KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCE

HPLC FINGERPRINT ANALYSIS OF AQUEOUS AND ETHANOLIC EXTRACT FOR TETRAPLEURA TETRAPTERA

A THESIS SUBMITTED TO THE DEPARTMENT OF

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CHEMISTRY

BY

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DECLARATION

It is hereby declared that this thesis is the outcome of research work undertaken by the Author and the experimental work described was carried out at the Department of Pharmaceutical Chemistry, KNUST. Any assistance obtained has been duly acknowledged. It is neither in part nor in whole been presented for another degree elsewhere.

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PROF. R. K. Adosraku
(Supervisor) ....

Signature

Date

DEDICATION

To my parents Mr. and Mrs. Martin Forson, and my lovely wife Irene Oteng Forson to whom I owe all that I am or hope to be,

To my siblings, Patricia Forson, Esther Forson and my dear brother Alfred Boateng Forson for his dedication to the course of human development.

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I cannot fail to appreciate the love and boost given me by my wife Irene Oteng Forson and my humble brother Alfred Boateng Forson. I also thank my parent Mr. and Mrs. Martin Forson, my sisters Patricia Ampomah Forson and Esther Forson for their prayers and support.

ABSTRACT

Based on the ever growing population, lack of access to modern medicine, and the increasing uses of traditional medicine as an alternative primary health care, attention on efficacy, safety, and quality have been the focal issue in the quality control (QC) of herbal medicines. Therefore, it is prudent to establish and encourage scientific, rational feasible quality control methods in enhancing the quality of our traditional medicines in general. With the advent of HPLC-fingerprint as a comprehensive quality control method for complex herbs, a total of eight (8) samples of Tetrapleura *tetraptera* from the two major market centres (i.e. Bantama Market and Central Market) in the Kumasi metropolis were selected. Four (4) samples each from the respective market centres under the same conditions were subjected to three (3) different extraction methods: i.e. aqueous method of extraction through decoction, ethanolic extraction with maceration processes and petroleum ether/aqueous fraction. The observations from the HPLC-fingerprints for all the extraction methods indicated that the petroleum ether/aqueous fraction method showed well resolved peaks with many peaks to characterize Tetrapleura *tetraptera* samples than the other extraction methods under the same chromatographic conditions. A suitable ternary mobile phase compositions of water-methanol-acetonitrile (85:5:10 v/v) was observed to improve peak shapes for the aqueous decoction extract while binary mixture of water-methanol (90:10 v/v) was noticed to enhance peak shapes and resolutions for the petroleum ether/aqueous fraction method (nonpolar and polar fractions) at a flow rate of 0.5 ml min^{-1}. The results revealed that changes in the method of extraction even for the same batch samples under the same conditions affect the nature of the chromatograms. However, there were obvious similar characteristic peaks across all the chromatograms for Tetrapleura *tetraptera* in spite of changes in the extraction methods.

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LIST OF ABBREVIATIONS

BM……Bantama

Market CDS……Chromatography Data System

CE……Capillary Electrophoresis

Cf……Chromatographic fingerprint

CM……Central

Market DAD……Diode Array Detector

DNA……Deoxyribonucleic acid

EMEA……European Medicines Agency

FDA……Food and Drugs Administration

FDB……Food and Drugs Board

GC……Gas Chromatography

HM……Herbal medicine

HMs……Herbal medicines

HPLC……High-Performance Liquid Chromatography

MS……Mass Spectroscopy

NMR……Nuclear Magnetic Resonance

Peak #……Peak Number

QC……Quality Control

RSD……Relative Standard Deviation

R_t / t_r/R.time……Retention time

SEM……Standard Error of the Mean

SN……Sample Number

T. tetraptera……Tetrapleura *tetraptera*

TLC……Thin Layer Chromatography

UPLCs……Ultra Performance Liquid Chromatography systems

UV……Ultraviolet

WHO……World Health Organization

CHAPTER ONE

1.1.0 INTRODUCTION

In recent years, interest in plant based drugs has increased considerably, and annual growth rate of 5-15% for plant based drugs and raw materials trade is an evidence of demand for herbal medicine (ICS-UNIDO, 2004) [1]. Despite major advances in medicinal chemistry and drug synthesis, traditional medicine from natural products has developed into a unique holistic health care system for treatment of some chronic diseases. Today herbal medicine is getting popularized in both developing and developed countries owing to its natural origin, consumer preference, concern regarding undesirable side effects of modern medicines and the belief that herbal drugs are free from side effects.

As per the traditional medicines programme of the World Health Organization (WHO) nearly 80% of the world's population uses phytoproducts, phytoconstituents of wild plants which play significant role in the health care of rural communities (Dubey et al.,2004) [2] . Due to the significant expansion of the use of this alternative medicine, it is very critical to develop a high standard of quality control in assessing the active components in raw plant materials to guarantee their identity, consistency and authenticity (WHO, 2000) [3]. With the growing needs for safer drug, attention should be geared towards quality, effective and standardization of traditional medicine preparations. But the quality control and quality assurances still remain a challenged due to several variations associated with herbal drugs and the complexity of the chemical components. Hence the need for developing a method for chromatographic fingerprint is becoming the primary tool for quality control of herbal medicine as suggested by

Lazarowych and Pekos et al., 1998 [4]. According to Liang Y.Z.et al., 2004, chromatographic fingerprint of a herbal medicine is defined in practice as chromatographic pattern of the extract of some common chemical components of pharmacologically active or chemically characteristics [5]. This can be properly achieved by harmonization and improvement of the process regulations which combine scientific studies and traditional knowledge. However, relatively few herbal drugs have been evaluated scientifically to prove their safety, potential benefits and effectiveness. The sources of raw material and good practice of manufacturing processes are certainly the essential steps for quality control of the herbal medicine.

Traditionally, oral herbal medicines are prepared by ethanol, water, or mixture of ethanol-water fluid extracts through decoction, infusion, maceration or cold extraction of the principal active component which are frequently unknown. Over the years, Ghana has seen significant changes in the regulation of the quality of allopathic medicines compared to the regulation of herbal medicines in the face of several challenges. This can be credited to the enactment of the Food and Drugs Law and the subsequent establishment of the Food and Drugs Board (FDB) on August 26, 1997 [6].

Herbal medicine, singularly or combine, contains myriad unknown components of which many are in low concentrations and in complex matrices with no single active constituents responsible for their efficacy. This may be due to the composite nature of the herbal medicines. Herbal medicines are produced as multi-component dosage regimens containing hundreds of constituents which may act synergistically to exert their pharmacological action. Unlike allopathic medicines, it has not received adequate formal recognition and attention to

permit the development of appropriate research methodologies for assessing their quality in many countries. Changes in the constituents of the herbal plant due to variations in geographic origins, harvesting time, prevailing climatic conditions, and processing techniques pose great challenges to its quality control and consistency. The regulation of the quality of herbal medicines in Ghana today, is based on the analysis of some few phytochemical constituents, macroscopic and microscopic examinations, test for heavy metals and toxicity levels.

Considering the composite nature of herbal medicines, the current approach used by regulatory bodies in Ghana is inadequate for their proper evaluation. In recent times, the concept of chromatographic fingerprint and chemometrics has been used to evaluate the quality and consistency of herbal products. This approach uses entire chromatographic fingerprints obtained from extracts of the herbal medicine which provides representative information about almost all the constituents in the herbal medicine.

In order to ensure the reliability and repeatability of pharmacological and clinical research of herbal medicines, it is necessary to determine majority of the constituents present rather than depending on only one or few phytochemical constituents for evaluation. This means that all constituents could be regarded as the active 'compound' in the quality control process. The fingerprint pattern is based on considering the whole chromatographic profile as a chemical feature covering the relative compositions. This appears to offer a more logical tool for evaluating the quality of traditional herbal preparations. At present, HPLC fingerprint technology is regarded as a primary means in the QC study of traditional medicine. It is sensitive, accurate, simple, fast, and suitable for almost all the compounds in herbal preparation.

The HPLC is a modern instrument used in pharmaceutical industry because it is easy to learn, use and is not limited by the volatility or stability of the sample compound, and therefore is essential in the control of herbal preparations. Hence Ghana's Food and Drug Board (FDB) is encouraging the use of HPLC for the quality management of all drug products where herbal preparations are no exception. However, in folk preparations where the active principal ingredient are unknown, marker substances are established for their analytical control which in most cases have never been tested to see whether they really accounted for the therapeutic action reported for the herbal drug. This and other limiting factors such as cost of pure reference standards, inaccessibility of marker compounds, absence of standardized methods of assay, absence of proper database for a particular herb and its preparation as in relation to its activity with respect to particular treatment has made the adoption of chromatographic fingerprint an alternative choice for herbal assay.

1.1.1 Justification

The concept of chromatographic fingerprint has been adopted and accepted by the European Medicines Agency (EMEA), the World Health Organisation (WHO), British Herbal Medicines Association, Indian Drug Manufacturer's Association and the Food and Drugs Administration (FDA). Currently, the use of HPLC techniques, chromatographic fingerprint and chemometrics for the quality assessment of herbal drugs has been fully embraced and applied by the world's regulatory bodies [7-10]. Therefore there is no doubt that chromatographic fingerprint will sooner or later become the primary tool for quality control of herbal medicines worldwide as suggested by Lazarowych and Pekos, 1998 [4].

The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of the herbal medicines and therefore be used for authentication and identification of the herbal products. Based on the concept of phytochemical screening, the chromatographic fingerprints of herbal medicines could be utilized for addressing the problems of quality control of herbal medicines in Ghana.

Notwithstanding the advantages chromatographic fingerprint offers and its acceptance by EMEA, FDA, WHO, SFDA and other organizations, there is no sign of its application for the assessment of the quality of herbal medicines in Ghana.

In spite of the fact that much research has been done on the plant Tetrapleura *tetraptera* by the following famous researchers *(*Adewunmi *et al*., 1989, 1991; Daiziel, 1984; Millard *et al*., 1989) (Adesina, 1982; Salako et al., 1990; Aka and Nwabie, 1993; Adebayo *et al*., 2000) there is no existing report on the chromatographic fingerprints of any part of this plant [11-19].

1.1.2 Main Objective

This project is being undertaken to encourage the use of chromatographic fingerprint as an alternative means of assessing and authenticating traditional herbal medicines in Ghana.

1.1.2 Specific Objectives

The specific objectives of the research are:

1. To determine the best HPLC conditions suitable for the method in aqueous and ethanolic extract of Tetrapleura *tetraptera*

2. To develop HPLC fingerprint for the quality control of aqueous and ethanol extract of Tetrapleura *tetraptera*- a herbal fruit commonly used in folkloric medicine

3. To compare aqueous fingerprint of the Tetrapleura *tetraptera* with that of the ethanolic extract.

CHAPTER TWO

2.1.0 LITERATURE REVIEW

2.1.1 Morphological Characteristics of Tetrapleura *Tetraptera*

Tetrapleura *tetraptera* is a large perennial tropical deciduous tree reaching about 20- 25m in height, with 1.5-3m girth. The bole is usually slender and older trees are virtually with very small, low, sharp buttresses. The bark is fairly smooth grey-brown with reddish slash and glabrous yound branchlets. The twigs and young foliage are practically glabrous or minutely hairy with a common stalk of about 15-30cm long. They are slightly channeled on the upper surface with 5-9 pairs of pinnae, of 5-10cm long mostly opposite but sometimes alternate. There are 6-12 leaflets alternately arranged on each side of the pinnae-stalk, about 12-25mm long, 6-12 mm broad and are slightly elongated and rounded at the both ends. The apex is sometimes slightly notched and the base usually unequal with slender stalks of about 2mm long. The lateral veins are indistinct and run at a wide angle to the prominent midrib (Keay 1989, Irvine 1961). [20,21]

Flowering appears after the new leaves have fully developed. They are pinkish or cream and densely crowded in spike-like racemes 5-20cm long which is normally in solitary in upper leaf axils. The individual flowers have slender stalks and ten (10) short stamens. The anthers have glands at the apex. (Hutchinson 1973, Keay 1986, Irvine 1961). [21, 22]

The picture of Tetrapleura *tetraptera* plant is shown in figure 2.1

Figure 2.1 Photograph of Tetrapleura *tetraptera* ("*prɛkɛsɛ*") plant

Fruits persistently hang at the ends of the branches on stout stalks. The fruit is shiny dark brown pod of about 15-25cm long by 5cm across, usually curved with 4 longitudinal wing-like ridges of 3cm broad. Two of the wings are woody containing small flat black hard rattling seeds, while the other two are filled with soft sugary pulp, oil and aroma. The oil contains oil and each fruit has up to 30 seeds in the pod depending on the length of the woody part of the fruit. (Blay 1997, Irvine1961). [21, 23] The generic name Tetrapleura *tetraptera* comes from a Greek word meaning "four ribs" referring to the ribbed fruit. Lateral picture of Tetrapleura *tetraptera* fruit is shown in figure 2.2

Figure 2.2 Photograph of Tetrapleura *tetraptera* ("*prɛkɛsɛ*") fruit.

2.1.2 Ecology and Distribution

The natural habitat of Tetrapleura *tetraptera* is on the fringe of the West African rainforest belt. The trees are widespread in tropical Africa, especially secondary forest, and they are at their best in rainforest. The species is found throughout the high forest zone, in riverain forest, in the southern savannah-woodlands and in the forest outliers in the African plains. It grows in deciduous forest from Senegal to west Cameroon and extends across West Africa to Chad, Sudan, Uganda and Zaire (Burkill, 1995) [24]. In Ghana the tree is common in forest outliers in the Afram plains and deciduous in December. Its pods are rip from September to December.

2.1.3 The family Mimosaceae

The plant belongs to the subfamily Mimosoidae of the Leguminosae family which are trees, shrubs, or herbs with legume or indehiscent winged fruit and seeds without endosperm are their features. They are mainly tropics and subtropics, often in dry regions and their frequently spiny with bipinnated leaves and small regular flowers. The calyx are tubular, 5-lobed or toothed with valvate petals which connate into a short tube. The stamen is equal in number to sepals or more and the anthers are small, about 2-celled opening lengthwise with deciduous gland at the apex with superior ovary of 1 carpel. The genera Tetrapleura have two varieties namely Tetrapleura thonningii and Adenanthera *tetraptera*. (Dalziel, J. M., 1984; Hutchinson, J. 1973). [14, 22]

2.1.4 Folkloric Uses

Traditionally, there two major categories in which Tetrapleura *tetraptera* is been used

a) Non-medicinal

b) Medicinal

2.1.5 Non-Medicinal Uses

Tetrapleura *tetraptera* is noted for its pleasant characteristic aroma and hence the plant has acquired the prestigious accolade of recognition in the Ashanti Kingdom. It is used in appellation to the 'Asantehene' in demonstration of his presence. The pulp extract of the fruit is used in flavouring wine. (Irvine, 1961; Abbiw, 1990) [21, 25]. The Ashantis of Ghana use the fruit in flavouring palm nut soup while the Yoruba's of Nigeria use it in spicing pepper soup. (Essien et al.1994; Adesina, 1982; Okwu 2003; Abbiw, 1990) [16, 25, 26, 27]. Economically, in some parts of Nigeria the fruit serves as flavour in the making of local powders and a booster for foaming ability of bathing soap.

It is believed in Congo that the fruit pod is used in certain ceremonies of exorcism while the Yoruba Odu of Nigeria use it in release of people tied by witches.(Burkill, 1995) [24].

The fruit is employed in fishing and grass-cutter poison as bush, whereas the seed is an ingredient in Gue're' arrow poison. (Irvine, 1961; Abbiw 1990) [21, 25].

The plant is grown as a shade tree for other crops as well as insect and snake repellents against coffee plants in Uganda. (Irvine, 1961) [21].

Tetrapleura *tetraptera* plant is hard with heavy wood which are used for firewood, building, and general carpentry works. (Irvine 1961) [21].

2.1.6 Medicinal Uses

There are wide ranges of medicinal use of T. *tetraptera* in West Africa where the plant can be predominantly found. In Ghana the stem bark is used as enema for constipation and the bark decoction as emetic. (Abbiw, 1990) [25]. The fruit has been used since time immemorial in various Ghanaian communities as sources of vitamins for nursing mothers to prevent postpartum contractions, and serves as a lactation aid (Nwawu and Alah, 1986) [28].

Infusion of the fruit is usually taken by convalescents to bathe in order to get relief from feverish conditions of malaria. (Irvine, 1961) [21]. Decoction of the pulp extracts of T. *tetraptera* is use in the treatment of tooth plaque and ache in Ghanaian local domestic homes. The bark of the plant is use as beverages and enema against gonorrhea by the Ivoirians. (Irvine, 1961) [21]. In Nigeria, the T. *tetraptera* is used to treat a variety of ailments such as inflammation, leprosy, rheumatoid pains, and particularly among the southwest Yoruba"s", herbal concoction with other plants are used in the management of convulsion in children. (Dalziel, 1985) [14].

2.1.7 Chemical Constituents of the Tetrapleura *tetraptera*

According to the Africa Phyto International, the T. *tetraptera* fruit contains essential oils, sapronoside, triterpens, coumarins, scopoletin, tannins, sugars, steroids triterpene glycoside. El- izzi et al (1990), reported the presence of flavonoids in the ethanolic extract of the stem bark.[29] The fruit was reported by Adewunmi et al 2002 to contain Cinnamic acids (1) Caffeic acids (2) and carbohydrates.[13]

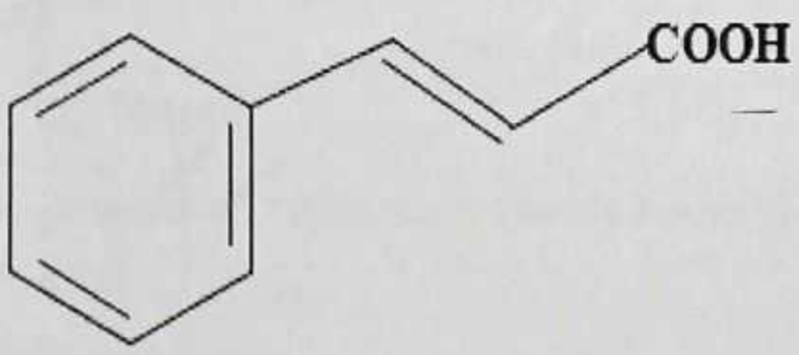
Dosunmu in 1997 reported that the fruit contains crude protein at very low content (2.21%), ascorbic acid (25mg-68mg), sucrose, glucose, fructose and mineral

elements such as potassium (K), iron (Fe), magnesium(Mg), phosphorus(P), sodium (Na), zinc (Zn), nickel(Ni),. [30]

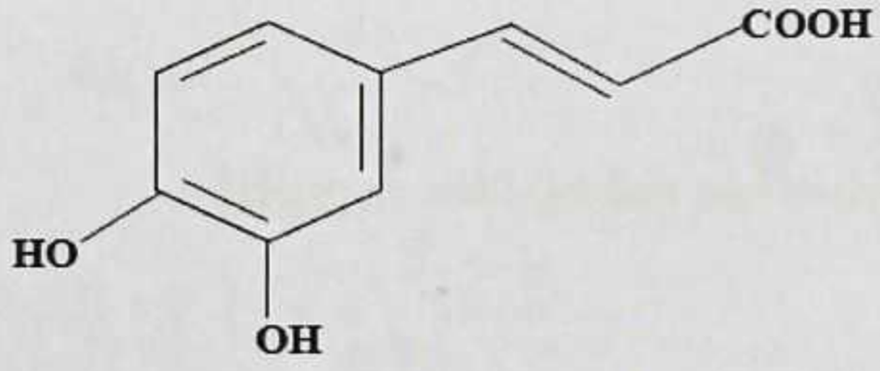
The bark is said to contain crystals of aminopropionic acid derivative called Mimosine (3) an alkaloid (Burkill 1995), Adesina and Sofowora (1979) isolate oleanic and tryglycoside [24, 31]. Ojewole et al (1983) obtained scopoletin (4), which in further studies was confirmed by Obidoa et al (1991) as an active principle in the traditional herbal infusion and a hypotensive non-specific spasmolytic agent [32, 33]. Maillard et al, (1989 and 1992) through activity-guided fractionation of the methanol extracted four (4) saponines exhibiting strong molluscicidal properties against schistomiasis transmitting snails" B. glabrata.[34,35]

These were shown to be, 3- O - {- 2- acetamido –2 deoxyl – β – D – glucopyranosyl} olean–12–en–28–ioc acid (5); 3–O-(2–acetamindo-2deoxyl–β – D – glucopyranosyl) echinocystic acid (6); 3-{ [O–β–D–glucopyranosyl–(1 → 6)– β –D- glucopyranosyl] - oxy} –27–hydroxyolean –12– en –28– oic acid (7); 3–hydroxy –oleanen –28– oic acid (8) and 3-O-{β-D-glucopyranosyl-1(1"-6)-2-acetamido-2'deoxyl-β-D- glucopyranosyl} olean-12-en-28-oic acid (9). Ngassapa et al, 1993 also isolated the new sulphated triterpene, echinocystic acid 3–O – sodium sulphate (10).

