.186		T		
4	4.686	148418	6055	0.340		TV		
5	8.043	93251	2612	0.214				
6	10.278	21126	1516	0.048		V		
7	12.488	57809	2979	0.132				
8	15.125	165514	6579	0.379		S		
9	20.481	19328	1127	0.044				
10	21.104	19873	1473	0.046				
11	22.808	3487469	224764	7.986		S		
12	24.951	281215	23825	0.644				
13	26.093	17918	1458	0.041				
14	26.590	19015	1323	0.044				
15	27.529	6416	1065	0.015				
16	27.708	134234	8870	0.307		V		
Total		43668388	1700677					

Figure 11: Chromatogram of Mojo

For Mojo, Content of safrole 577.5 µg/250 ml

4.2.6 Virgin

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

Here, A_s = GC peak area of safrole = 2969

A_{is} = GC peak area of DHB = 12340

W_s = weight (µg) of safrole

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

RRF = 2.97

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

$W_s = (2969/12340) \times (4.76 \times 10^{-3}$ µg /2.97)

$= 3.86 \times 10^{-4}$ µg

0.1 µl contains 3.86×10^{-4} µg of safrole

1 µl contains 38.6×10^{-4} µg of safrole

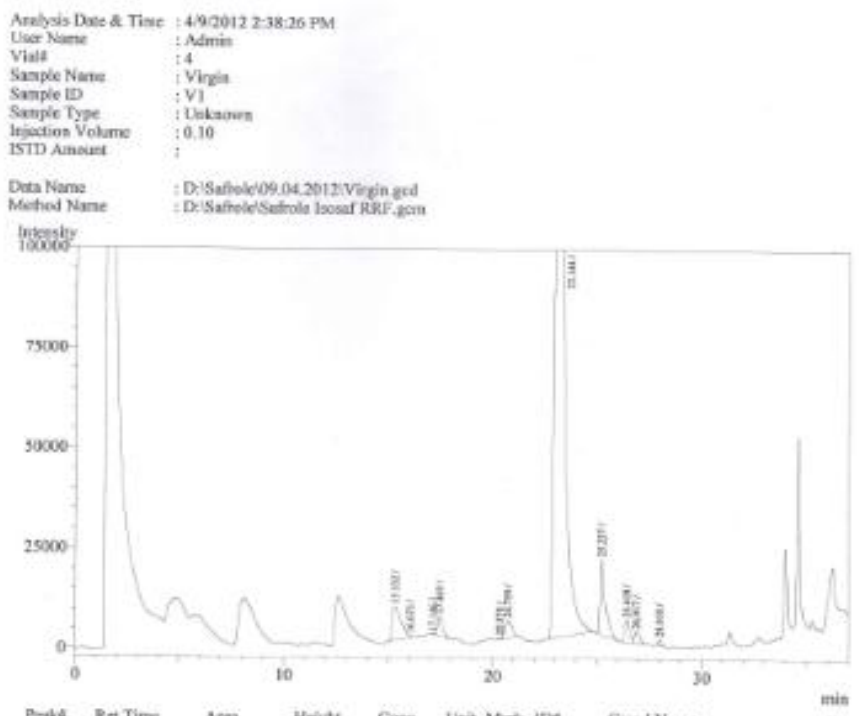
Therefore, 1000 µl or 1 ml contains $38.6 \times 10^{-4} \times 1000$ µg of safrole

= 3.86 µg of safrole

So, 250 ml contains 250× 3.86 µg of safrole

=965 µg of safrole

For Virgin, Content of safrole, 965 µg/250 ml



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	15.332	238806	8392	3.451				
2	16.073	2070	491	0.030		V		
3	17.146	1794	145	0.026				
4	17.469	79707	4873	1.152		V		
5	20.370	2969	436	0.043				
6	20.759	103722	4502	1.499				
7	23.144	6006680	246499	86.799		SV		
8	25.237	329694	18893	4.764				
9	26.418	89600	5532	1.295				
10	26.917	52828	2946	0.763		V		
11	28.030	12340	1508	0.178				
Total		6920210	294217					

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Figure 12: Chromatogram of Virgin

