IDENTIFICATION OF A BIOMARKER AND DEVELOPMENT OF AN HPLC METHOD FOR QUALITY CONTROL OF THE SEEDS OF VOACANGA AFRICANA (FAM: APOCYNACEAE)

A THESIS SUBMITTED IN
FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE
OF

MASTER OF PHILOSOPHY

In the

Department of Pharmaceutical Chemistry,
Faculty of Pharmacy and Pharmaceutical Sciences

By

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FEBRUARY, 2013

DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmaceutical Chemistry, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

Voacanga seeds obtained from the plant voacanga africana have been an export commodity for some West African countries including Ghana, Cote d'Ivoire, Cameroon and DR Congo. Due to its stable market since 1980s, hundreds of tonnes of seeds have been exported from these countries and the seeds are bought mainly by pharmaceutical companies in countries such as France and Germany. However, there are no known analytical procedures for controlling the quality of these seeds if they have to be developed as an official non-traditional export commodity in Ghana. A validated method has been developed for the quantification of a biomarker in the seeds of voacanga africana. Isolation of biomarker was achieved by crude extraction of the total alkaloid which was subjected to column chromatography using gradient elution. Preparative TLC was further used to separate and purify the bulked fractions to obtain the pure biomarker. The pure isolate was used as a bio marker and a reverse phase isocratic HPLC UV detection method was developed for the quantification of the biomarker in the whole seeds. Resolution was achieved using a Phenomenex Kromasil 5µm C_8 column of dimensions 250 × 4.60mm and mobile phase used to elute the biomarker was methanol/water (**90:10 v/v**) with the retention times approximately **4.70**, **6.0** and **10.0 minutes** at flow rates of **1.0**, **0.8** and **0.5ml/min** respectively. Detection was at a wavelength of **257nm**. A calibration curve was plotted and it was linear over the concentration range **1.4µg/ml - 14µg/ml** with a coefficient of regression, $\mathbf{R^2}$ = **0.9984.** The **LOD** and **LOQ** were determined to be **0.84µg/ml** and **2.55µg/ml** respectively. The developed method was used to quantify the biomarker in the whole seeds. The crude extract from the whole seeds was found to contain between **0.02 –**

0.035% of the biomarker with the precision not exceeding 10% (RSD < 10%). The retention time was confirmed by injecting two different concentrations of the extract which were spiked with the pure biomarker and average recoveries obtained were **79.29%** and **80.38%** respectively.

ACKNOWLEDGEMENT

"I will rather take that bold step forward and fail rather than regretting I never took that step", was my guiding post throughout this project. It is the Lord who gives wisdom and from Him comes knowledge. I thank the Lord for how far he has brought me. Indeed the terrains have never been smooth but I have seen favour in the eyes of the Lord.

My acknowledgement first goes to my supervisor Mr. Samuel Asare Nkansah and also Mr. Samuel Oppong Bekoe for their immense assistance and guidance throughout the project. My sincere thanks also go to my circle of friends especially Daniel Afosah, Emmanuel Kenneth Cudjoe, Ernest Obese, Derrick Afful, Edmund Ekuadzi and all my course mates. Lastly I want to express my gratitude to Edward Ofori and the department of Pharmaceutical Chemistry, Florida Agriculture and Mechanical University for running my spectral data.

Lastly my gratitude also goes to my parents, Mr George Kweku Affram and Mrs Mabel Yartey, my siblings and to that special person in my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Plants from ancient days have been the storehouse of a myriad of medicinal components which has been extracted in various methods to treat diseases. The use of medicinal plants has been embraced in many disciplines which encompass botany, chemistry, horticulture, agriculture etc. A perpetual understanding of the biosynthesis and chemistry of the medicinal components of plants has led to discovery of many useful drugs but that notwithstanding, the number of medicinal components in plants that stands to be investigated far supersedes what has been explored and characterized. The medicinal components are in the form of secondary metabolites which serve as important phytotherapeutic agents in drug design. These metabolites in the form of alkaloids, glycosides, flavonoids, essential oils, tannins etc have been a source of inspiration which serves to drive research into the design of new drugs.

Africa has been a treasure land for a vast wealth of medicinal plants. Though the export of medicinal plants continues to grow globally, Africa's contribution towards the global trade in natural product is little and hence a minor player in the trade. Furthermore, medicinal plants exported are those which are of interest to the international market. Medicinal plants are exported in the form of raw bulk material or semi processed materials. Limited resources have been devoted towards research in this avenue. This has led to the poor or inadequate quality control and standardization of these medicinal plants. Voacanga Africana is a popular plant in West Africa which has a wide medicinal use. The root bark, stem bark and seeds are exported mainly from West Africa but a difficulty in the quality control of these products is due to unavailability of commercial

standards of specific alkaloid (**Simon.J.E et al, 2007**).The seeds of voacanga Africana has become of great interest by the pharmaceutical companies due to the tabersonine, the major alkaloid found in the seeds. Use of tabersonine has been made by readily converting it to vincamine which is used in medicinal preparations for the geriatrics (**www.prota.org**). The seeds contain a wide array of indole alkaloids including voacangine which is the principal and important alkaloid found in most parts of the plant. The pharmacological properties of voacangine have well been investigated for decades and it is a promising lead compound yet to be endorsed by the regulatory bodies as a safe drug for human consumption.