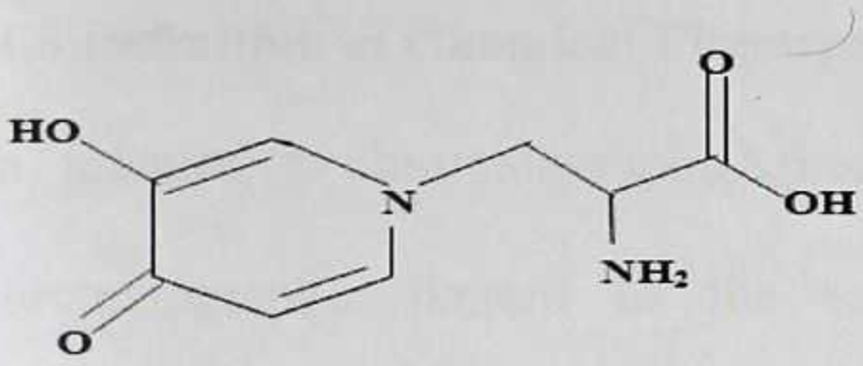
Structures of the Chemical Constituents



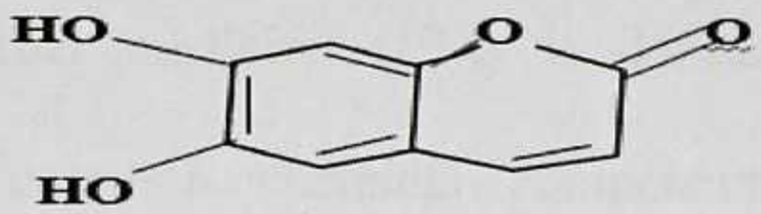
CINNAMIC ACID (1)



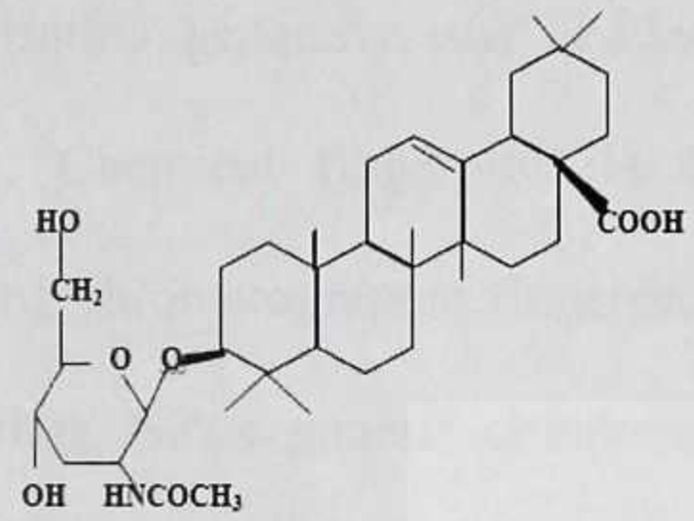
CAFFEIC ACID (2)



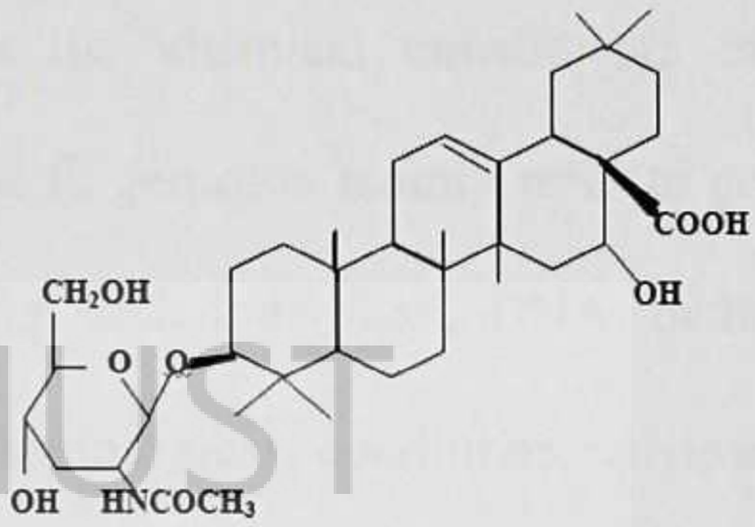
MIMOSINE (3)



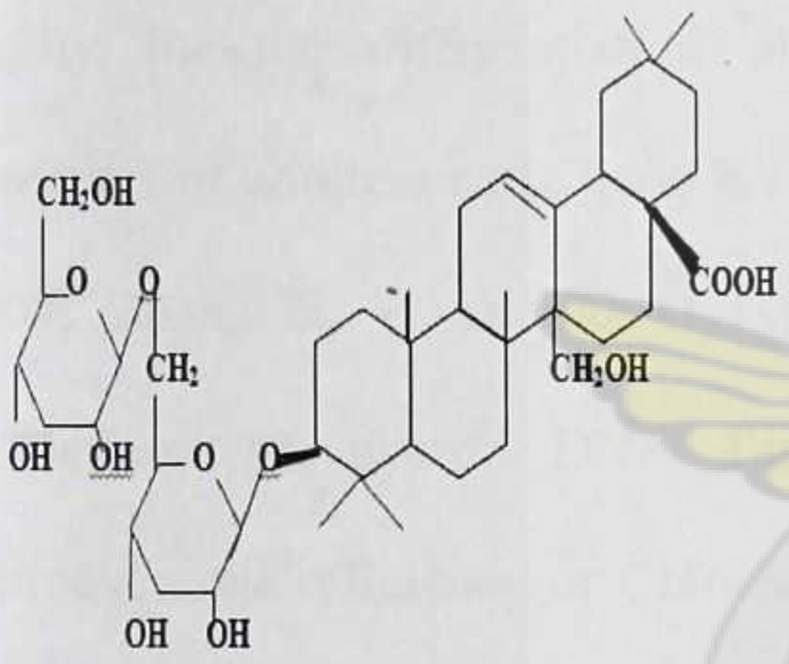
SCOPOLETIN (4)



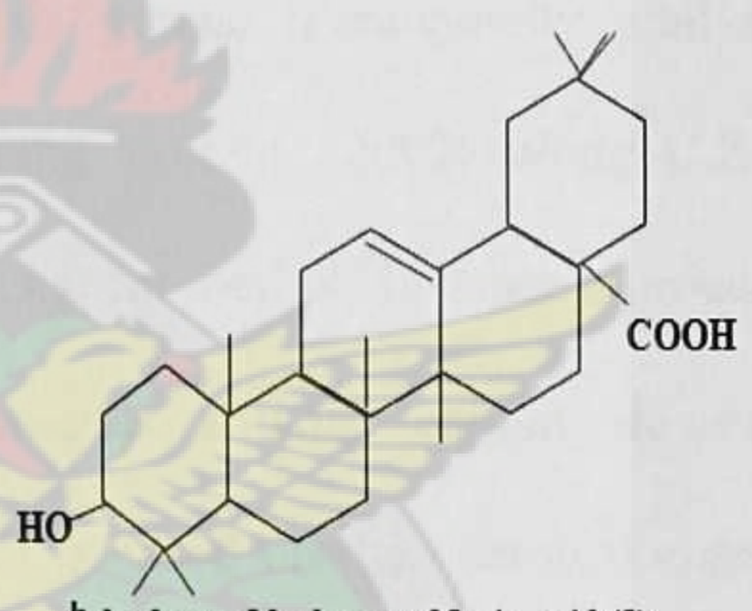
3-O-{-2-acetamido-2'deoxyl-β-D-glucopyranosyl}olean-12-en-28-oicacid (5)



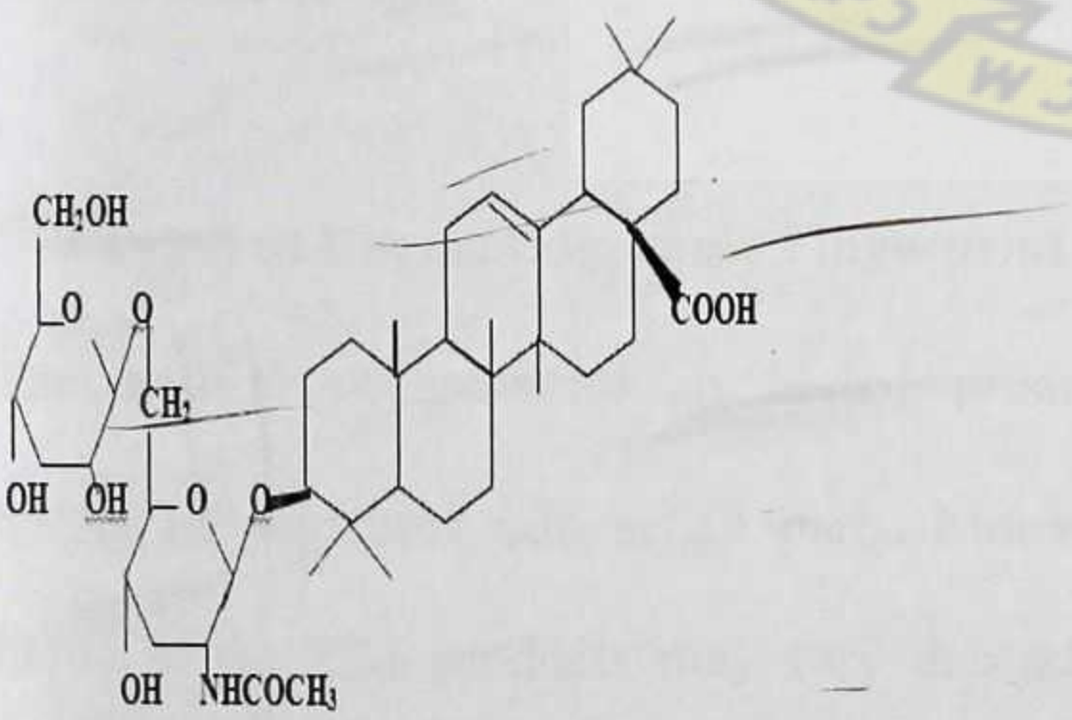
3-O-{-2-acetamido-2'deoxyl-β-D-glucopyranosyl} echinocystic acid (6)



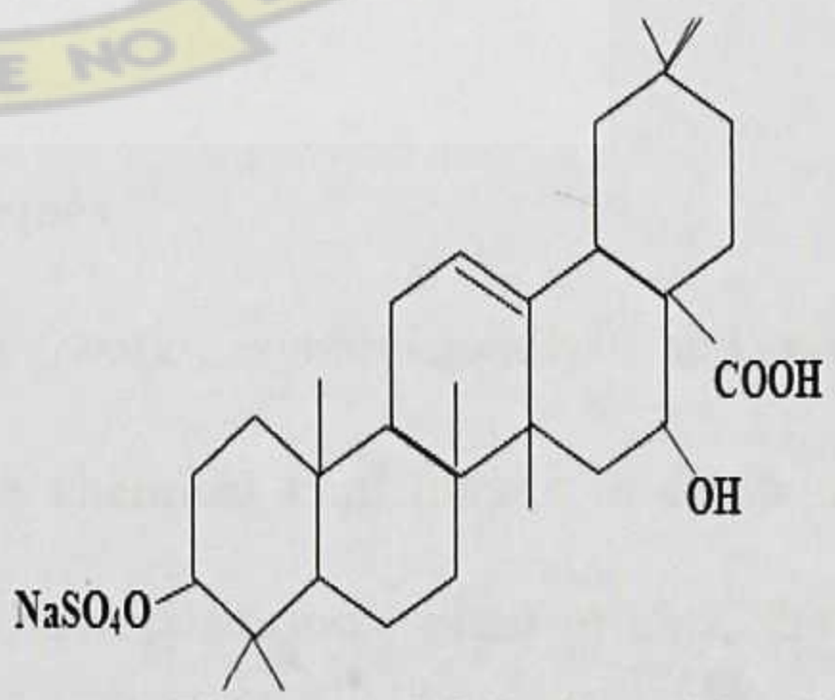
β-{[O-β-D-glucopyranosyl-(1→6)-β-D-glocopyranosyl]-oxy}-27-hydroxyolean-12-en-28-oic acid (7)



β-hydroxy-12-oleanen-28-oic acid (8)



3-O-{β-D-glucopyranosyl-1(1''-6')-2-acetamido-2'deoxyl-β-D-glocopyranosyl} olean-12-en-28-oic acid (9)



Echinocystic acid 3-O-sodium sulphate (10)

2.1.8 Definition of Chemical Fingerprint

In practice a chromatographic fingerprint of a herbal medicine (HM) is defined as chromatographic pattern of the extract of some common chemical components of pharmacologically active and or chemically characteristics [59-60].

Fingerprinting, generally, was divided into chemical and biological fingerprint patterns. Chemical fingerprint is used to analyse the chemical constituents in HMs, consisting chromatographic fingerprint, the biological fingerprints mainly refer to genomics fingerprints. Since genetic composition is unique for each individual, DNA methods for HMs" identification are less affected by age, physiological conditions, environmental factors, harvest, storage and processing methods. Genomic fingerprint has been used widely for the differentiation of plant individual, genus, homogeneity analysis, and detection of adulterants (Cheng KT et al., 1997; Huang Y et al., 2002; Wang C.Z. et al., 2007; Zhang X. et al., 2006) [52-56]. However, as for herbal instances processed or extractions of plants, DNA fingerprinting techniques usually cannot do anything. Moreover, the efficiency of Chinese herbal medicines is based on the chemical components they contain, chemical analysis therefore better reflect the intrinsic quality of medicine. Consequently, DNA fingerprinting should be used as a complement tool of other quality control techniques.

2.1.9 Types of Chromatographic Fingerprint Techniques

The multiple constituents in herbal product may work „synergistically" and could hardly be separated into active parts. Moreover, the chemical constituents in component herbs in the HM products may vary depending on harvest seasons, plant origins, drying processes and other factors. Hence, it seems necessary to determine most of the phytochemical constituents of herbal products in order to ensure the

reliability and repeatability of pharmacological and clinical research, to understand their bioactivities and possible side effects of active compounds and to enhance product quality control [40–41]. Thus, several chromatographic techniques, such as thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE), and high-performance liquid chromatography (HPLC) can be applied for chromatographic fingerprint. In this way, the full herbal product could be regarded as the active 'compound'.

2.2.0 Thin layer chromatography (TLC)

TLC is a simple, low-cost, versatile and specific method for the identification of herbal medicines. The unique feature of picture-like image of TLC supplies an intuitive visible profiling and, due to this TLC is used by various pharmacopoeias such as American Herbal Pharmacopoiea and Pharmacopoiea of the Peoples" Republic of China to provide initial characteristic fingerprints of herbs. TLC serves as a simple method for screening of herbal medicines and possesses many-fold detection possibilities [41]. However, TLC analysis also has shortcomings such as low resolution, low sensitivity and the difficulty of detection of trace components which limits it acceptance as a universal method.

2.2.1 Gas chromatography (GC)

In the case of herbal product containing volatile compounds, gas chromatography is the separation technique that gives a reasonable fingerprint for reliable characterization, identification and authentication of the herbal medicine [41-42]. Moreover, the high selectivity of capillary columns enables separation of many volatile compounds simultaneously within very short time. However, it is not

convenient for the analysis of samples of polar, non-volatile and heat-labile ingredients (Liang Y.Z. et al., 2004) [43]

2.2.2 Capillary electrophoresis (CE)

Another important separation technique that can be used is capillary electrophoresis (CE). CE is a powerful analytical technique capable of separating charged compounds. It is good for producing fingerprints of herbal medicines [41]. Alkaloids and flavonoids have been extensively studied with this technique [44-47]. Capillary electrophoresis (CE) method was established to evaluate one herb drug in terms of specificity, sensitivity, precision, shorten analysis time and lessen solvent consumption. However, due to poor resolutions resulting from overlapping peaks in complex samples and irreproducible migration times makes HPLC better choice comparatively. (Christophe Tistaert et al., 2011; Wang L.C., et al., 2005) [48-49]

2.2.3 High-performance liquid chromatography (HPLC)

In this twenty-first century, HPLC has become the popular method for the analysis of herbal medicines because it is easy to learn, use and is not limited by the volatility or stability of the sample compound. Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry, these fields currently comprise only about 50% of HPLC users. Currently HPLC is used by a variety of fields including cosmetics, energy, food, and environmental industries [50]. One of the main advantages of HPLC is that many detectors can be coupled with it, such as UV, DAD, ELSD, FLD, RID, MS, and NMR, etc., which supplies much more possibilities for detecting different

constituent types. Because of its advantages and popularization, HPLC fingerprint analysis has been regarded as the first choice.

In this work, the emphasis was put on HPLC/UV chemical fingerprinting of T. *tetraptera* a herbal fruit used in the management several diseases in Ghana and other parts of Africa.

2.2.4 HPLC/UV-System

The modern form of column liquid chromatography has been called high - performance, high resolution, high-pressure and high-speed liquid chromatography. However, the abbreviation HPLC is now universally understood to describe the technique that separates mixtures on columns filled with small particles by elution with a liquid under high pressure. Basically, HPLC is a separation technique that can be used for the analysis of organic molecules and ions. It is based on mechanisms of adsorption, partition, ion exchange or size exclusion, depending on the type of stationary phase used. It involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases. HPLC has been used to assess the purity and/or determine the content of many pharmaceutical substances [62]. It has also been used in determined enantiomeric composition, using suitably modified mobile phases or chiral stationary phases.

The equipment consists of an eluent reservoir normally referred to as mobile phase reservoir, a degasser, a high-pressure pump, an injector for introducing the sample, a stainless steel column containing the packing material, a detector with data

processor, and a chart recorder. HPLC equipment can be obtained as a complete system or assembled from individual modules [61].

Mobile Phase Reservoir

This is usually a glass container or an inert flask of volume 1-5litres use to store the mobile phase during analysis. The glass container normally comes along with an inert tube of 1/16 inch internal diameter which is in contact with the mobile phase. The end of the tube is fitted with a 10μm filter commonly made of glass or stainless steel which remove any particulate matter from the reservoir and also act as a "sinker" to hold the tube at the bottom of the container. Generally the number of mobile phase reservoir depends on the number of lines available on the instrumentation. However, for a reversed phase gradient elution, more than one line is required in order to be able to increase the mobile phase proportion throughout the analysis. Thus isocratic (1line), binary (2 lines), ternary (3 lines) and quaternary (4 lines) systems are mostly common. For a binary system the mobile phase reservoir are labeled A and B. [63]

Degasser

Most liquids dissolve quite amount of atmospheric gases that may be a major cause of practical problems in HPLC, specifically affecting the operation of the pump and the detector. This problem can be eliminated by degassing the mobile phase.

Modern HPLC have a vacuum degasser in the flow path of the mobile phase prior to the pump. The vacuum degasser allows gases to penetrate into the surrounding vacuum through semiporous polymer membranes. This is a way of removing dissolved gases from the mobile phase to reduced errors resulting from pump

malfunction and retention time shift. After Degassing the mobile phase is then transferred from the degasser to the pump. [64]

Pumping system

Pump refers to the device that forces the mobile phase through a liquid chromatography column at pressures up to 10,000 psi and varies in pressure capacity [67]. Their performance is measured on their ability to yield a consistent and reproducible flow rate. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns that are less than 2 micrometres. These "Ultra Performance Liquid Chromatography" systems (UPLCs) can work at up to 15,000 lbf/in² (~100 MPa or about 1000 atmospheres) [68]. HPLC pumping systems are required to deliver metered amounts of mobile phase at a constant flow rate. Pumping systems that deliver solvent from one or more reservoirs are available [62]. The flow rate through the column ranges between 0.3and 10 ml/min, with pressures ranging from 500 to 4000Ib/ in2 (3.4-27.6MPa). The pumps must show minimum fluctuations within these ranges to achieve maximum stability of the detector response and reproducible retention data.

The simplest pump consists of the direct application of gas pressure on to the surface of the eluent contained in a pressure bottle or holding coil. Such devices are relatively cheap but have the disadvantage that the pressure limit is low typically 1500Ib/in2, 10.3MPa. The gas dissolved in the eluent may reappear as bubbles in the detector flow-cell, and a change of eluent requires the apparatus to be dismantled, washed and refilled. Commercial HPLC pumps use pistons or diaphragms to displace eluent, hence avoiding problem of dissolved gas [61].

Modern computer- or microprocessor-controlled pumping systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying (gradient elution) composition, according to a defined programme. In the case of gradient elution, solvent mixing can be achieved on either the low or high-pressure side of the pump(s). Depending on the flow rate and composition of the mobile phase, operating pressures of up to 42000kPa (6000 psi) can be generated during routine analysis [62].

Injectors

The primary goal for any injection is to introduce the sample into the column. The liquid samples are injected with small glass-syringes whose volume is between 1 10μL for capillary columns and 50μL for packed columns. The sample is normally dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10μl to over 500 μl depending on how much packing material is in the column. In modern HPLC systems, the sample injection is typically automated [64].