4.2.7 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

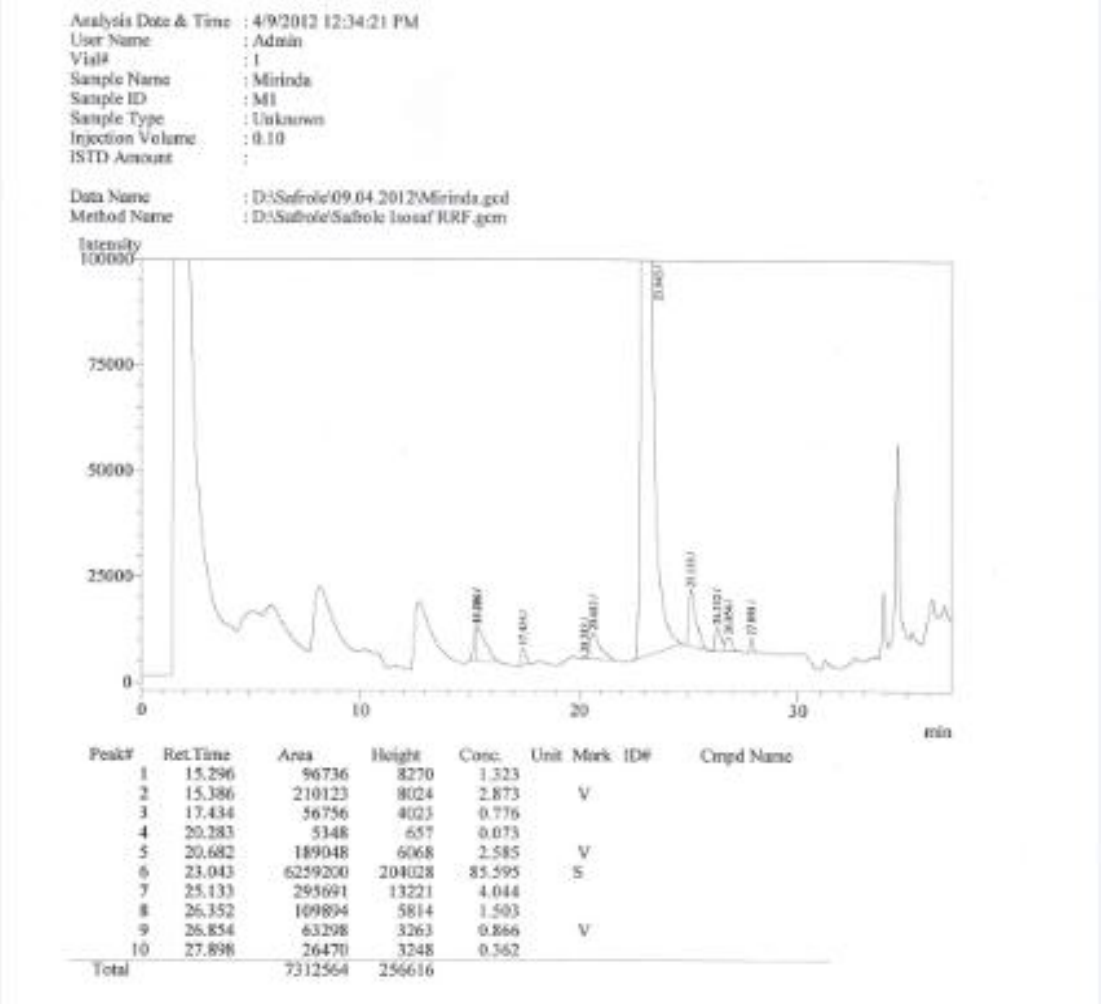
Therefore, 1000 µl or 1 ml contains $32.4 \times 10^{-4} \times 1000$ µg of safrole

= 3.24 µg of safrole

So, 250 ml contains 250× 3.24 µg of safrole

=810 µg of safrole

For Mirinda, Content of safrole, 810 µg/250



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 13: Chromatogram of Mirinda

Chapter 5-Result and Discussion

5.1 Results

In the analysis we have found safrole in these soft drinks. **For Speed,** Content of safrole, 95 µg/250 ml, **Pepsi** 1482.5 µg/250 ml, **Black Horse** 1072.5 µg/250 ml, **Tiger** 1407.5 µg/250 ml, **Mojo** 577.5 µg/250 ml, **Virgin** 965 µg/250 ml and for **Mirinda**, Content of safrole is 810 µg/250 ml, which was confirmed in recovery test. 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 validation test. One reason for this, isosafrole may be used after derivitization.