Voacanga Africana plant can supply up to about 5grammes per kilogramme of the alkaloid voacangine whiles ibogaine obtained mainly from tabernanthe Iboga supplies up to 3grammes per kilogramme of the alkaloid ibogaine. Moreover, voacanga Africana is a common plant and easily accessible and the harvesting of the bark for medicinal purposes is quite advantageous rather gathering the roots reducing the incidence of plant destruction **(US Patent)**

Voacanga seeds have been an export commodity for some West African countries namely Ghana, Cote d'Ivoire, Cameroon and DR Congo. It has enjoyed a stable market since 1980s and hundreds of tonnes of seeds have been exported from these African countries. The seeds have been bought mainly by Pharmaceutical companies in France and Germany. In 2004, exports of voacanga from Cameroon sold per Kg were as follows; stem bark US$14, stem bark powder US$18, roots US$14, root powder US$18, root bark US$47, root bark powder US$51, seeds US$6. In 2005, prices coated on the internet in the United States were as follows; 30g seeds US$20, 30g root bark US$24, 115g root bark US$80, 450g root bark US$280 and 1kg root bark US$400

(**www.prota.org**). Voacanga seeds and griffonia simplicifolia have been reported to be the most leading medicinal export commodity in Ghana. In 2008, US$ 15 million was accrued in earnings from the exportation of medicinal products for which 80% was derived from the export of voacanga seeds and griffonia seeds **(Andel et al, 2012).**

Chapter 2

LITERATURE REVIEW

2.1 VOACANGA AFRICANA

***Botanical Name*:** Voacanga africana

Common Names: Voacanga

Active Constituents: voacangine, voacristine, voacamine (voacanginine), tabersonine etc.

Voacanga Africana is a small tropical tree which grows in Africa. It grows to a height of about 10 – 25m with pale gray-brown bark **(Li Ping-Tao et al, 1995)**. The voacanga africna plant is a deciduous, mesophytic, sap-wody, perennial, aborescent shrub which grows in the primary and secondary forest, within the tropical rain forest and Guinea savannah woodland belt. A milky latex is produced by the slash. Leaves are simple in nature, petiolate and arranged in decussate manner. Inflorescence, terminal, lax, pedunculate, cyme. The flowers are pedicellated and have a mild scent upon smelling. It has a corolla lobe with overlapping aestivation. Stamen are pentamerous and epipetalous in description. The ovary is superior and bicarpellary. The fruit is globose berry with brownish –white blotches (**Duru et al, 2010**). The seeds are dark brown and obliquely ellipsoid, 7-10 × 3.5-5 mm **(Li Ping-Tao et al, 1995).**