Chromatographic column

Columns are usually made of polished stainless steel, of about 50 to 300mm long, with internal diameter of between 2 and 5mm and an outside diameter of 6.3mm. They are commonly filled with a stationary phase with a particle size of 5 - 10μm [62]. The outlet is terminated by a stainless steel mesh disk to retain the packing material. The mesh disk is held in position by the end-fitting with the end of the tubing to the detector flush against the center. This tubing should have an internal diameter of 0.25mm or less and be as short as possible to minimize peak broadening [61]. Columns with internal diameters of less than 2 mm are often referred to as micro- bore columns. Ideally, the temperature of the mobile phase and the column should be kept constant during an analysis. Most separations are performed at ambient temperature, but columns may be heated to give better efficiency. Normally, columns are not heated above 60 °C because of the potential for stationary phase degradation or changes occurring to the composition of the mobile phase [62]. However, recent advances in technology is popularising the use of high temperature HPLC up to 200 °C. The most efficient columns produce the sharpest peaks, which give better separation by minimizing band spreading [65].

There are two main classes of column, normal and reversed phase columns. The normal phase columns are most usually packed with silica gel and are used in partition or adsorption chromatography. Reversed phase columns are packed with a chemically bonded octadecylsilyl coated silica; such columns are referred to as C-18 and are very non-polar. This is the most popular column used in HPLC [66].

There are various columns that are secondary to the separating column or stationary phase. They are: Guard, Derivatizing, Capillary, Fast, and Preparatory Columns (67).

Today most modern HPLC have column compartment which house the column for a proper temperature control.

Detectors

A detector is a component in the chromatographic system which senses the presence of a compound passing through it, and provides an electronic signal to a recorder or computer data station. The output is usually peaks known as the chromatogram [65].

It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The detector functions with a data processor normally referred as chromatography data system (CDS) which is computer software that converts generated peaks from the detector into useful analytical results. This facilitates naming peaks, integration of peaks and the production of quantitative data by the use of functions. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled [69]. With this chromatograms can overlaid with each other and a range of statistical functions may be applied. Most CDS enables conversion of results into user defined reports and also integration of data with other software such as Microsoft Excel. There are many types of detectors that can be used with HPLC. Some of the more common detectors include: Ultra-Violet (UV), Refractive Index (RI), Fluorescent, Radiochemical, Electrochemical, Near-Infra Red (Near-IR), Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Light Scattering (LS).

There are four types of detectors that have found widespread of application; an important factor in the choice of a detector is the amount of peak broadening which

occurs as the eluted compounds pass through. This is largely controlled by the volume and geometry of the detector flow-cell but is also influenced by the connecting tubing between the column and the detector [61].

2.2.5 Factors Affecting Chromatographic Fingerprint.

In herbal drug analysis, a large number of chemical components of which many are in low concentration are considered for chemical fingerprint. Hence chromatographic instruments and experimental conditions are difficult to reproduce during real analysis. Conditions that affect the chromatographic technique also affect the quality of the chromatographic fingerprint (Cf).

Several factors associated with chromatographic fingerprints such as the extraction method, the chromatographic parameters, measurement conditions, geographical and climate conditions of herbal plants makes it quite challenging task. Consequently, to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components is not an easy or trivial work.

2.2.6 Methods of Extraction and Sample Preparation

Methods of extraction and sample preparation are of great importance in preparing good fingerprints of herbal medicines. As a single herbal medicine may contain a great many natural constituents, and a combination of several herbs might give rise to interactions with hundreds of natural constituents during the preparation of extracts, the fingerprints produced by the chromatographic instruments, which may present a relatively good integral representation of various chemical components of

herbal medicines, are mainly concerned.

The traditional herbal medicines (HM) and their preparations have been widely used for centuries in many traditional settings. However, one of the characteristics of these herbal medicine preparations is that almost all the herbal medicines, either presenting as single herb or as collections of herbs in composite formulae, are extracted with boiling water during the decoction process. This may be the main reason why quality control of oriental herbal drugs is more difficult than that of western drug. As suggested in "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines (World Health Organization, 2000)" [39]

2.2.7 Chromatographic Parameters

This relates to the setup of HPLC system suitable for a particular analysis. This typically include:

Column Type

Though selection of appropriate column for analysis is usually based on prior experience or personal preferences. It is advisable to explore selectivity differences between C18 and C8 polar-embedded cyano or phenyl-bonded phase prior to analysis. Consulting Column selectivity chart to select equivalent columns based on its hydrophobicity or silanol activity is also recommended.

Column selection must be able to improve reproducibility, stability and versatile to ensure better assay consistency. Column parked with high purity (3 or 5µm) silica bonded phase with wider PH ranges from reputable manufacturers are often preferred [64].

Mobile Phase Composition

The choice of the mobile phase and the proportion of different solvent act to adjust the polarity in order to achieve proper separation within a practical time scale.

Binary mixtures is the most common types of mobile phase proportion, however, ternary mixtures can also be used. Normally, water is used with organic solvent such as methanol form miscible mixtures of preferred proportion in a reversed phase HPLC. This is essential, since the mobile phase composition controls the retention and the selectivity of the separation and can be conveniently and continuously adjusted. Lowering the solvent strength increases retention and the resolution of the analytes for a reversed phase HPLC. Thus an increase in the proportion of the organic solvent in the mobile phase composition reduces the retention time of the analytes. This is because the analyte will usually be more soluble in the organic phase and therefore spend more time in the mobile phase thus reducing the time spent on the stationary phase [63].

Flow rate

This refers to the flow of the mobile phase through the column and its usually measured in ml/min. On the other hand the flow rate can be obtained by collecting the liquid for a known period, and thereafter, either measuring the volume or weighing it physically periodically at the column outlet. It is important to stabilise the flow rate since an increase in the flow rate tends to elute the analyte faster. Operating at high flow rate increases column back pressure but reduces retention time and the certainly the resolution of analyte. Fluctuations in the flow rate will therefore introduce variation into the measured areas. Low flow rate increases the resolution as well as the enhancing separation of multi-complex peaks [64].

Injection volume

Most problems of poor peak area reproducibility has been associated to variability in the injection volume resulting from several factors. One of such source is variability in the syringe. For example, when syringe plunger is moved by an auto sampler motor there is an inherent variability in the positioning of the plunger which translate into variation in the volume of delivered and thus affecting peak height and peak area. Hence it is important to select a syringe of appropriate volume otherwise the injection can contribute to the uncertainty in the measured peak area significantly. Further details about injection volume and chromatographic performance can be found in the reference [68].

Temperature

In HPLC, difficult separations may be achieved by increasing the temperature carefully, but this must be done initially on a hit and trial basis since the maintenance of strict „temperature control" plays a vital role in measuring the retention-data correctly and precisely. This makes the temperature control an important variable and in reality, higher temperatures lower the viscosity of the mobile phase (i.e. column back pressure) and usually have significant effects on retention, efficiency and selectivity [64].

Detection settings

Detector settings are very important aspect in the chromatographic analysis especially in the case of UV-visible detector where detection is strictly based on absorption of ultra violet light by analyte. Therefore detection is applicable only to analytes with this property. It is important to note that even compounds which have

this property do not absorbed at the same extent or at the same wavelength and therefore this is a selective detector. Hence it is essential to know the appropriate settings of the detector"s wavelength at which a particular analyte will absorb. Improper settings cause several variations in the quantification of many analytes.

Another problem associated with detector settings is the optional use and configuration of „Reference wavelength" value software programmes in modern UV- vis detectors which functions are unknown to most chromatographer"s. For example there is always variation resulting from change in solvent property and temperature over time during gradient analysis noticeable baseline drift. This relative drift occurring from starting baseline causes variations in both retention time and the peak areas since it is easy to integrate flat baseline than slopes. In order to circumvent this problem, it is advisable to run the same method again without the sample (blank) and subtract from it [69].

2.2.8 Geographical and Climate Condition

The qualities of herbs are closely related to their producing areas, for this reason the determination of producing regions are critical. For example, the concentration and contents of the same kind of herbs originated from different regions or from the same region collected at different times may change distinctly. Several research reports have proved that herbal medicines of the same kind of plant will usually differ from each other greatly. Hence the concentration of their active components may vary from batch to batch thus affecting their efficiency in treating diseases. Even the quality of the same batch herbal medicine stored under different conditions may also vary significantly. It is vital to put this factor into consideration during chemical finger

printing to obtain a chromatogram of adequate information for a particular plant since growth of herbs is easily affected by geographical and climate conditions [71].

CHAPTER THREE

3.1.0 EXPERIMENTAL METHODS, MATERIALS AND REGENTS

3.1.1 Chemical and Reagents

All reagents and chemicals employed in this experiment were of analytical and HPLC graded obtained from the Pharmaceutical chemistry Department's chemical store (KNUST-Faculty of Pharmacy) in college of Health Science Kumasi.

Acetonitrile and methanol were HPLC grade, ethyl acetate, petroleum ether spirit, chloroform, 96% ethanol, Ammonium chloride, potassium hydroxide, sulphuric acid, Fehling"s solutions A and B, ferric chloride, Mayer"s reagent, Draggendorff"s reagent were of analytical grade and doubly distilled water were used throughout.

3.1.2 Instrumentation

The HPLC apparatus was a Shimadzu chromatographic system with two LC-20AB prominence liquid chromatography, variable wavelength programmable UV–vis detector SPD-20A, DGU-20A3 prominence Degasser (Shimadzu). The analysis was carried out on two different columns i.e. a reversed phase Luna C8 column (150 ×4.6mm i.d., particle size 5 µm); Bonclone 10 C18 column (300 x 3.9mm i.d., particle size 5 µm);and HPLC system was monitored by software "LC solution GPC version 1.2 (Shimadzu)". A model SL-164 UV–vis spectrophotometer was employed for spectrophotometric study.

3.1.3 Collection and Authentication of Plant Material

The fruits of Tetrapleura *tetraptera* were randomly obtained from the two (2) major market centres (i.e. Bantama Market and Central Market) in Kumasi. The Bantama Market samples were known to have been harvested from Ahafo in the Brong Ahafo region, while samples from the Central Market were harvested from Bogu in the Asanti Akim area. It was authenticated in the Department of Pharmacognosy, Faculty of Pharmacy and voucher specimen has been deposited at the pharmacy Herbarium of Kwame Nkrumah University of Science and Technology, Kumasi- Ghana.

A total of 8 samples of Tetrapleura *tetraptera* were collected with 4 samples from each sampling site. The collected specimen were manually washed with distilled water, sun-dried for 3 days, and separated into two different sections of the fruit (i.e. fleshy pulp, and woody shell containing the seeds). The fleshy pulps of the fruit were dried to constant weight in a conventional oven at 40^{o}C. The samples were ground into fine powder and stored in airtight plastic bags for further works.

3.1.4 Aqueous Extract of the Fine Powder (Decoction)

Approximately 4g of the powder was weighed into a glass stoppered conical flask. 100ml of distilled water was then added and weighed. The flask was then shaken and allowed to stand for 1hour. A reflux condense was attached to the flask and boiled for 4hours. After 4 hours, the flak was cool, weighed, and the original weight was then readjusted with distilled water. It was then shaken and filtered rapidly through a dry filter paper. 1ml of the filtrate was quantitatively transferred into 100ml

volumetric flask with distilled water which was then filtered through a syringe membrane filter of pores size 0.45 µm. 20µm of each of these solutions were subjected to HPLC analysis.

3.1.5 Ethanolic Extraction of the Fine Powdered Sample (Cold Extraction)

Approximately 4g of the powder was weighed into a glass stoppered conical flask. 100ml of 96% ethanol was added and the stopper was inserted. The flask was then shaken frequently for the first 6 hours before allowing it to macerate for 72 hours. The supernatant extract was rapidly filtered with precaution against any loss of ethanol. 1ml of the filtrate was quantitatively transferred into 100ml volumetric flask with 96% ethanol which was then filtered through a syringe membrane filter of pores size 0.45 µm. 20µm of each of these solutions were subjected to HPLC analysis.

3.1.6 Aqueous/ Petroleum Ether Fractions

Approximate weight of 4g of each fine powdered sample was extracted separately with 100ml distilled water for 4hours by heating under reflux. After cooling, the supernatant filtrate was then shaken with 100ml petroleum ether spirit in a separating funnel. This was repeated for four times with 50ml portions of the petroleum ether spirit to ensure complete extractions of the non-polar from the aqueous layer. The pairs of immiscible solvents were separated and the petroleum ether portion was evaporated to dryness. The constant weight of the petroleum ether fraction was completely dissolved in 100ml of 96% ethanol. 1ml aliquot of the dissolved fraction was quantitatively transferred into 100ml volumetric flask with 96%

ethanol which was then filtered through a syringe membrane filter of pores size 0.45 μm. 20μm of each of these solutions were subjected to HPLC analysis.

3.1.7 Phytochemical Test

Both aqueous and ethanolic extracts of the T. Tetraptera fruit samples were subjected to various phytochemical tests respectively to establish the presence or absence of some specific active principles through qualitative analysis.

3.1.8 Test for Alkaloid

0.5g of extract was diluted to 10ml with acid alcohol (1:9 portions of ammonia and ethanol respectively), boiled and filtered. To 5 ml of the filtrate was added 2ml of dilute ammonia. 5ml of chloroform was added and shaken gently to extract the alkaloidal base.

The chloroform layer was extracted with 10ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other.

The formation of a reddish brown precipitate (with Mayer's reagent) or (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

3.1.9 Test for flavonoids

Two different methods were used to test for flavonoids in both the Aqueous and Ethanolic extract

I. First 5ml of both the aqueous and ethanolic extract in their respective test tubes were heated with 10ml of ethyl acetate each over a steam bath for 5min. The respective mixtures were filtered and 4ml each of the filtrate were shaken with 1ml of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

II. 5ml each of dilute ammonia were added to 3ml of the aqueous and the ethanolic filtrate of the extract in their separate test tubes.1ml concentrated sulphuric acid were added to the content of the test tubes respectively. A yellow colouration that disappears on standing indicates the presence of flavonoids. Alkaline acid test (Harborn, 1973)

3.2.0 Test for reducing sugars (Fehling's test)

About 0.5g of the powdered fruit was boiled with about 20ml of 1% H_2SO_4 and filtered.

The filtrate was rendered alkaline with dilute KOH. Few drops of Fehling"s solutions A and B were added and the mixture heated on water bath for about 5min. and observed for brick-red precipitation.

3.2.1 Test for saponins

The combined froth and emulsion test was used to test for the presence of saponin in the sample.

2ml of the aqueous extract was mixed with 10ml of distilled water in a test tube. The mixture was shaken vigorously for about 1min and observed for stable formation of

froth (foam) as a positive result. However, this was confirmed by the addition of few drops of olive oil and shaken again. The formation of an emulsion confirmed the presence of saponin in the test sample.

3.2.2 Test for Steroids/Triterpens

The Dragendert's test was used. A portion of the ethanolic extract was mixed with acetyl anhydride in a test tube. The presence of a brown colouration at the interface between the two layers indicated a positive result for steroid.

3.2.3 Test for Tannins (Ferric Chloride test by Harborn, 1973)

5mls of the aqueous extract mixed with equal volume of distilled water in a test tube and drops of diluted ferric chloride solution. The presence of dark green precipitate is a positive indication of the presence of tannin in the extract.

3.2.4 Determination of Extract Yield

25ml of the ethanolic extract filtrate was evaporated to dryness in a flat bottomed shallow dish at 105^{o}C to constant weight. Subsequently, the dish and its content were reweighed. The content in mg/g of ethanol extract mater in the test specimen was calculated. By difference, the weight of extract was calculated using the formula below:

$$\text{Extract yield in mg/g} = (w_2\text{-}w_1/w_t)^* \ 1000/25$$

wt = weight of sample

w1 = weight of empty evaporation dish

w2= weight of dish + extract

The weight of extract (w2-w1) was multiplied

by 1000 to convert it from grams to milligrams

aliquot in 25ml

CHAPTER FOUR

4.1.0. RESULTS, DISCUSSIONS AND CONCLUSION

4.1.1 Phytochemical Test Results and Extraction Yield of Tetrapleura *Tetraptera*

Table 4.1 shows the results of the basic phytochemical test performed on aqueous and ethanolic extracts of the Tetrapleura tetraptera fruit extract. On this table the Symbol (+) indicates the presence of a particular constituent while the sign (-) signifies absence of a constituent.

Table 4.1: Results of basic Phytochemical test of Tetrapleura Tetraptera sample in water and ethanol.

Phytochemical constituent	Water	Ethanol
Alkaloids	(-)	(+)
Flavonoids	(+)	(+)
Reducing Sugars	(+)	(+)
Saponins	(+)	(+)
Steroids/ Triterpens	(+)	(+)
Tannins	(+)	(+)

Table 4.2 Weight of powdered sample of T. *tetraptera*, Sampling Site and Date of Collection

Sample number (SN)	Weight of powdered sample of T. tetraptera in grams (g) *Wt*	Collection location (Sampling Site)	Date of Sampling
01	4.0012	Bantama Market (BM)	14th Sept. 2011
02	4.0011	Bantama Market (BM)	14th Sept. 2011
03	4.0000	Bantama Market (BM)	14th Sept. 2011
04	4.0010	Bantama Market (BM)	14th Sept. 2011
05	4.0001	Central Market (CM)	14th Sept. 2011
06	4.0021	Central Market (CM)	14th Sept. 2011
07	4.0011	Central Market (CM)	14th Sept. 2011
08	4.0012	Central Market (CM)	14th Sept. 2011

W1 is weight of empty evaporation dish = 72.9117g

W2 = weight of dish + extract

Volume of Extract evaporated = 25ml

Table 4.3 Corresponding weights of dish+ aqueous extract and their relative weight in grams (g)

SN	(W2) in grams	(w2-w1) in gram
01	73.1919	0.2802
02	73.1826	02709
03	73.1824	0.2707
04	73.1927	0.2810
05	73.1910	0.2793
06	73.1927	0.2810
07	73.1825	0.2708
08	73.1921	0.2804

Calculation of content in mg/g of aqueous extract of T. tetraptera from (BM)

Extract yield for *SN01* = (0.2802/4.0012)*1000/25

= 2.8012mg/g

Extract yield for *SN02* = (0.2709/4.0011)*1000/25

= 2.7082mg/g

Extract yield for *SN03* = (0.2707/4.0000)*1000/25

= 2.7070mg/g

Extract yield for *SN04* = (0.2810/4.0010)*1000/25

= 2.8090mg/g

Table 4.3 (a) Percentage deviation of content yield for aqueous extract samples from Bantama Market (BM).

SN	weight in mg/g	Deviation	% Deviation
01	2.8012	0.04485	1.627151849
02	2.7082	-0.04815	-1.7468754
03	2.7070	-0.04935	-1.79041123
04	2.8090	0.05265	1.91013478

Mean ( ) =2.75635

Standard Deviation =0.056383774

SEM =0.028191887

RSD =2.05%

Calculation of content in mg/g of aqueous extract of T. tetraptera from (CM)

Extract yield for *SN05* = (0.2793/4.0001)*1000/25

= 2.7929mg/g

Extract yield for *SN06* = (0.2810/4.0021)*1000/25

= 2.8085mg/g

Extract yield for *SN07* = (0.2708/4.0011)*1000/25

= 2.7073mg/g

Extract yield for *SN08* = (0.2804/4.0012)*1000/25

= 2.8031mg/g

Table 4.3.(b) Percentage deviation of content yield for aqueous extract samples from Central Market (CM).

SN	weight in mg/g	Deviation	%Deviation
05	2.7929	-0.01495	-0.538167
06	2.8085	-0.03055	-1.099732
07	2.7073	0.07065	2.543242
08	2.8031	-0.02515	-0.905344

Mean (□) = 2.77795

Standard Deviation = 0.047542087

SEM = 1.388975

RSD = 1.71%

Table 4.4 Corresponding weights of dish+ ethanolic extract and their relative weight in grams (g).

SN	(W2) in grams	(w2-w1) in gram
01	73.2373	0.3256
02	73.2368	0.3251
03	73.2349	0.3232
04	73.2361	0.3244
05	73.2359	0.3242
06	73.2378	0.3261
07	73.2371	0.3254
08	73.2372	0.3255

Calculation of content in mg/g of ethanol extract of T. tetraptera from (BM)

Extract yield for *SN01* = (0.3256/4.0012)*1000/25

= 3.2550mg/g

Extract yield for *SN02* = (0.3251/4.0011)*1000/25

= 3.2501mg/g

Extract yield for *SN03* = (0.3232/4.0000)*1000/25

= 3.2320mg/g

Extract yield for *SN04* = (0.3244/4.0010)*1000/25

= 3.2432mg/g

Table 4.4 (a) Percentage deviation of content yield for ethanolic extract samples from Bantama Market (BM).