Table: 2 Concentration of Safrole and Isosafrole in the samples

Sample	Conc. of Safrole	Conc. of Isosafrole
Speed	95 µg/250 ml	Not found
Pepsi	1482.5 µg/250 ml	Not found
Black Horse	1072.5 µg/250 ml	Not found
Tiger	1407.5 µg/250 ml	Not found
Mojo	577.5 µg/250 ml	Not found
Virgin	965 µg/250 ml	Not found
Mirinda	810 µg/250 ml	Not found

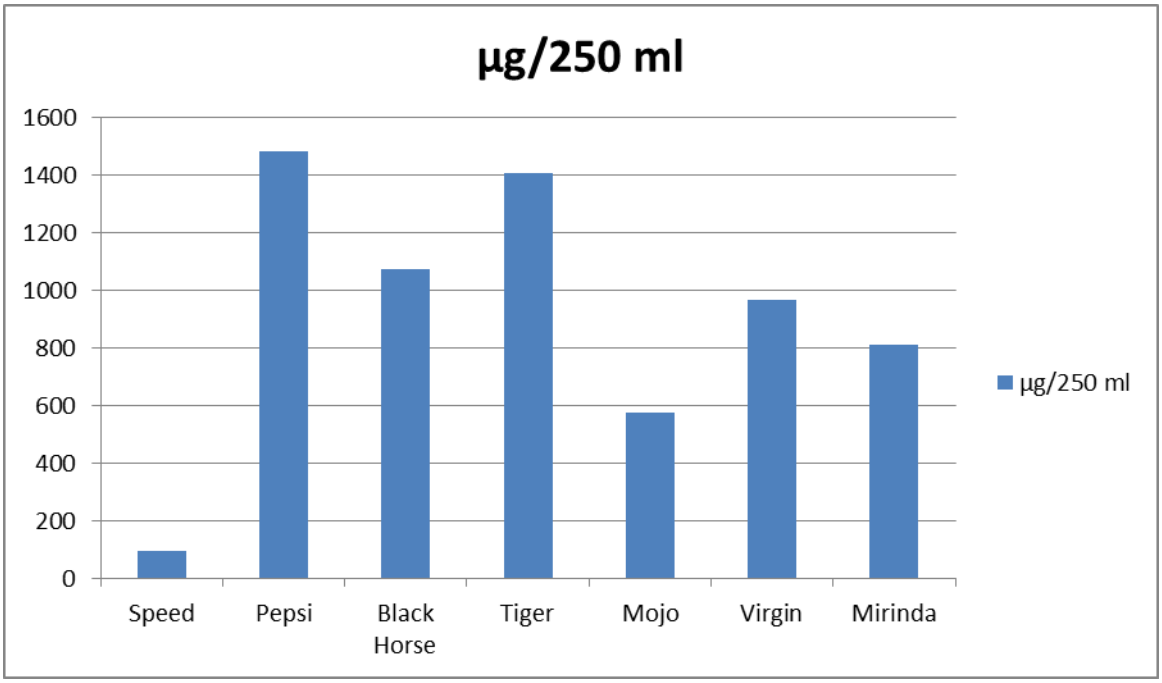


Figure: Comparison of conc. of safrole between different soft drinks in (µg/250 ml)

From the figure we can see that Tiger soft drink contains the maximum amount of safrole, then Pepsi and Speed contains the minimum amount of safrole.

5.2 Discussion

Rtx 625 column was used for trial with respect to the selection of the analytical column. Results demonstrated that the Rtx 625 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 (1-13), 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 Speed, Pepsi, Black Horse, Tiger, Mojo, Virgin and 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 6-Conclusion

6.1 Conclusion

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 (0.38-5.93 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 iso-safrole, and most exceeded 1-5 times the regulated concentration of 1 µg/mL

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