SN	Weight in mg/g	Deviation	% Deviation
01	3.255	0.009925	0.305848093
02	3.2501	0.005025	0.154850042
03	3.232	-0.013075	-0.402918268
04	3.2432	-0.001875	-0.057779866

Mean ($\bar{x}$) = 3.245075

Standard Deviation = 0.009970415

SEM = 0.004985207

RSD = 0.31%

Calculation of content in mg/g of ethanol extract of T. tetraptera from (CM)

Extract yield for SN05 = (0.3242/4.0001)*1000/25

= 3.2419mg/g

Extract yield for SN06 = (0.3261/4.0021)*1000/25

= 3.2593mg/g

Extract yield for SN07 = (0.3254/4.0011)*1000/25

= 3.2531mg/g

Extract yield for SN08 = (0.3255/4.0012)*1000/25

= 3.2540mg/g

Table 4.4(b) Percentage deviation of content yield for ethanolic extract samples from Central Market (CM).

SN	weight in mg/g	Deviation	% Deviation
05	3.2419	-0.010175	-0.312877163
06	3.2593	0.007225	0.222165848
07	3.2531	0.001025	0.031518338
08	3.254	0.001925	0.059192977

Mean (□) =3.252075

Standard Deviation =0.007314085

SEM =0.003657042

RSD =0.23%

Table 4.5 (a) Mean and Relative standard deviation of extraction yield for aqueous sample of T. tetraptera from Bantama Market (BM) and Central Market (CM) Respectively.

Sample Sites	Bantama Market (BM)	Central Market (CM)
Mean(□) ± SEM	2.7564 ± 0.0282 mg/g (n=4)	2.7780 ± 1.3890 mg/g (n=4)
RSD	2.05%	1.71%

*SEM is the Standard Error of the Mean

**RSD is the Relative Standard Deviation

Table 4.5 (b) Mean and Relative standard deviation of extraction yield for ethanolic extract sample of T. tetraptera from Bantama Market (BM) and Central Market (CM) Respectively.

Sample Sites	Bantama Market (BM)	Central Market (CM)
Mean(□) ± SEM	3.2451 ± 0.0050 mg/g (n=4)	3.2521 ± 0.0037 mg/g (n=4)
RSD	0.31%	0.23%

*SEM is the Standard Error of the Mean

**RSD is the Relative Standard Deviation

The result of extract yield and qualitative phytochemical test showed, flavonoid, reducing sugar, saponin, steroids/ triterpens and tannin, were found present in the aqueous extract while alkaloids, flavonoid, reducing sugar, saponin, steroids/ triterpens and tannin were found present in the ethanolic extract. Refer Table 4.1. These groups of chemical constituents and others - perhaps yet unknown - form the active principles that confer the pharmacological use of the extract by many in Ghana. The above test in Table 4.1 confirms the presence of several phytochemical constituents of T. *tetraptera* as indicated in literature by renowned African researchers. From Table: 4.5. (a), it was observed that the aqueous extract yield of

T. *tetraptera* from (BM) was found to be 2.7564 ± 0.0282 mg/g weight of the dried powdered T. *tetraptera* for four (4) different samples from the same site. This corresponds to 2.05% RSD value. On the other hand, the aqueous extract yield of T. tetraptera from (CM) was found to be 2.7780 ± 1.3890 mg/g weight of the dried powdered T. *tetraptera* for four (4) different samples from the (CM) site. This alternatively corresponds to 1.71% RSD value. Comparatively, the aqueous extract yield from (BM) and (CB) are found to be precise since their respective %RSD values are smaller even less than 3.00%, and closer to 0.00% RSD value for ideal situation which is practically impossible for any laboratory work. However, the aqueous extract yield of T. *tetraptera* from (CM) with %RSD value of 1.71% shown to be more precise than that of the (BM) with 2.05%. The mean obtained for both samples from (BM) and (CM) under the same extraction method -aqueous extraction by decoction- expressed in their respective standard error of the mean (SEM), were used to show that, for every 1g of the dried samples of the T. *tetraptera* taken through this extraction procedure, the extracted aqueous solution is likely to contain 2.7564mg and 2.7780mg of dried constant weight of all the principle constituent

within the errors of ±0.0282mg and ±1.3890mg respectively. Although the respective quantities of the various phytochemical constituents in the T. *tetraptera* were not determined but the total weight of all the chemical constituents were calculated in order to estimate quantitatively the weight of the phytochemical constituents present in a gram of dried T. *tetraptera*. This is significant because it can be used to estimate a weight of chemical constituents that will be present in a known weight of the dried

T. *tetraptera* on aqueous decoction extraction.

The extraction yield for ethanolic extract samples of T. *tetraptera* from both (BM) and (CM) were found to be 3.2451 ± 0.0050mg/g and 3.2521 ± 0.0037mg/g weight of the dried powdered T. *tetraptera* for four (4) different samples in each sampling site respectively. Refer table 4.5(b). Their corresponding %RSD value were determine to be 0.31% and 0.23% indicating that extracted sample from (CM) was much precise than that of the (BM). Comparatively, the ethanolic extract show relatively small

%RSD values than that of the aqueous extract yield, suggesting that the extraction method with ethanol was precise and effective than that with distilled water. More over the extraction yield for ethanolic extract was higher than that in the aqueous extract. This is can be attributed to the extra alkaloids that were found present in the ethanolic extract during the phytochemical screening of the T. *tetraptera*.

Research has shown that the presence of Saponin can control human cardiovascular disease and reduce cholesterol, however, excess saponin has been shown to homolyse red blood cells by destroying the erythrocyte membrane (Sodipo and Akiniyi, 2000; Abii and Elegalam, 2007) [72, 73]. This suggest that oral intake of the extract dose should be put under experimental studies in order to determine significant tolerant regimen required for effective treatment in spite the fact that

phytochemicals in T. *tetraptera* fruit extract has not yet shown any adverse effect on liver function.

The presence of flavonoids has antioxidant property while Tannins may provide protection against microbiological degradation of dietary proteins in the semen (Aletor, 1993, OKwu 2004) [27, 70]. This in effect has generate versatile wide range of local therapeutic applications of the plant T. *tetraptera* as laxative, analgesic, antifungal, antibacterial and anti-inflammatory across some part of Africa (Faruq *et al*., 2004, Olafinmihan, 2004) [71].

4.1.2 Optimization of the Extraction Method

In order to achieve a satisfactory extraction efficiency, extraction methods and extraction solvents were investigated based on the two major extraction methods

(i.e. decoction and maceration for oral preparation) employed in folkloric medicine. Two (2) different solvents were selected for the extraction of the constituents in T. *tetraptera.* These were distilled water and 96% ethanol. In the first extraction method, samples of dried T. *tetraptera* was shaken and refluxed with distilled water as described in Chapter 3 section 3.1.4. In the second extraction method, samples of dried T. *tetraptera* were macerated with 96% ethanol of equivalent volume as that of the distilled water in four (4) proportions for 72 hours. Refer Chapter 3, section 3.1.5. A third extraction method was developed by shaken and reflux T. *tetraptera* with distilled water. This was then followed by addition of petroleum ether spirit. The immiscible solution was then separated into polar and nonpolar as described in Chapter 3, section 3.1.6. to separate the polar fraction from the nonpolar fraction.

Comparing various extractions methods with their respective chromatographic profiles as in the Figures; 4.0, 4.1, 4.2, 4.3, 4.4 and 4.5, and their corresponding

retention times, it was observed that the solvent extraction method condition was suitable and effective. From the various chromatograms, it was observed that, the solvent extraction method gave well resolved peaks with many peaks to characterized T. *tetraptera* samples than the other extraction methods.

The method of extraction is important in fingerprinting because it is able to give enough chromatographic profile information require for detailed analysis. Furthermore, these two extraction methods are the most dominant processes commonly used by traditional medicinal practitioners in the extraction of most herbal medicine for oral treatments, and hence cannot be ignored in the analysis by any contemporary method. However, the choice of extraction method depends on the targeted principle constituents (i.e. polar or nonpolar) as well as the kind of diseases meant to be treated.

4.1.3 Optimization of Chromatographic Condition

To achieve chromatograms with adequate chemical information and better resolution within a reasonable analysis time for qualitative fingerprint analysis, the column, mobile phase, detection wavelength, column temperature and elution conditions were investigated. The chromatographic conditions were optimised to obtain a suitable fingerprint containing sufficient information of constituents with good resolution of peaks within short analysis time. Two (2) different types of chromatographic columns namely; a reversed phase Luna C8 column (150 ×4.6mm i.d., particle size 5 µm) and Bonclone 10 C18 column (300 x 3.9mm i.d., particle size 5 µm) were screened.

The extraction methods showed different retention on these columns. The Luna C8 column was found to be more suitable for good separation and adequate chemical information for the extracts.

The effect of mobile phase composition on chromatographic separation was investigated and an obvious distinction between water-methanol-acetonitrile and water-methanol was found. Considering the good peak separation and sharp peaks, the ternary mixture of water-methanol-acetonitrile (85:5:10 v/v) was found to improve the peak shape for aqueous extraction method (Decoction) as shown in figures 4.0 and 4.1. On the other hand, the binary mixture of water-methanol (90:10 v/v) was observed to enhance the peak shape for the solvent extraction method (nonpolar and nonpolar fractions) as shown in figures: 4.2, 4.3 for polar fractions and figures:

4.4 and 4.5 for nonpolar fraction. The flow rate was modified to obtain well resolved chromatogram with maximum number of peaks at 0.5ml/min for both mobile phases. However, both mobile phases were not able to separate the ethanolic extraction method (maceration) for the T. *tetraptera* sample.

Selection of detection wavelength was one of the key factors contributing to a reliable HPLC fingerprint in complex samples. Hence, full scan UV-vis absorption was carried out within the region of 200nm-800nm for all the extraction method samples to determine maximum absorption after using their respective solvent for extraction to run a blank. It was clearly observed that the maximum absorption for all the extraction methods at low concentrations occurred at 280nm and 310nm. However, it was considered that detection at 280nm would favour sensitivity and precision for the determination of all the extraction methods over detection at higher wavelengths 310nm since absorbance at 280nm was found to be better and

common in all the extraction methods than that at 310nm. Therefore, 280nm was selected as ultraviolet detection wavelength for the programmable UV–vis detector SPD-20A.

Fig.4.0 (e) and Fig.4.1 (e) Overlaid chromatograms for T. *tetraptera* samples from BM and CM respectively under the same extraction method and chromatographic conditions -Aqueous Decoction Method of Extraction-

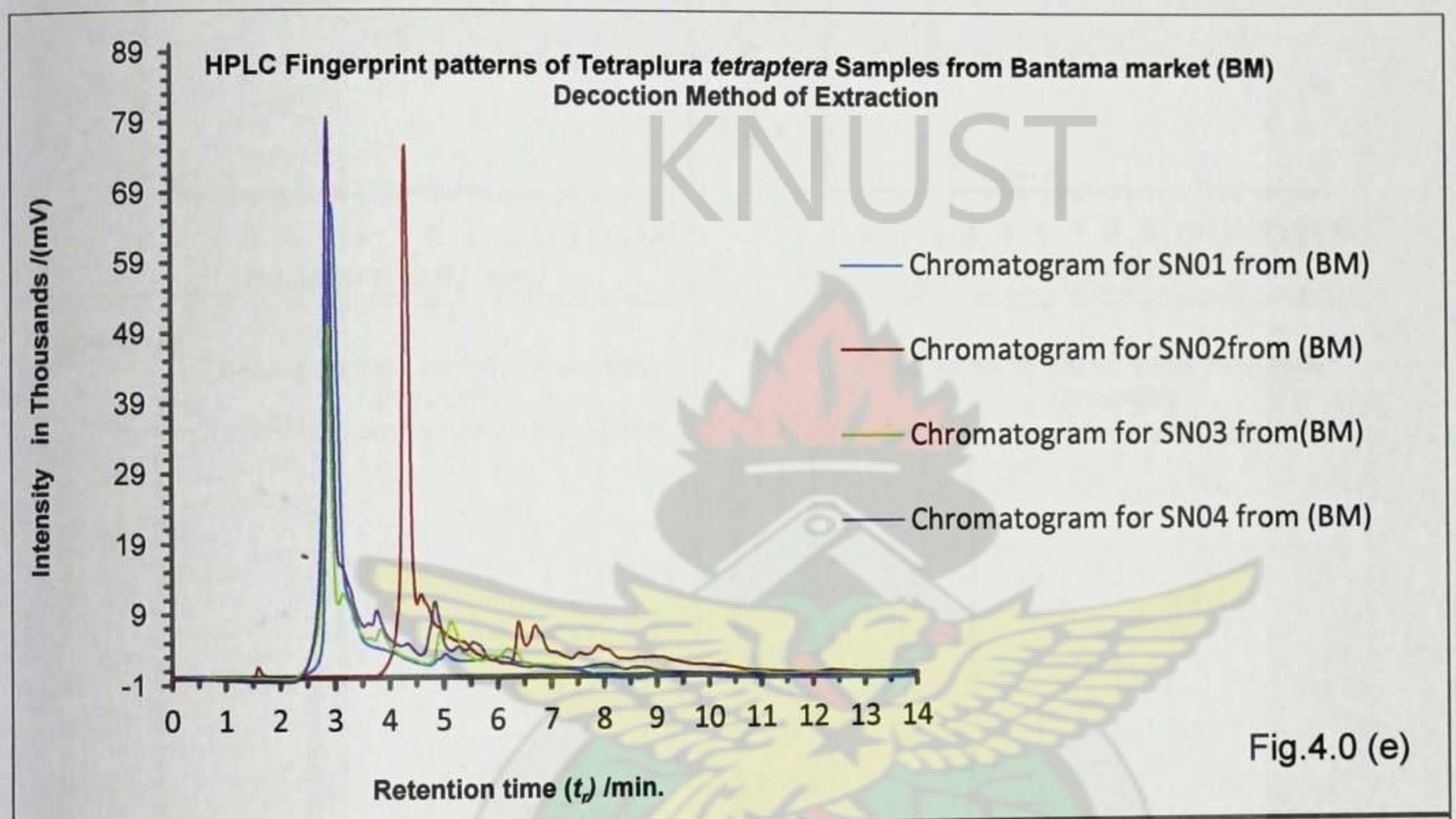


Fig.4.0 (e)

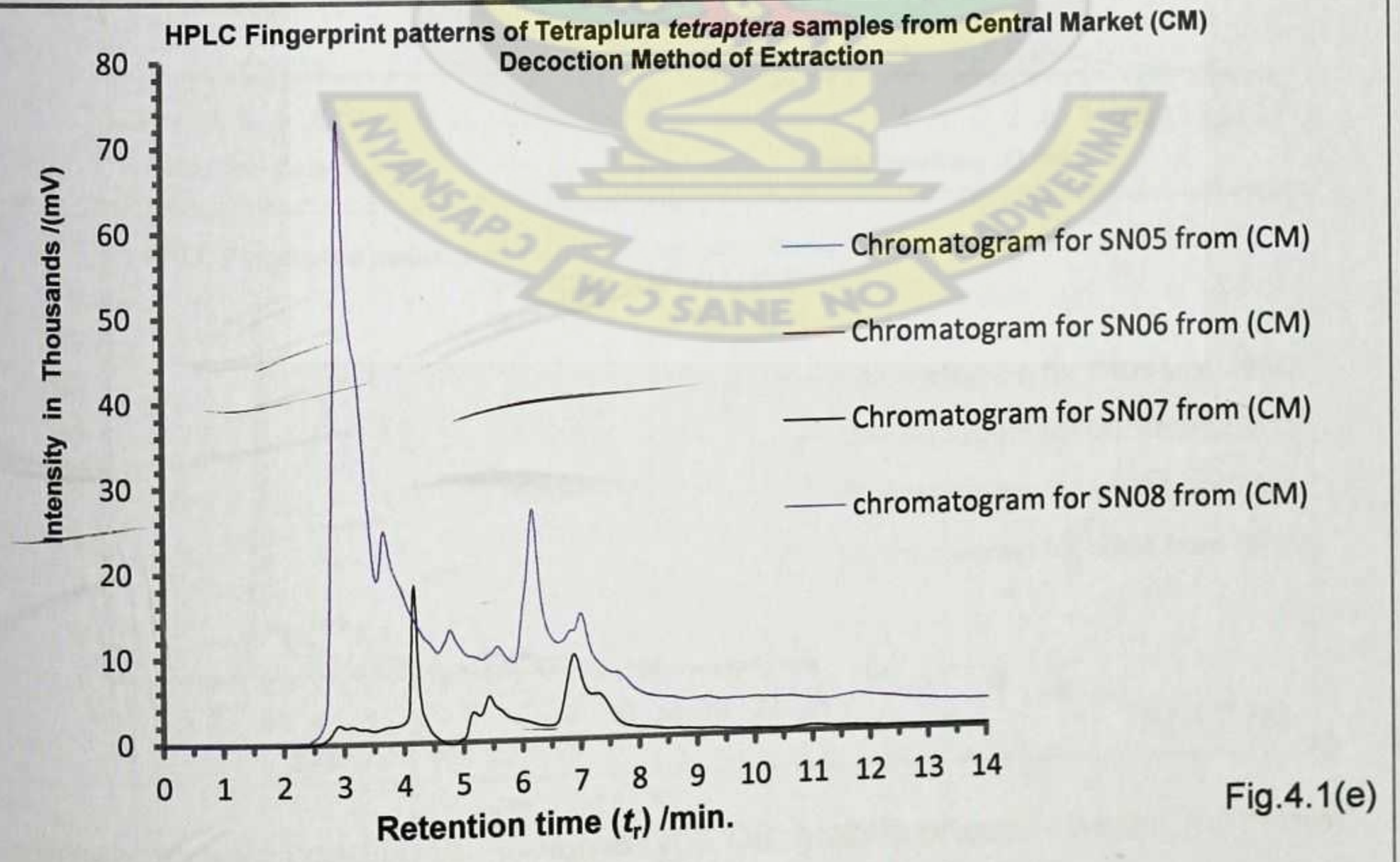


Fig.4.1(e)

Figure 4.0 Chromatograms for T. *tetraptera* samples from Bantama Market (BM) Aqueous extract-Decoction Method of Extraction

Fig.4.0(a)

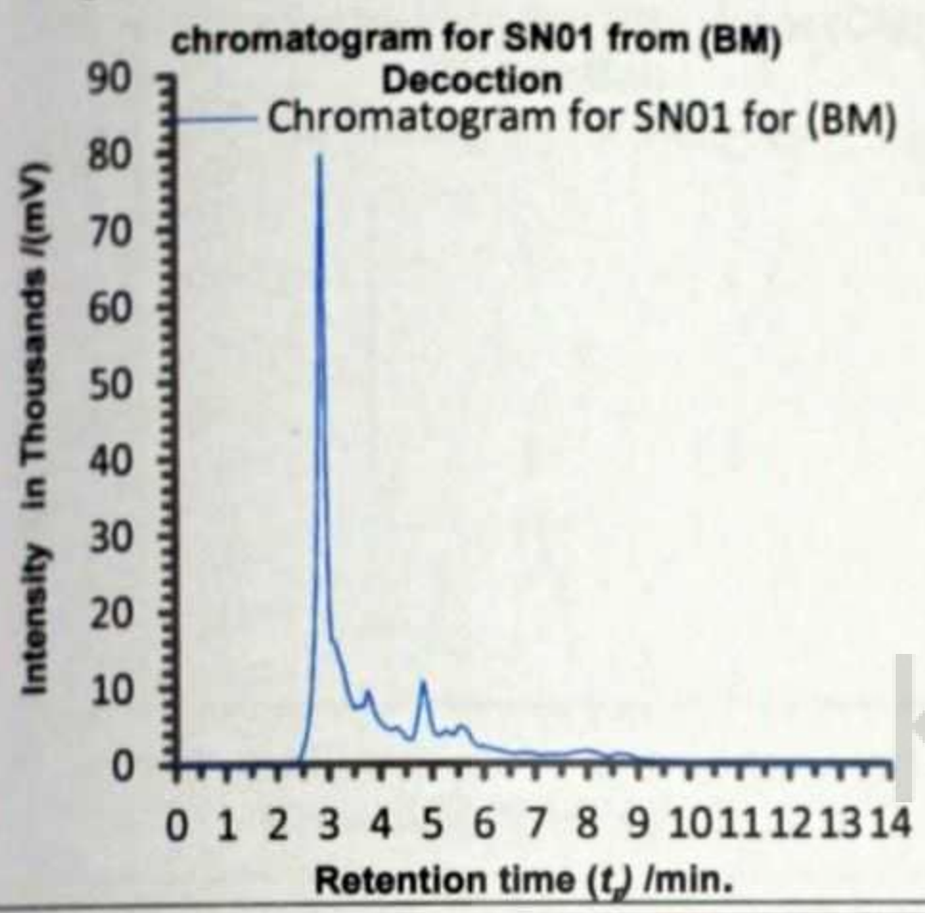


Fig.4.0(b)

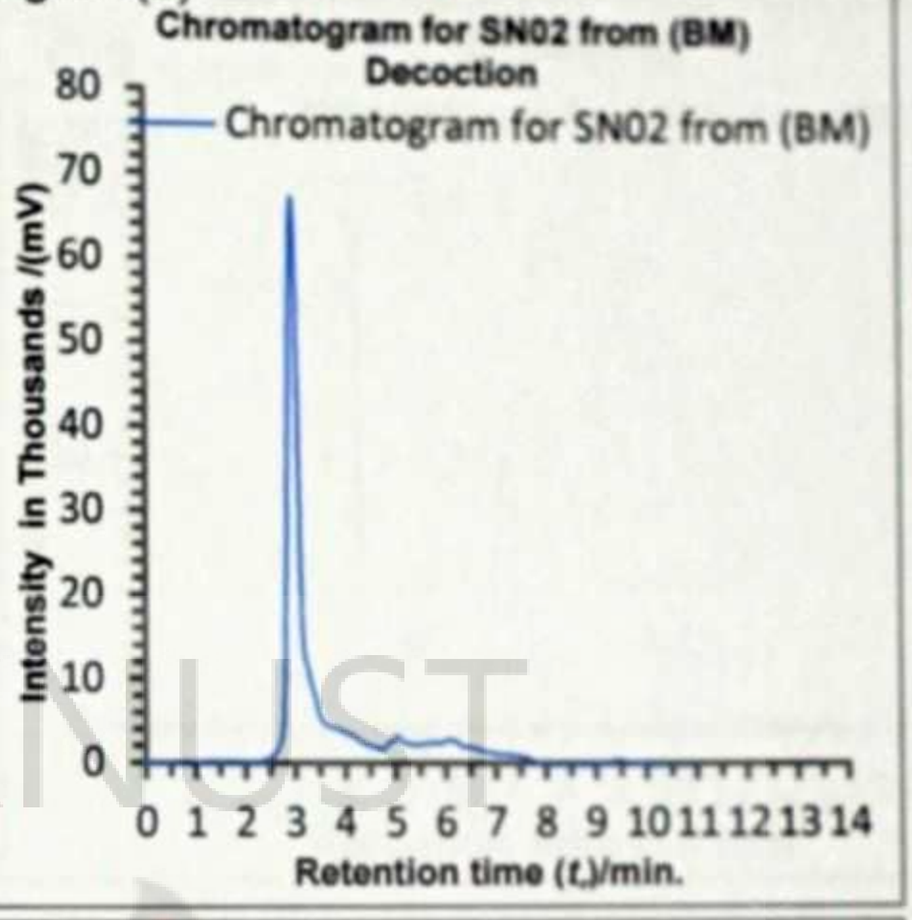


Fig.4.0 (c)

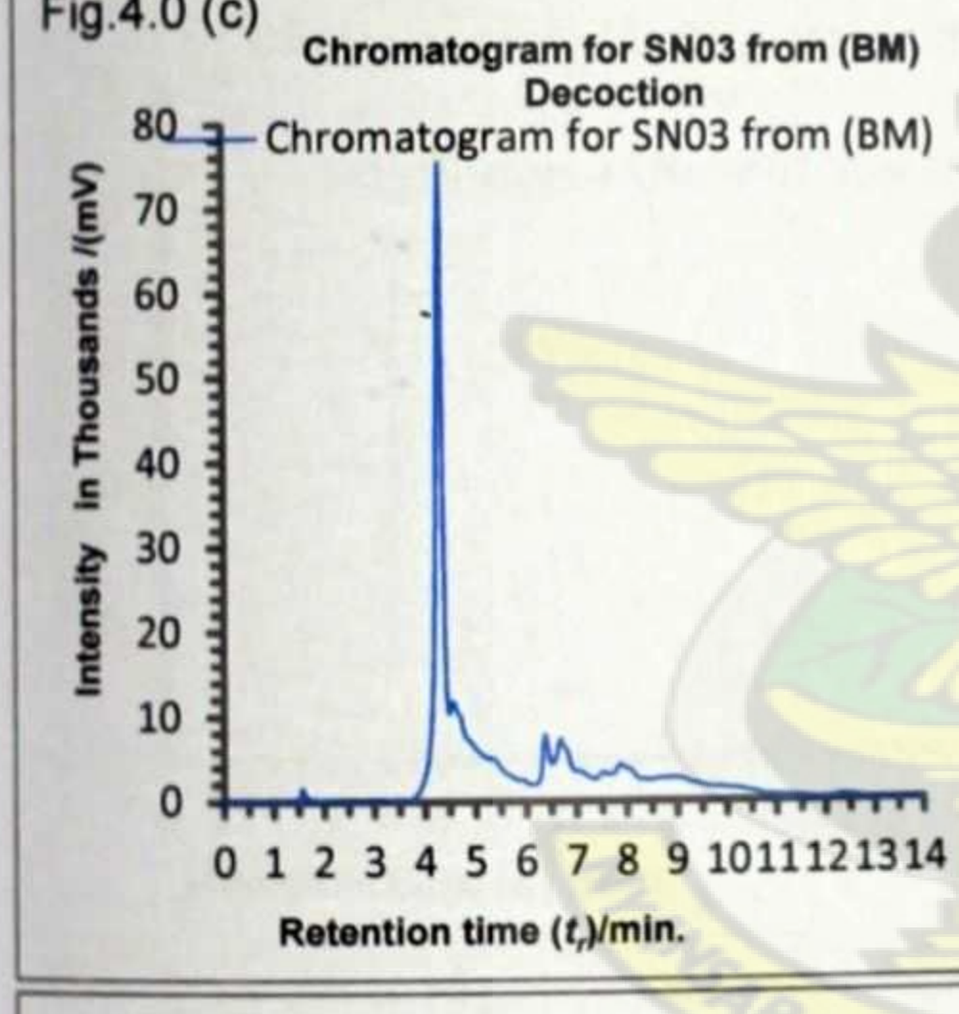
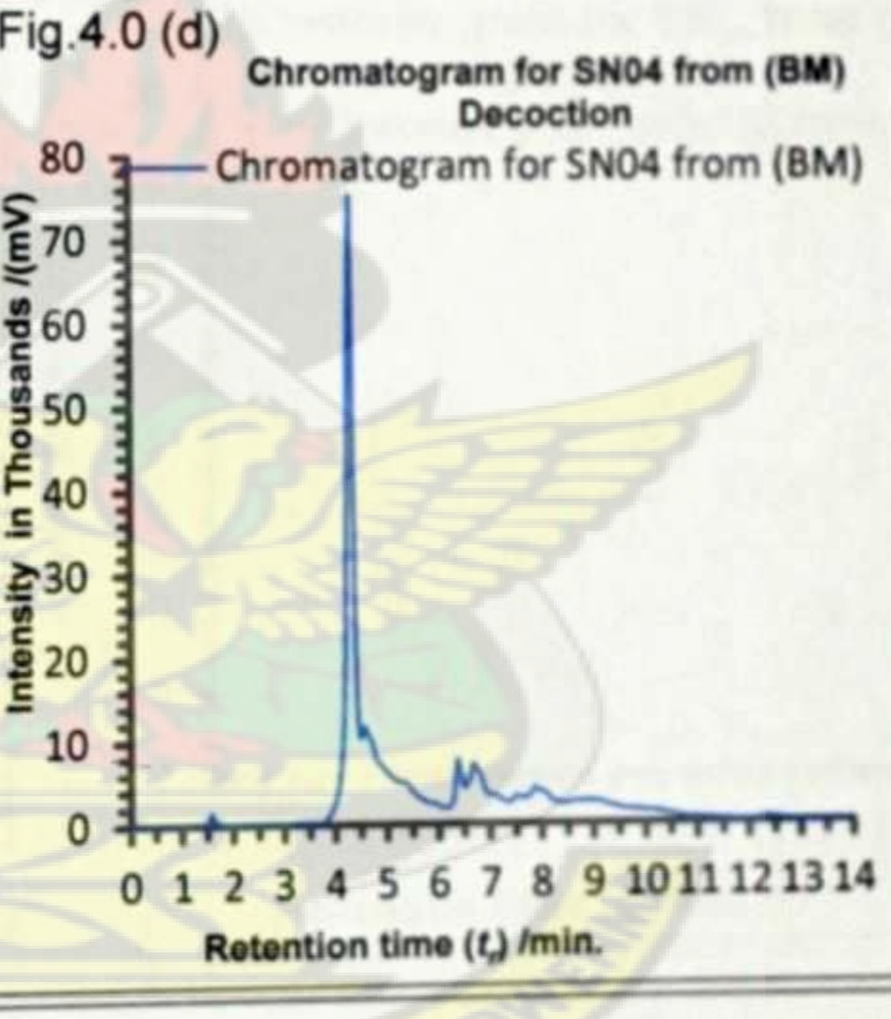


Fig.4.0 (d)



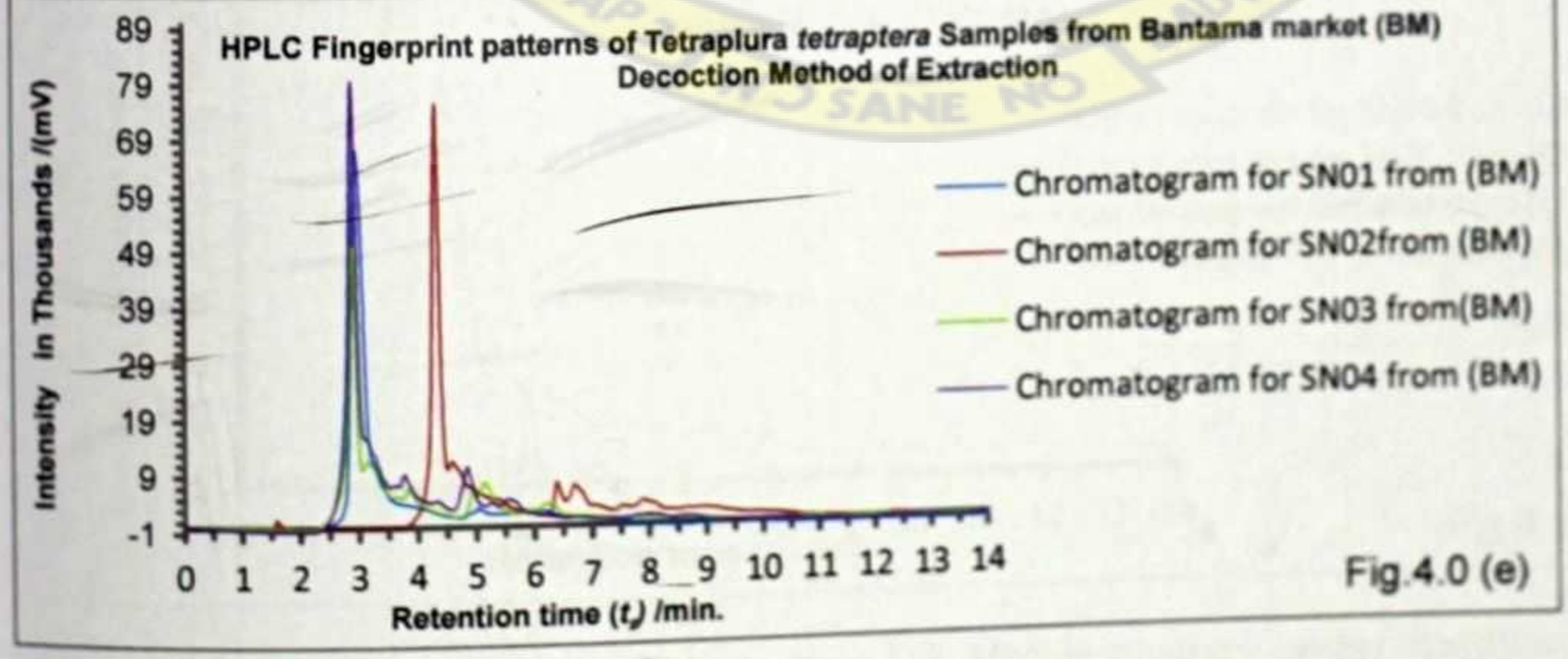
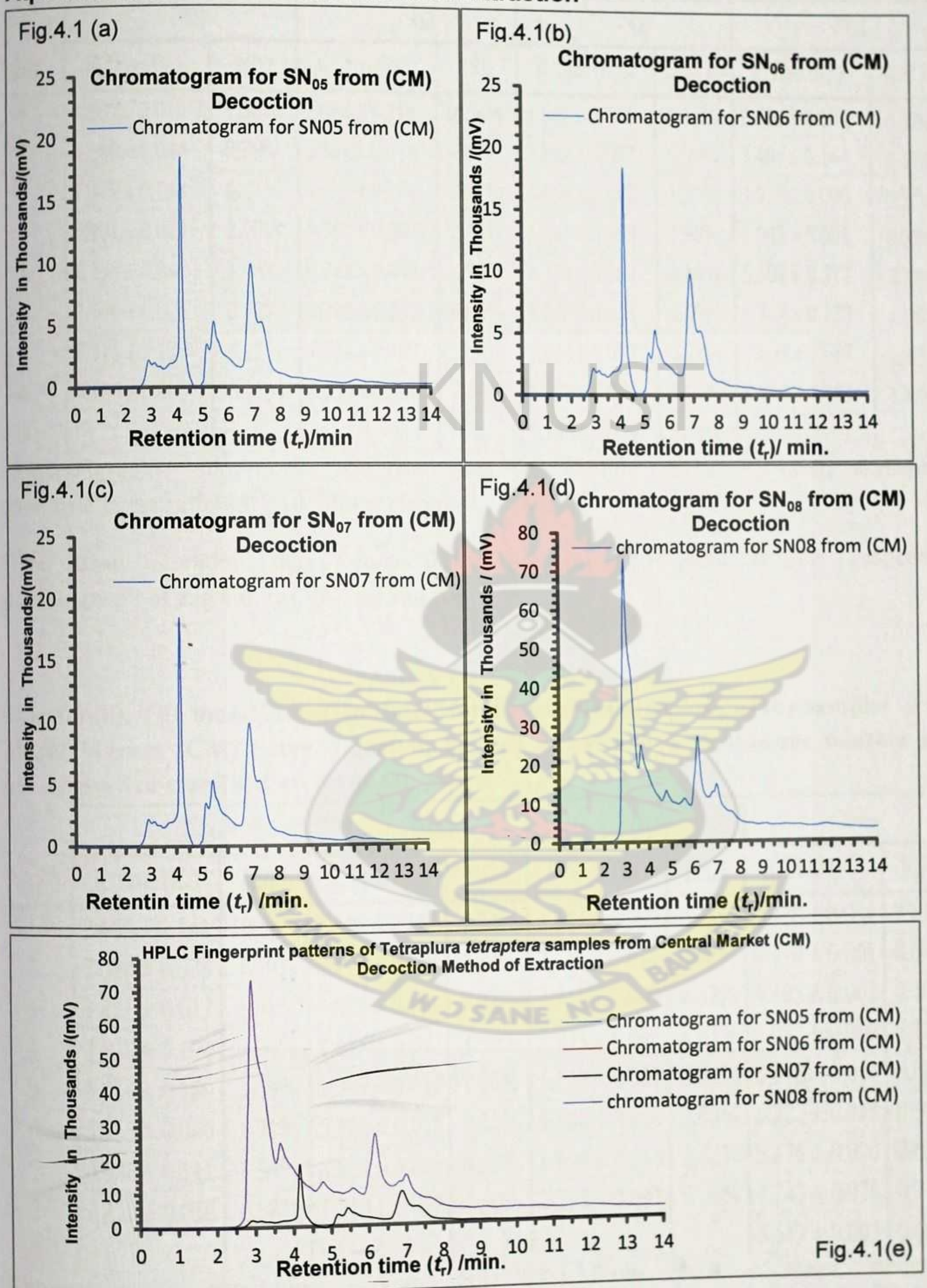


Fig.4.0 (e)

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol, acetonitrile in the ratio 85:5:10, at wavelength λ = 280nm

Figure 4.1 Chromatograms for T. *tetraptera* samples from Central Market (CM) Aqueous extract-Decoction Method of Extraction



*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol, acetonitrile in the ratio 85:5:10, at wavelength λ = 280nm.

Table 4.6 (a). The mean retention times' for 4 replicates injections of the samples from Bantama Market (BM) using decoction method of extraction in aqueous solution and their respective standard error of the mean.

	N_{01}		N_{02}		N_{03}		N_{04}	
Peak#	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD
1	2.867 ± 0.019	1.38%	2.664 ± 0.218	16.30%	1.661 ± 0.073	8.79%	2.886 ± 0.001	0.06%
2	3.689 ± 0.044	2.39%	3.337 ± 0.076	4.57%	3.519 ± 0.007	0.39%	3.404 ± 0.144	8.46%
3	4.579 ± 0.148	6.47%	4.520 ± 0.208	9.21%	4.454 ± 0.072	3.23%	3.839 ± 0.006	0.31%
4	5.407 ± 0.079	2.90%	5.368 ± 0.067	2.46%	5.643 ± 0.169	5.98%	4.943 ± 0.001	0.04%
5	6.798 ± 0.049	1.44%	6.748 ± 0.044	1.30%	6.184 ± 0.067	2.16%	5.503 ± 0.212	7.70%
6	7.309 ± 0.026	0.72%	7.296 ± 0.032	0.87%	6.536 ± 0.088	2.69%	6.324 ± 0.132	4.17%
7	8.311 ± 0.176	4.22%	7.704 ± 0.103	2.67%	7.614 ± 0.087	2.28%	7.669 ± 0.087	2.26%
8	9.506 ± 0.005	0.11%	8.514 ± 0.087	2.04%	8.789 ± 0.071	1.61%	8.624 ± 0.081	1.87%
9	–	–	–	–	9.735 ± 0.056	1.15%	–	–

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol, acetonitrile in the ratio 85:5:10, at wavelength λ = 280nm

**The mean retention times from Table 4.6 (a) correspond to the respective chromatograms of Fig 4.0. (a), (b), (c) and (d)

Table 4.6 (b). The mean retention times' for 4 replicates injections of the samples from Central Market (CM) using decoction method of extraction in aqueous solution and their respective standard error of the mean.

	N_{05}		N_{06}		N_{07}		N_{08}	
Peak#	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD
1	2.669 ± 0.141	10.54%	2.616 ± 0.063	4.84%	2.741 ± 0.099	7.23%	2.220 ± 0.031	2.81%
2	3.090 ± 0.028	1.79%	3.449 ± 0.021	1.20%	3.117 ± 0.011	0.68%	3.470 ± 0.001	0.04%
3	3.321 ± 0.017	1.01%	3.803 ± 0.002	0.07%	3.346 ± 0.009	0.52%	4.405 ± 0.002	0.10%
4	3.829 ± 0.190	9.94%	4.035 ± 0.072	3.56%	3.933 ± 0.096	4.87%	5.052 ± 0.009	0.34%
5	5.014 ± 0.180	3.59%	5.067 ± 0.032	1.26%	5.128 ± 0.021	0.84%	5.578 ± 0.017	0.62%
6	5.355 ± 0.090	1.71%	5.366 ± 0.015	0.55%	5.340 ± 0.054	2.03%	6.222 ± 0.018	0.59%
7	6.686 ± 0.111	3.34%	6.870 ± 0.008	0.22%	6.694 ± 0.116	3.47%	6.476 ± 0.020	0.62%
8	7.228 ± 0.020	0.58%	7.210 ± 0.018	0.50%	7.233 ± 0.001	0.04%	7.243 ± 0.026	0.73%
9	–	–	–	–	–	–	8.517 ± 0.003	0.06%

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol, acetonitrile in the ratio 85:5:10, at wavelength λ = 280nm.

**The mean retention times from Table 4.6.(b) correspond to the respective chromatograms of Fig 4.1. (a), (b), (c) and (d)

4.1.4 Comparison of HPLC Fingerprint Chromatograms for T. *tetraptera* Samples from BM and CM under the Same Chromatographic conditions (Aqueous Decoction Extraction Method)

The chromatographic peaks of extracts from each sampling site and their extraction methods were compared to see variations in them. Their corresponding retention times as well as common characteristic peaks were compared to observe variation in their respective SEM and %RSD.

This was done to see how various retention times for their corresponding chromatograms spread around their respective mean. Comparison of chromatograms from BM and CM sampling sites measured by the same kind of column, the same extraction method, the same flow rate, the same wavelength but different samples to see how they vary from each other, and the variations in their retention times as well as common characteristic peaks which run through all the chromatogram. Figure 4.0, indicate chromatograms of T. *tetraptera* samples from BM extracted by aqueous decoction method of extraction. Four (4) samples from BM labeled SN01, SN02, SN03 and SN04 corresponding to fig.4.0 (a), fig.4.0 (b), fig.4.0(c) and fig4.0(d) chromatographic profiles respectively. Refer to figure 4.0

Visible observations show a tall prominent peak eluting between times (2.5 – 4.5 minutes). This particular peak ~~was found~~ running through all the injected samples from BM under aqueous decoction method of extraction. Though, the exact constituent was not identified by further qualitative test or spectroscopic methods such as IR-spectra, NMR-spectroscopy and Mass spectroscopy after elution. However, it was further observed that the characteristic peak is likely to be peak number (peak #) 2 with retention times; 3.689 ± 0.044, 3.337 ± 0.076, 3.518 ± 0.007

and 3.404 ± 0.144 for samples SN01, SN02, SN03 and SN04 respectively according to their corresponding peak areas. Refer table 4.6 (a). In fig.4.0 (e), the 4 chromatograms were overlain to compare and see various shift and similarities in their fingerprint patterns. In general, 8 peaks were observed for samples SN01, SN02, and SN04 from BM using decoction method of extraction in aqueous solution except SN03 which gave 9 peaks. Compering all the peaks for various samples from BM- decoction method- it was found out that %RSD ranges from 0.04% to 16.30%. Refer table 4.6 (a)

Figure 4.1 show chromatograms samples from CM under the same conditions as figure 4.0. Four (4) samples from the CM labeled SN05, SN06, SN07 and SN08 representing the various chromatographic profiles of figure 4.1; (a), (b), (c) and (d) respectively. There were clear indications that the chromatogram of fig.4.1 (a) of SN05 is similar to that of fig.4.1(b) and fig.4.1(c) of samples SN06 and SN07. However fig.4.1(d) showed unique chromatogram even upon several runs of the same sample injection. In order to be sure of this anomaly, the chromatograms were overlain as shown in fig.4.1(e) to be subjected to critical observations. It was observed that all the first 3 chromatograms in figure 4.1 show the same similarity and even superimposed on each it, making it quite difficult to distinguish between them, while the chromatogram of SN08 is distinct. Refer Figure 4.1(e). Though there were slight similarities in the peaks shape which drew concern for further investigations. Hence, number of chromatographic peaks generated from each sample and their retention times were compared. Comparatively, 8 numbers of peaks were observed in all the first 3 samples from CM (i.e. SN05, SN06, and SN07) with relatively close retention times while SN08 reveals 9 peaks with very low %RSD values. Refer Table 4.6(b).

Comparison between figure 4.0 and figure 4.1 show clearly, characteristic tall peak occurring between times 2.5 - 4.5 min, indicating common constituent present in the T. *tetraptera* samples irrespective of its geographical area.

Fig.4.2(e) and Fig.4.3(e) Overlaid chromatograms for T. *tetraptera* samples from BM and CM respectively under the same extraction method and chromatographic conditions – petroleum ether fraction (nonpolar-

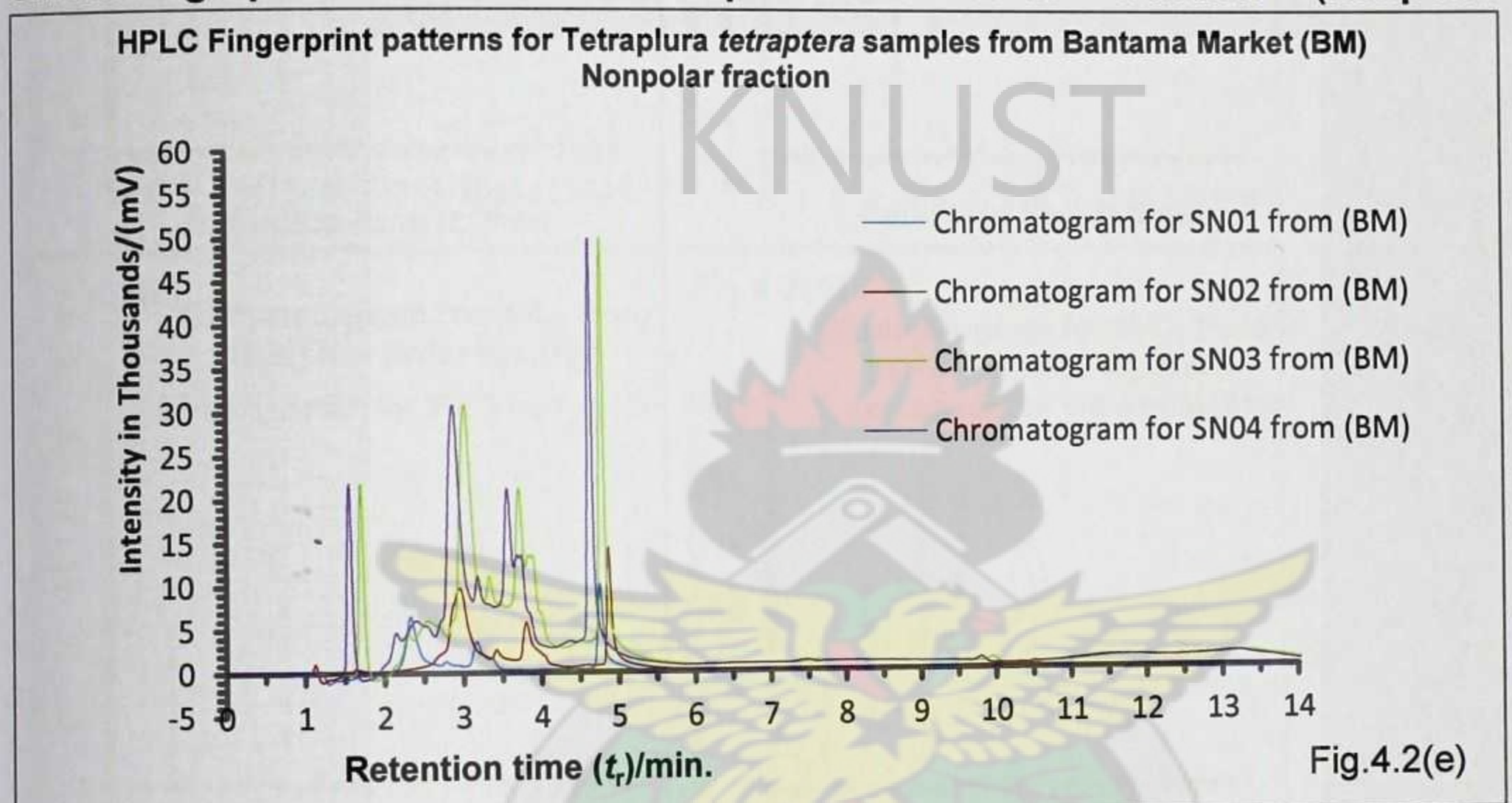


Fig.4.2(e)

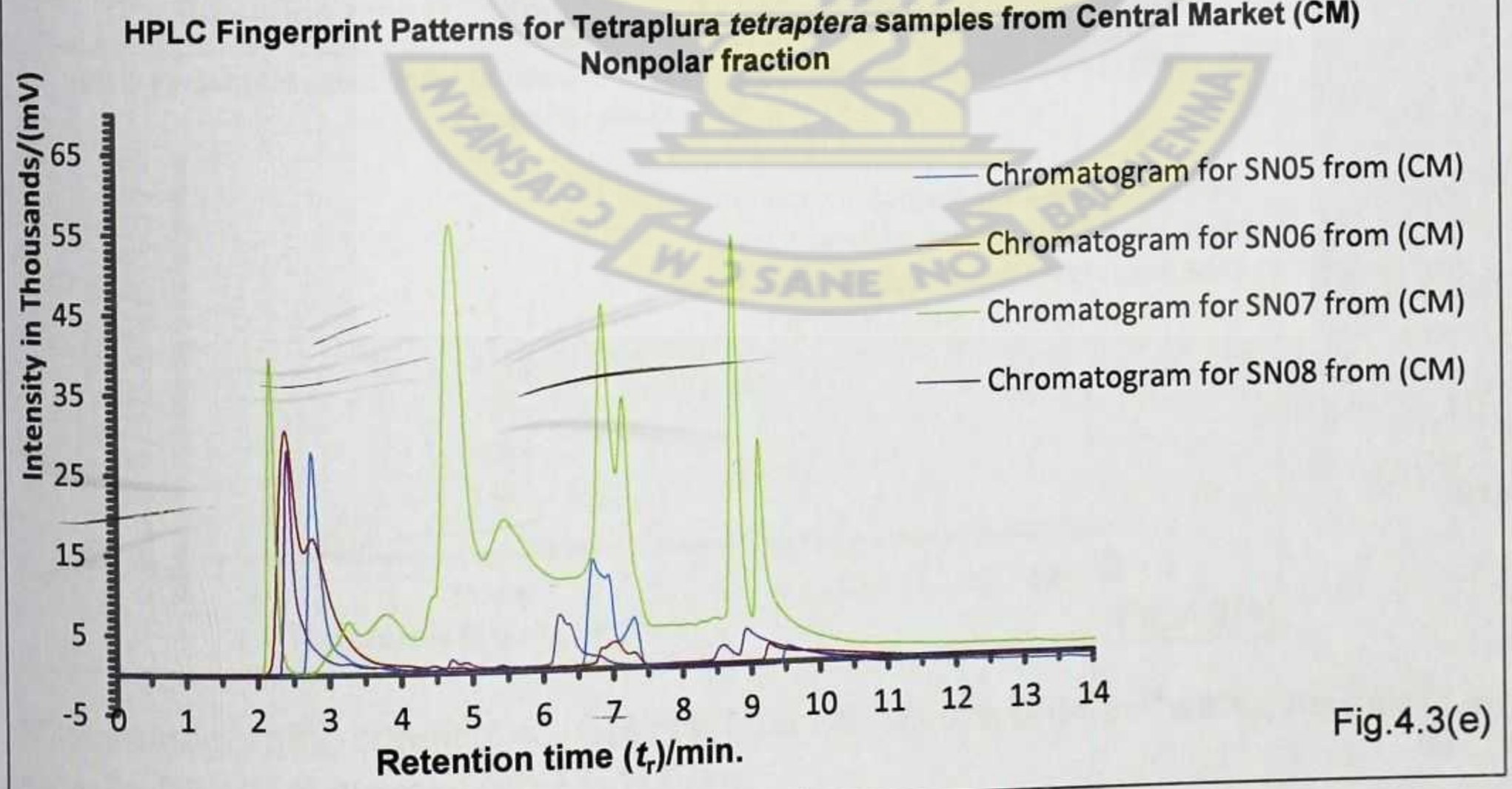
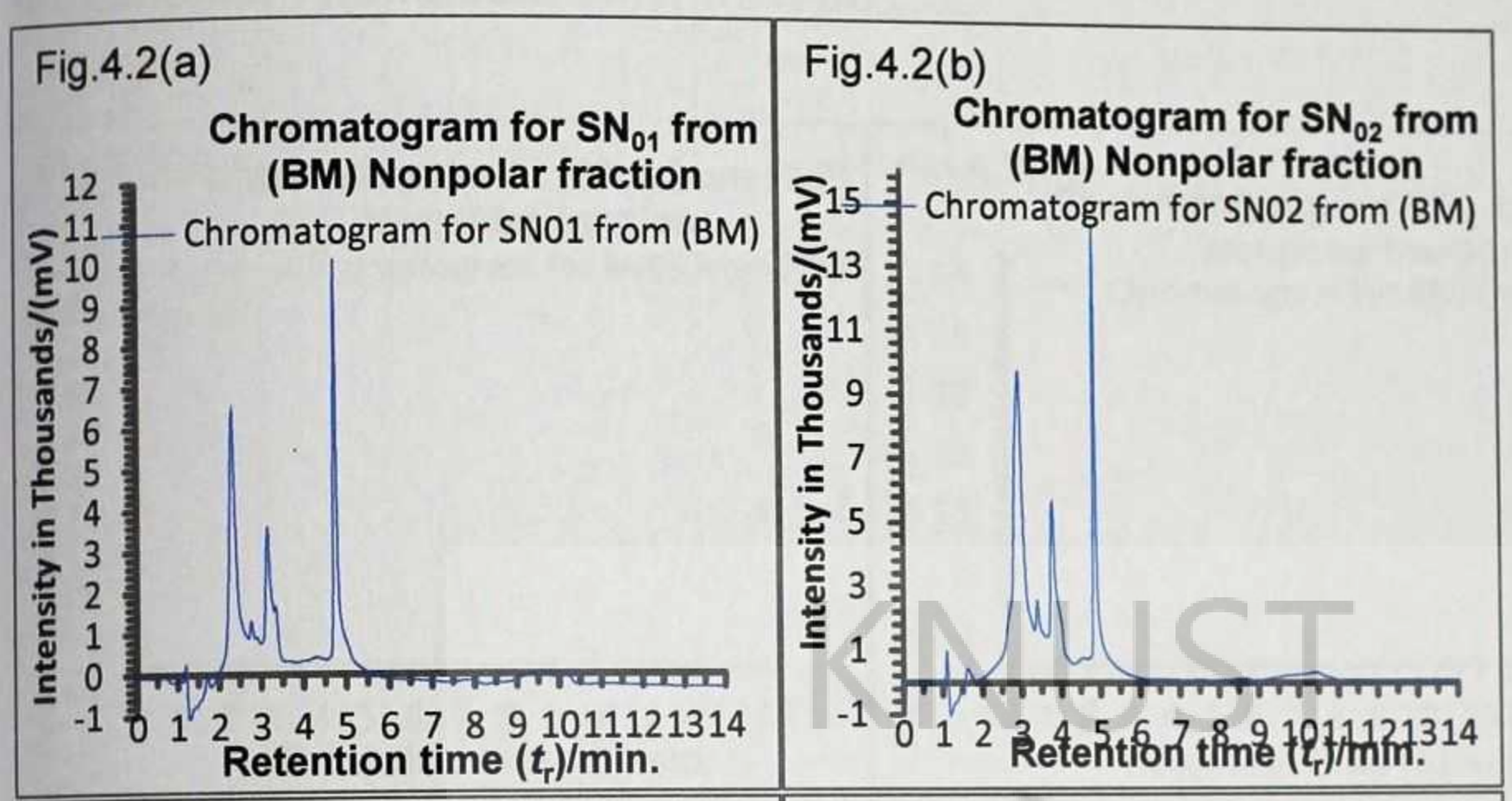


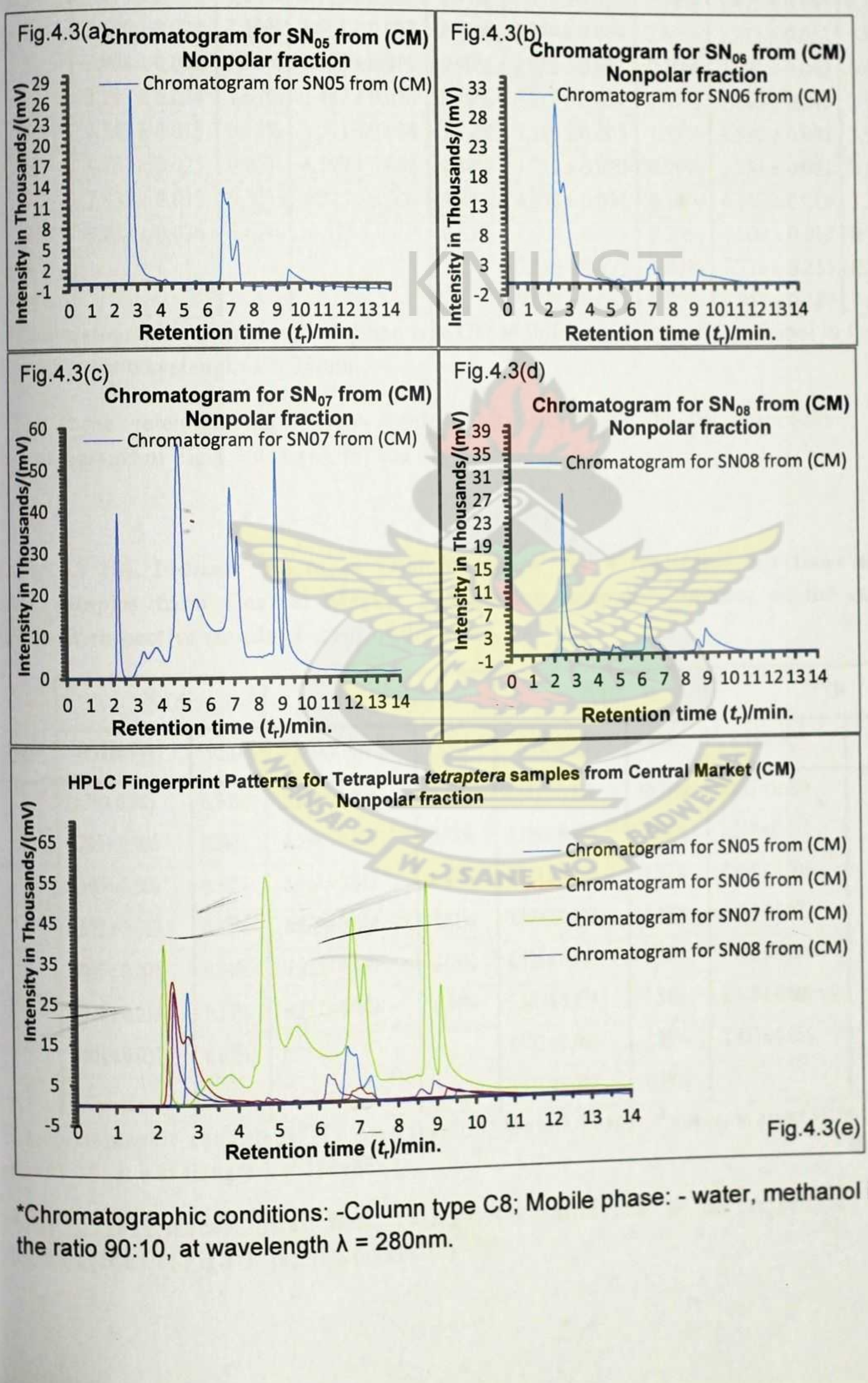
Fig.4.3(e)

Figure 4.2 Chromatograms for T. *tetraptera* samples from Bantama Market (BM) for Nonpolar - petroleum ether fraction



*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

Figure 4.3 Chromatograms for T. *tetraptera* samples from Central Market (CM) for nonpolar - petroleum ether fraction



*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

Table 4.7 (a). The mean retention times' for 4 replicates injections of the same samples from Bantama Market (BM) using nonpolar fraction of the extract and their respective standard error of the mean.

	SN_{01}		SN_{02}		SN_0		SN_0	
Peak#	R.Time (R_t)	RSD	R.Time (Rt)	RSD	R.Time (Rt)	RSD	R.Time (R_t)	RSD
1	1.473 ± 0.035	5.54%	1.122 ± 0.005	0.97%	1.511 ± 0.023	2.98%	1.472 ± 0.045	6.17%
2	2.696 ± 0.024	3.10%	1.655 ± 0.008	0.99%	2.239 ± 0.029	2.82%	2.211 ± 0.061	5.95%
3	2.904 ± 0.002	1.61%	2.926 ± 0.013	0.90%	2.828 ± 0.021	0.30%	2.792 ± 0.042	0.65%
4	3.297 ± 0.024	1.67%	3.447 ± 0.036	2.10%	3.217 ± 0.024	1.47%	3.260 ± 0.049	2.98%
5	4.543 ± 0.013	0.13%	3.771 ± 0.024	1.29%	3.546 ± 0.005	1.52%	3.538 ± 0.001	2.99%
6	4.751 ± 0.023	0.65%	4.599 ± 0.008	0.30%	3.731 ± 0.030	0.29%	3.781 ± 0.061	0.56%
7	7.933 ± 0.013	0.33%	4.825 ± 0.020	0.84%	4.358 ± 0.004	0.18%	4.352 ± 0.016	3.21%
8	9.837 ± 0.036	0.62%	10.525 ± 0.033	0.63%	4.609 ± 0.005	0.20%	4.600 ± 0.012	0.72%
9	–		–		7.589 ± 0.073	1.92%	7.716 ± 0.255	0.51%
10	–		–		9.879 ± 0.045	0.92%	9.957 ± 0.180	3.62%

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

**The mean retention times from Table 4.7(a) correspond to the respective chromatograms of Fig 4.2. (a), (b), (c) and (d)

Table 4.7 (b). Indicate the mean retention times' for 4 replicates injections of the same samples from Central Market (CM) using nonpolar fraction of the extract and their respective standard error of the mean.

	SN_{05}		SN_{06}		SN_{07}		SN_{08}	
Peak#	R.Time(R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD
1	2.670±0.085	6.34%	2.618±0.089	6.76%	2.571±0.132	10.31%	2.453±0.039	3.21%
2	4.205±0.006	0.28%	4.598±0.087	3.78%	3.459±0.126	7.29%	3.529±0.011	0.62%
3	5.500±0.008	0.30%	5.424±0.011	0.42%	4.714±0.040	1.71%	4.808±0.006	0.27%
4	6.697±0.013	0.37%	6.829±0.014	0.41%	5.473±0.012	0.43%	6.253±0.023	0.73%
5	6.931±0.001	0.04%	7.325±0.129	3.53%	6.826±0.010	0.30%	6.606±0.061	1.84%
6	7.326±0.011	0.31%	9.222±0.042	0.91%	7.463±0.134	3.58%	8.570±0.009	0.20%
7	9.501±0.031	0.65%	–		8.472±0.096	2.27%	8.833±0.055	1.24%
8	–		–		9.131±0.027	0.59%	–	

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

**The mean retention times from Table 4.7(b) correspond to the respective chromatograms of Fig 4.3. (a), (b), (c) and (d)

4.1.5 Comparison of HPLC Fingerprint Chromatograms for T. *tetraptera* Samples from BM and CM under the Same Chromatographic conditions (nonpolar fraction)

Figure 4.2 indicate chromatograms of T. *tetraptera* samples from BM which was separated through liquid-liquid extraction to obtain nonpolar fraction (Solvent extraction method). The Chromatograms of T. *tetraptera* samples from BM- nonpolar fraction showed drastic difference from the aqueous decoction extraction method of the same sample site but different mobile phase. There were 4 distinct peaks in BM- nonpolar fraction solvent extraction method which concentrated between 1.5 – 5min. elution times. These distinct peaks were seen in all the chromatograms of nonpolar fraction BM extract samples. Refer to figure 4.2

By visual comparison of fingerprint of BM-nonpolar fraction for samples; SN01, SN02, SN03 and SN04, it was obviously showing similarities in them. In order to substantiate the similarity in their chromatographic profiles, figure 4.2(e) was used to show the overlaid fingerprints of the BM-nonpolar fraction extract. Table 4.7(a) shows 8 peaks for sample SN01 and SN02 while SN03 and SN04 recorded 10 peaks. It was obvious from Table 4.7(a) that most of the chromatographic peaks were packed within elution retention times 1.122 ± 0.005 to 4.825 ± 0.020, suggesting that the respective retention times were close to each other. This made it quite difficult to confirm whether peaks with similar or same retention time(s) represent the same eluate compound, since there were no known marker compounds whose relative retention time"s (RRT) and relative peak areas (RPA) could be used as parameters for evaluation and validation of the data.

From figure 4.3, chromatograms of T. *tetraptera* samples from CM-nonpolar fraction solvent extraction method show similarities except fig.4.3(c) of sample SN07 which

reveals different chromatogram clearly distinct from the rest of the same sample site.

Vivid expatiation is seen from the summary of the chromatograms in fig. 4.3.(e).

This was supported by their retention times' and %RSD values of the peaks in Table 4.7. (b)

Fig.4.4(e) and Fig.4.5(e) Overlaid chromatograms for T. tetraptera samples from BM and CM respectively under the same extraction method and chromatographic conditions - polar fraction.

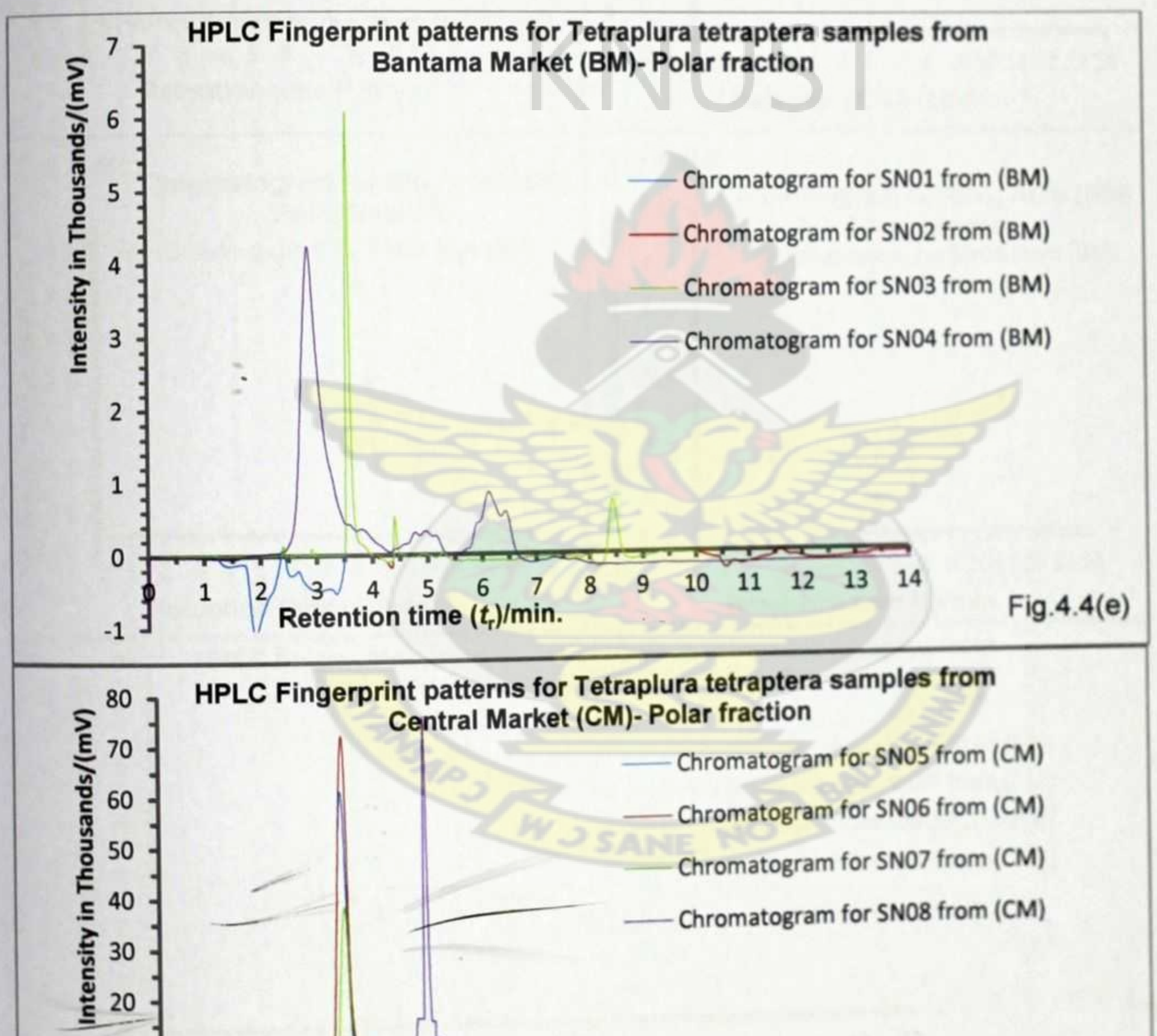


Fig.4.4(e)

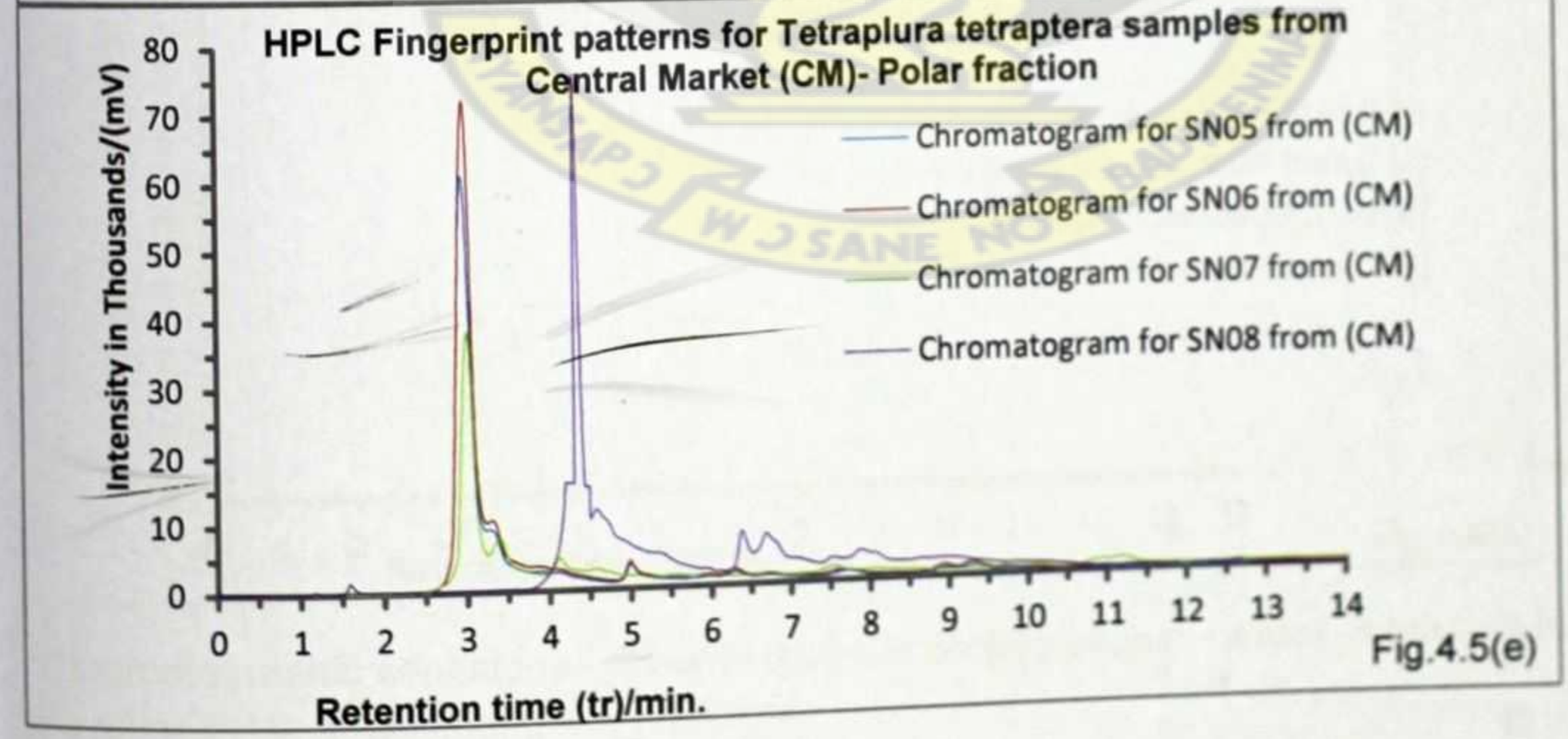
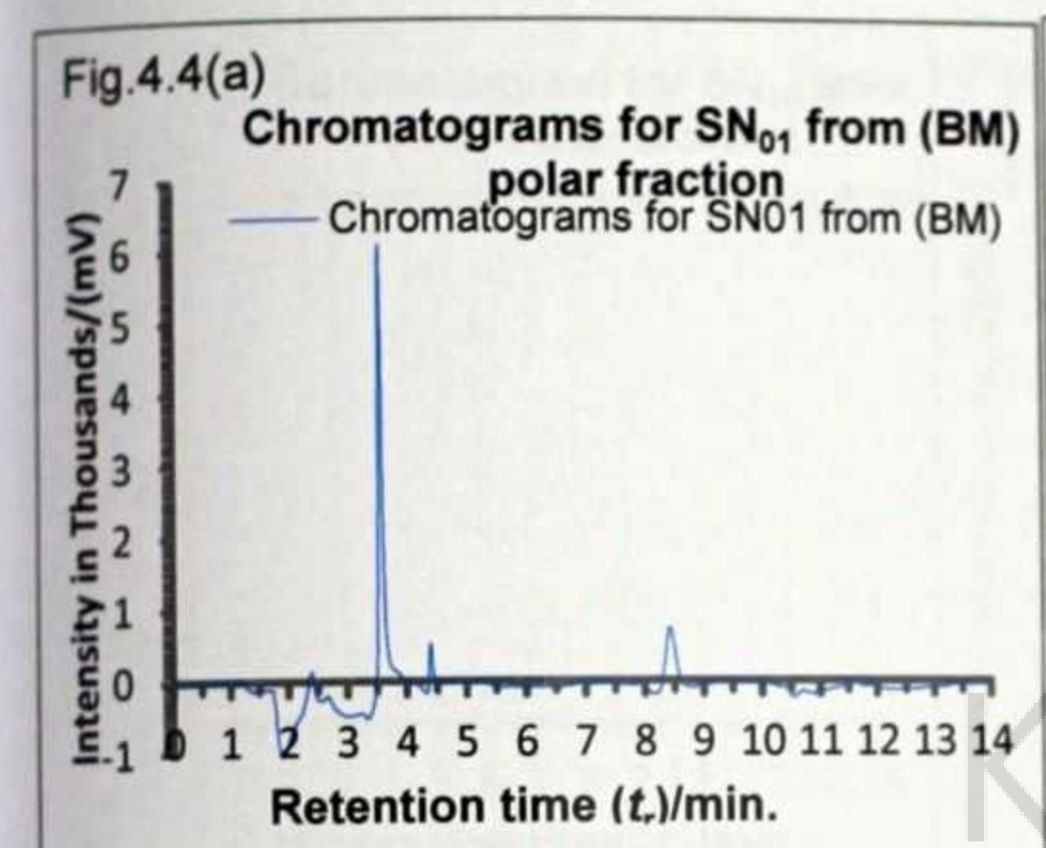
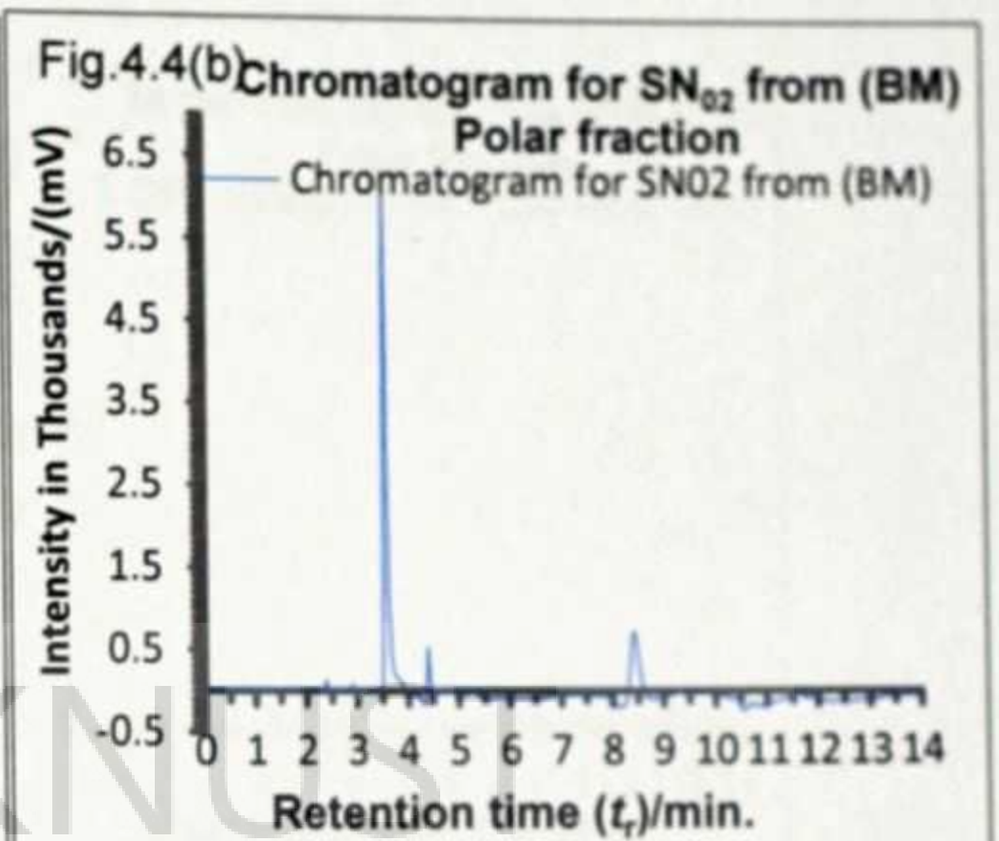
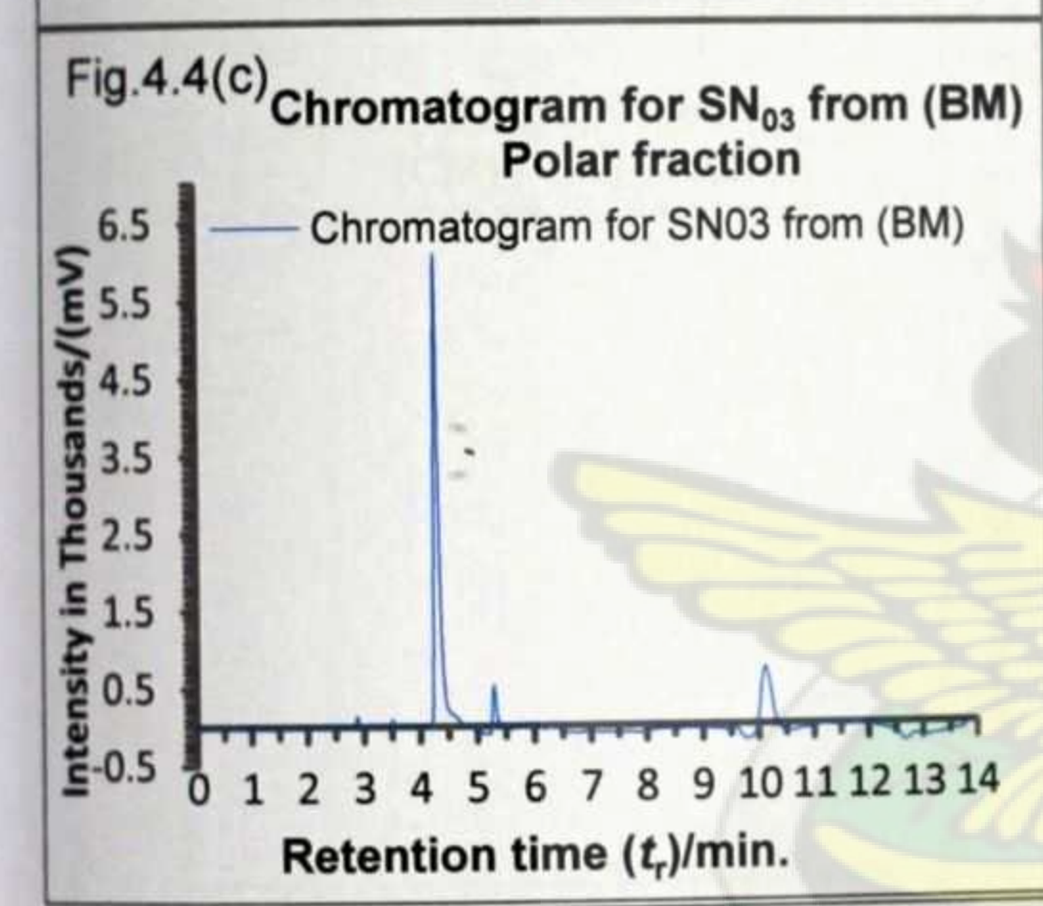


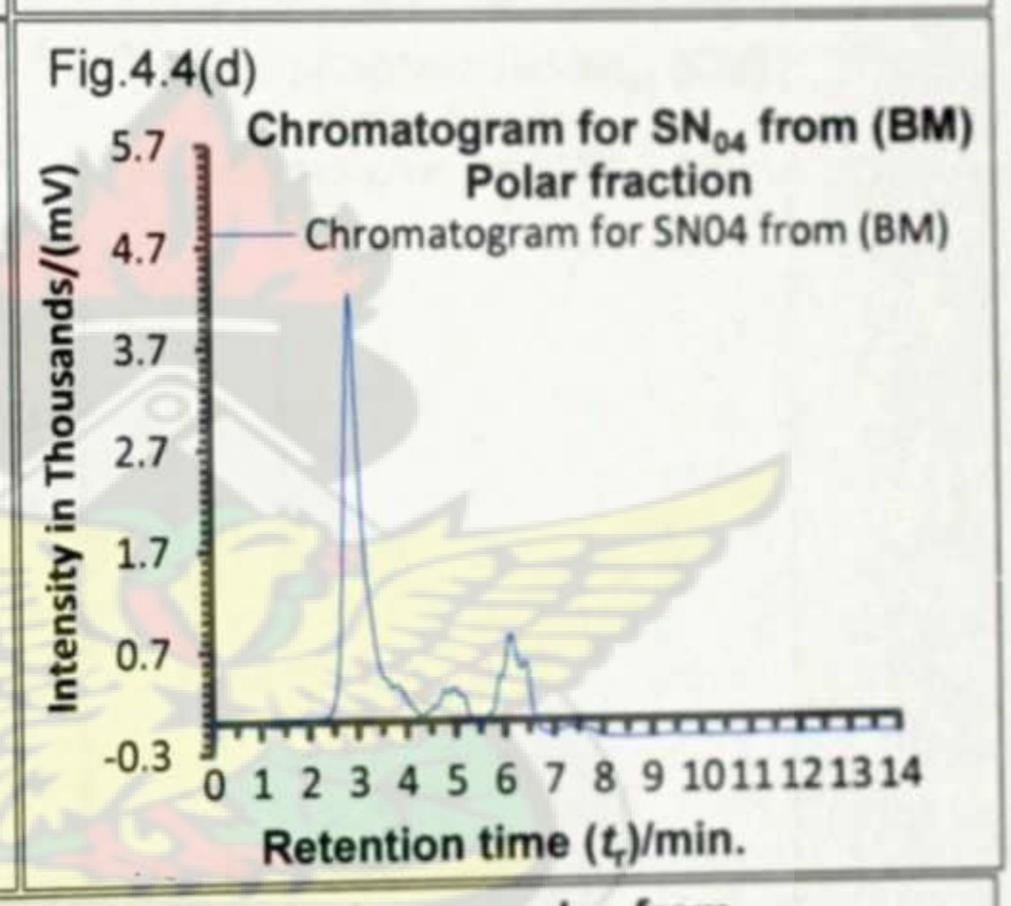
Fig.4.5(e)

Figure 4.4 Chromatograms for T. *tetraptera* samples from Bantama Market (BM) for polar fraction









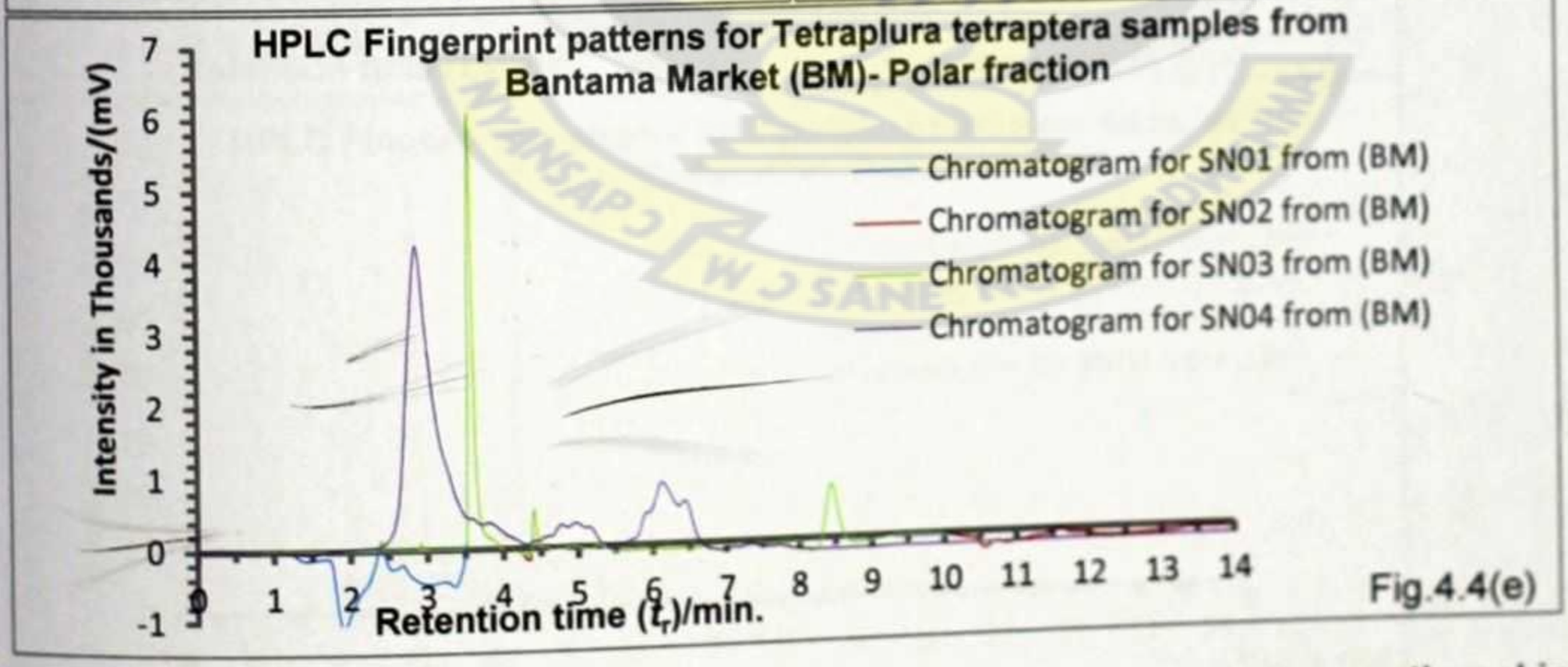
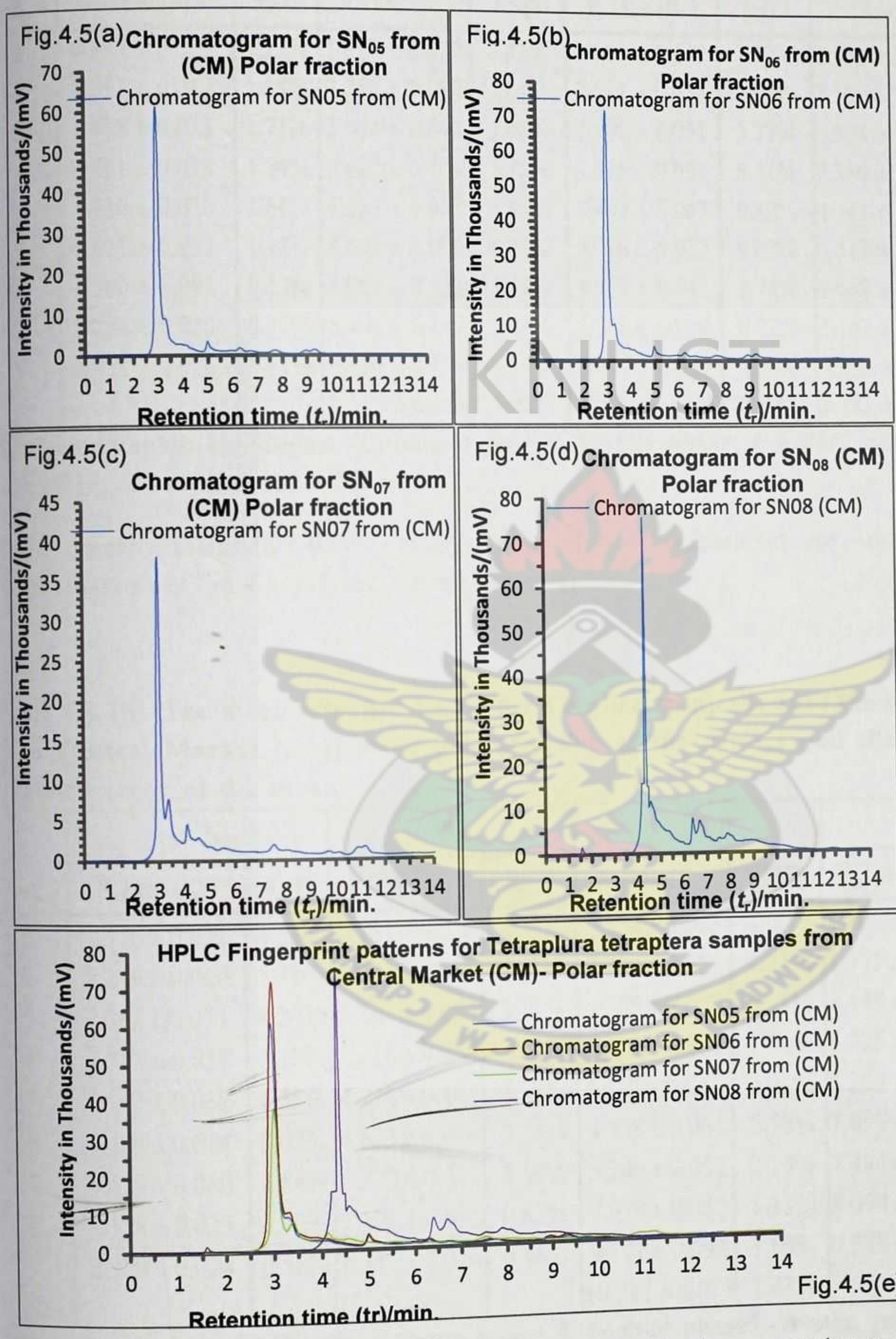


Fig.4.4(e)

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

Figure 4.5 Chromatograms for T. *tetraptera* samples from Central Market (CM) for polar fraction -



*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

Table 4.8.(a). The mean retention times' for 4 replicates injections of the samples from Bantama Market (BM) using polar fraction of the extract and their respective standard error of the mean.

	N_{01}		N_{02}		N_{03}		N_{04}	
Peak #	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD
1	1.637 ± 0.026	3.23%	1.613 ± 0.028	3.47%	1.985 ± 0.027	2.72%	1.892 ± 0.059	6.24%
2	2.391 ± 0.017	1.39%	2.356 ± 0.090	7.64%	2.512 ± 0.037	2.94%	2.550 ± 0.066	5.17%
3	2.658 ± 0.023	1.71%	2.668 ± 0.042	3.15%	2.701 ± 0.051	3.77%	3.033 ± 0.083	5.47%
4	3.591 ± 0.028	1.54%	3.620 ± 0.104	5.75%	3.475 ± 0.094	5.41%	3.350 ± 0.114	6.80%
5	4.430 ± 0.036	1.61%	4.523 ± 0.087	3.84%	4.441 ± 0.007	0.32%	4.342 ± 0.096	4.42%
6	4.611 ± 0.032	1.38%	4.693 ± 0.094	4.01%	4.716 ± 0.022	0.93%	4.517 ± 0.091	4.03%
7	4.900 ± 0.003	0.13%	4.909 ± 0.123	5.01%	4.937 ± 0.043	1.74%	4.683 ± 0.109	4.66%
8	5.234 ± 0.020	0.77%	5.166 ± 0.183	7.08%	5.308 ± 0.019	0.72%	5.167 ± 0.152	5.88%
9	8.411 ± 0.026	0.61%	5.450 ± 0.139	5.10%	8.477 ± 0.087	2.05%	5.525 ± 0.170	6.15%
10	_	_	8.516 ± 0.165	3.88%	10.632 ± 0.033	0.62%	8.783 ± 0.050	1.14%

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

**The mean retention times from Table 4.7(a) correspond to the respective chromatograms of Fig 4.4. (a), (b), (c) and (d)

Table 4.8. (b). The mean retention times' for 4 replicates injections of the same samples from Central Market (CM) using polar fraction of the extract and their respective standard error of the mean.

	N_{05}		N_{06}		N_{07}		N_{08}	
Peak #	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD	R.Time (R_t)	RSD
1	1.376 ± 0.031	4.53%	1.367 ± 0.030	4.35%	1.204 ± 0.065	10.85%	1.301 ± 0.058	8.96%
2	2.249 ± 0.060	5.33%	2.226 ± 0.055	4.97%	2.200 ± 0.052	4.71%	3.375 ± 0.029	1.72%
3	3.365 ± 0.071	4.27%	3.261 ± 0.083	5.11%	3.288 ± 0.052	3.15%	4.448 ± 0.035	1.57%
4	3.575 ± 0.057	3.17%	3.616 ± 0.048	2.66%	3.622 ± 0.032	1.74%	5.723 ± 0.120	4.17%
5	4.163 ± 0.032	1.55%	4.218 ± 0.105	4.98%	4.122 ± 0.041	2.01%	6.268 ± 0.024	0.75%
6	4.489 ± 0.014	0.62%	4.633 ± 0.076	3.26%	4.748 ± 0.061	2.58%	7.099 ± 0.019	0.52%
7	5.629 ± 0.080	2.84%	5.670 ± 0.045	1.59%	5.205 ± 0.052	2.14%	7.484 ± 0.055	1.46%
8	7.178 ± 0.026	0.72%	7.101 ± 0.022	0.62%	7.079 ± 0.042	1.18%	8.099 ± 0.008	0.19%
9	8.379 ± 0.024	0.58%	8.381 ± 0.024	0.56%	9.178 ± 0.092	1.99%	9.330 ± 0.013	0.27%
10	_	_	_	_	10.281 ± 0.073	1.41%	_	_

*Chromatographic conditions: -Column type C8; Mobile phase: - water, methanol in the ratio 90:10, at wavelength λ = 280nm.

**The mean retention times from Table 4.7(a) correspond to the respective chromatograms of Fig 4.4. (a), (b), (c) and (d)

4.1.6 Comparison of HPLC Fingerprint Chromatograms for T. *tetraptera* Samples from BM and CM under the Same Chromatographic conditions (polar fractions)

Figure 4.4 illustrate chromatograms for T. *tetraptera* samples from BM for polar fraction portion of the solvent extraction method for SN01, SN02, SN03 and SN04. Figures; 4.4(a), 4.4(b) and 4.4(c) of sample numbers; SN01, SN02, and SN03 gave similar chromatographic patterns with three common identical peaks occurring at virtually the same retention times in their respective samples. These 3 peaks were well resolved and distinct in appearance from the other peaks which were present. In comparison, there was one particular single peak found to be taller than all the others within all the chromatograms of figure 4.4. This presupposed that the tallest peak is a phytochemical constituent present in the T. *tetraptera* in high concentration. The rest of the peaks were observed to be small, suggesting that those constituent were present in smaller quantities. However, figure 4.4(d) of SN04 revealed broad peaks of similar retention times but different chromatographic profile from visual observation. This was further confirms with overlaid chromatograms in figure 4.4(e), given a clear distinction in SN04 and the others. Table 4.8(a) recorded 10 peaks for sample numbers": SN02, SN03, and SN04, with exception of SN01 which registered 9 peaks for its four injections. It was also noticed from Table 4.8(a) that, the retention times for all the various samples were clustered between 4.342 ± 0.096 to 4.937 ± 0.043 for samples from BM. Although, there were other retention times registered on Table 4.8(a) whose peaks could not be seen on their respective chromatograms by visual observation.

On the other hand figure 4.5 indicates fingerprints of T. *tetraptera* samples from CM for polar fraction portion of the solvent extraction method. This revealed similar peaks across all the chromatograms in figure 4.5; however, their respective retention times recorded in Table 4.8(b) shows variations in positions of the individual constituent forming the chromatograms. Figure 4.5(a) and figure 4.5(b) of samples SN05 and SN06 respectively gave similar fingerprint patterns but the prominent peak in figure 4.5(b) was observed to be slightly taller than that in figure 4.5(a). A clear distinction was observed of the chromatographic profiles in figure 4.5(e).

Table 4.8(b) recorded 9 peaks for SN05, SN06 and SN08 with relatively similarities in their respective corresponding %RSD values. SN07 recorded 10 peaks with relatively higher %RSD values compared to %RSD values of SN05, SN06 and SN08.

4.1.7 Conclusion

There is no reported method for obtaining fingerprint of any part of Tetrapleura *tetraptera* and therefore, this thesis seems to be the first attempt in determine conditions and extraction methods feasible for thorough utilization of the chromatograms as a means for quality control of herbal medicine. The results obtained clearly show that, the aqueous/petroleum ether fraction gave well resolved peaks with many peaks to characterized T. *tetraptera* samples than the other extraction methods employed. Thus the impact of the methods of extraction on the chemical profile of T. *tetraptera* fruit samples from two main major market centers within Kumasi metropolis has been clearly demonstrated. Suitable chromatographic conditions were also established for the extraction methods for T. *tetraptera*. The results and the discussions, suggested

that HPLC fingerprints could help distinguished samples of the same genus from others of different geographical location. The constituent in T. *tetraptera* have not traditionally been fully exploited with HPCL, despite the fact that focus on the use of the plant is on the increase in our local pharmaceutical industries. Hence, this research can serve as the basis of acquiring chromatographic fingerprints for Tetrapleura *tetraptera* in the folkloric oral preparation methods.

In this twenty-first century, where emphasis on quality, safety and efficacy of herbal medicine with recent increasingly demand for alternative healthcare becoming sensitive. The need to establish global or regional regulatory mechanisms will no doubt be legislated, and sooner or later HPLC fingerprint will be the primary tool for quality control of herbal medicine. Hence it is critical to train and encourage the use of HPLC chromatographic fingerprint and chemometrics in the analysis and evaluation of herbal medicines in Ghana.

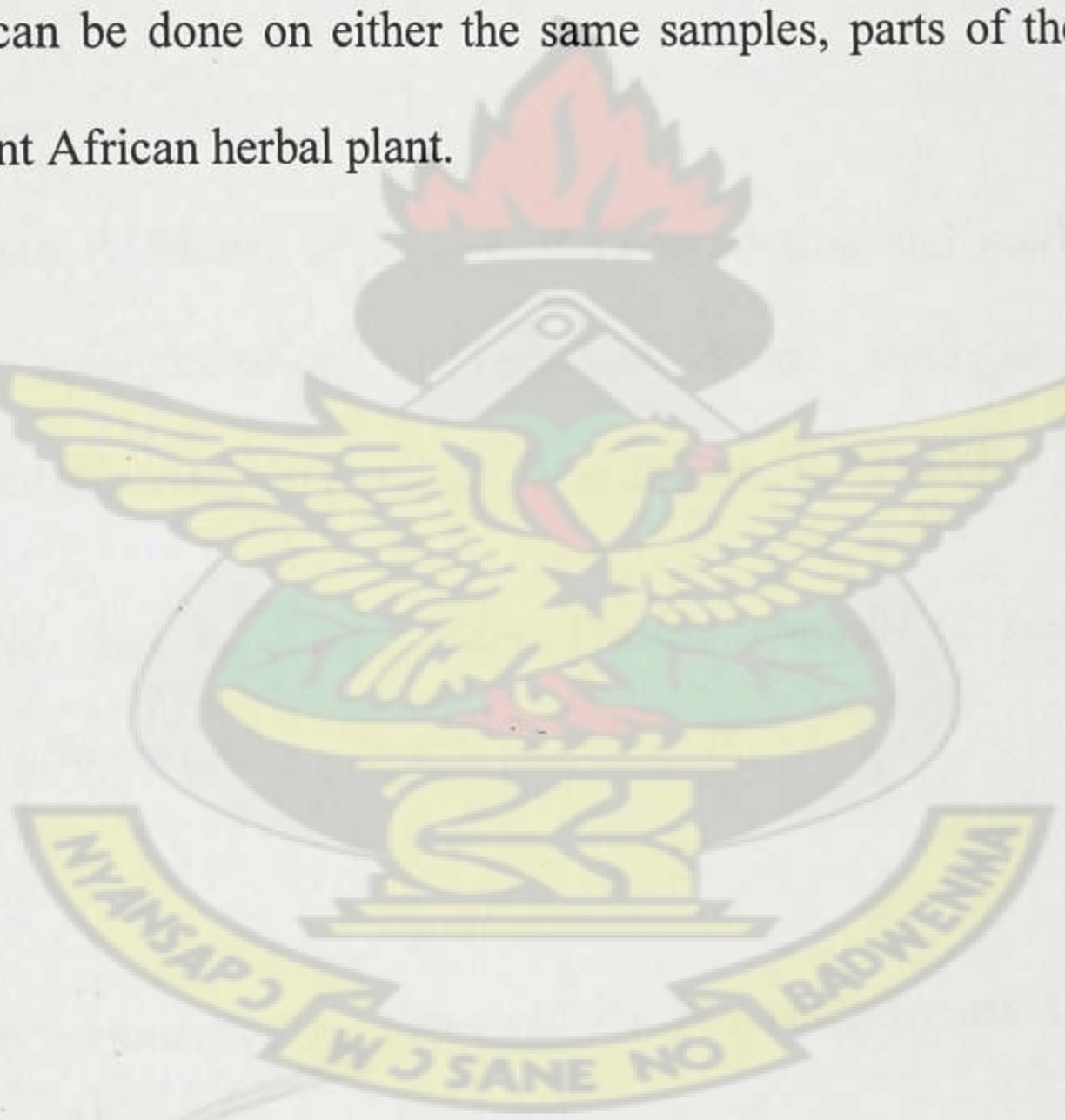
4.1.8 Recommendation

It worthwhile, to note that chemical fingerprint of any plant is not set in stones and could change over a certain period of time depending on its surroundings. Therefore it is advisable to carry out several researches on a particular plant from different geographical areas with standardised procedures to at least generate enough HPLC fingerprints to monitor various chemical profiles they exhibit.

Research fields of quality control of herbal medicine is an interdisciplinary task which needs crossover of chemistry, pharmacology, medicine and even statistics to provide a platform for efficacy, consistency, safety and quality. This can be adopted in piloting research programmes for higher learning in the various faculties to improve quality of Ghanaian herbal drugs. Of course, the research works on chemical

fingerprint in establishing efficacy for the purpose of quality control of herbal medicines is far from sufficient to meet required regulatory standards. However, to set up a successful quality control for traditional medicine as effective means of ensuring quality for herbal medicine demands the establishment of characteristic fingerprint for various herbal medicines to create detailed database, which allows comparison and serve as references for evaluation.

Based on this work, further studies of thorough quantification with standard markers, chemometrics, suitable chromatographic-spectroscopic analytical system with software capable of performing pattern recognition and classification analysis (e.g. MATLAB, Unscrambler or Sirius) can be done on either the same samples, parts of the T. *tetraptera* plants, or entirely different African herbal plant.



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