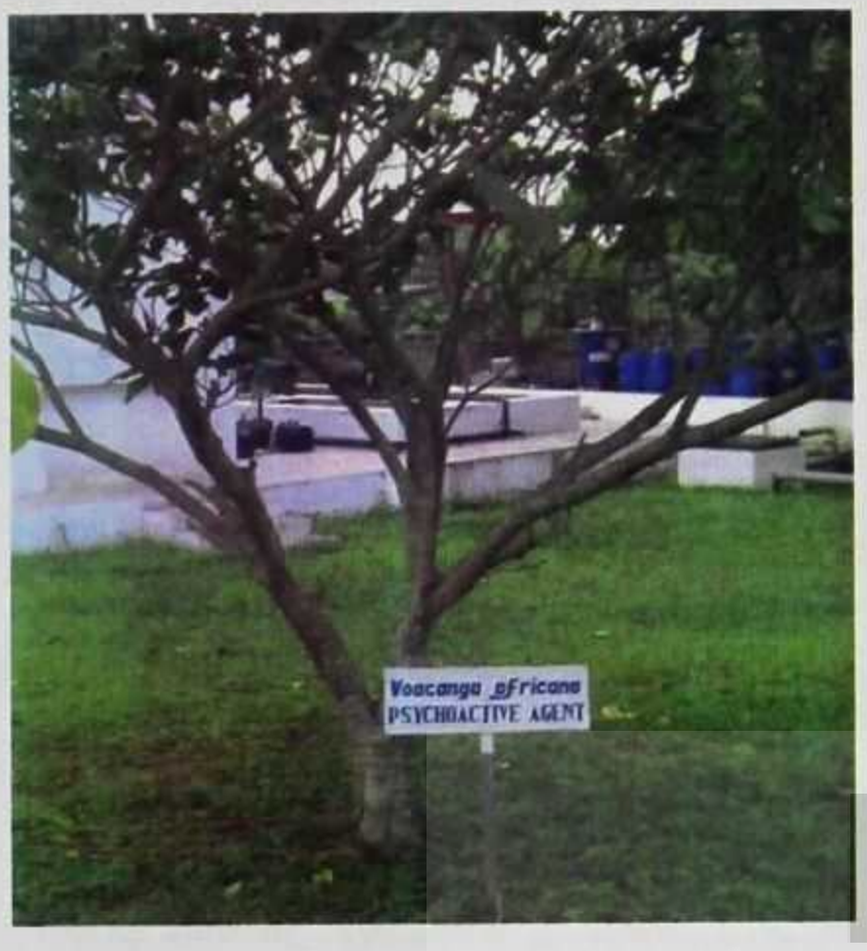

Figure 2-1 (a) Voacanga Africana plant (b) whole seeds of voacanga africana

2.1.1 Uses of Voacanga Africana

Voacanga Africana is a common plant which has been adopted in herbal medicine for the treatment of various ailments. Extracts obtained through decoction has been used for various ailments or ethnomedical practices such as leprosy, oedema, diarrhoea convulsions and madness figures. Ethnomedical medicine has also employed it in the cure orchitis, ectopic testes and gonorrhea. It has also been reported to be used as stimulants in ethnics of Gabon, West Africa which makes them feel high

There is the belief among Africans that the hanging of the leaves and fruits over a house-door drive away witches. Fruit can be poisonous upon ingestion and this warrants the use of the bark as arrow poison. The magic healers in Africa keep V. Africana as a well guided secret. Its preparations are used as medium for ritual and visionary purposes though knowledge on its utilization in this perspective is little. Some magicians ingest as much as 50 seeds to improve their visionary abilities. It has also been cultivated for its latex which is used as a rubber adultrant (**Macebeo et.al, 2009**).

2.2 THE INDOLE ALKALOIDS OF VOACANGA

There are many species of voacanga and contain a wide array of closely related indole alkaloids have been shown to be highly concentrated in the different parts of the plant such as the leaves, roots, stem, seeds and branches. The alkaloid content of the root bark and stem bark have been reported to be 9.2% and 3.9% respectively. Among the alkaloids isolated include coronaridine, decarbomethoxyvoacamine, ibogaine, ibogamine, iboluteine, voacamine, voacangine, voacangine hydroxyindolenine, 3-epi-a-yohimbine, iboxygaine, N-oxyvoacamine, perakine, reserpine,voabasine, voacryptine, voacristine, voacorine, vobtusine and many more. All these compounds have been characterized to determine their structures. The stem bark of V. Africana contains mainly voacamine, voacangine, voacorine, voacristine and vobasine. The total alkaloid. The alkaloids found in the leaves voacamine, vobtusine, desoxyvobtusine lactone, folicangine, isovoafoline, voafoline, voafolidine, voaphylline, voaphylline-diol, voaphylline hydroxyindolenine and vobtusine lactone **(Inga et al, 1981)**

2.2.1 SOME MONOTERPENE ALKALOIDS IN VOACANGA AFRICANA

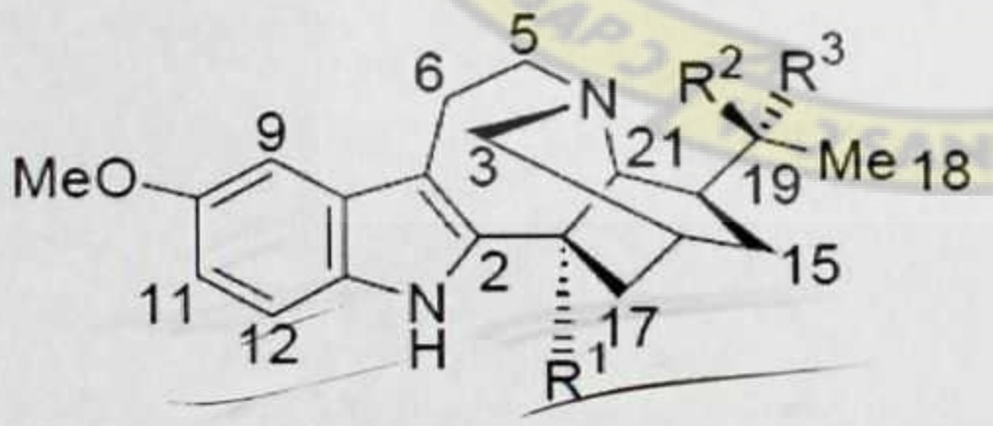

Figure 2-2 Iboga Alkaloid

Table 2-1 Indole Alkaloids of Voacanga Africana

INDOLE ALKALOID	R^1	R^2	R^3
Voacangine	COOMe	H	H
Voacristine	COOMe	OH	H
Iboxygaine	H	OH	H

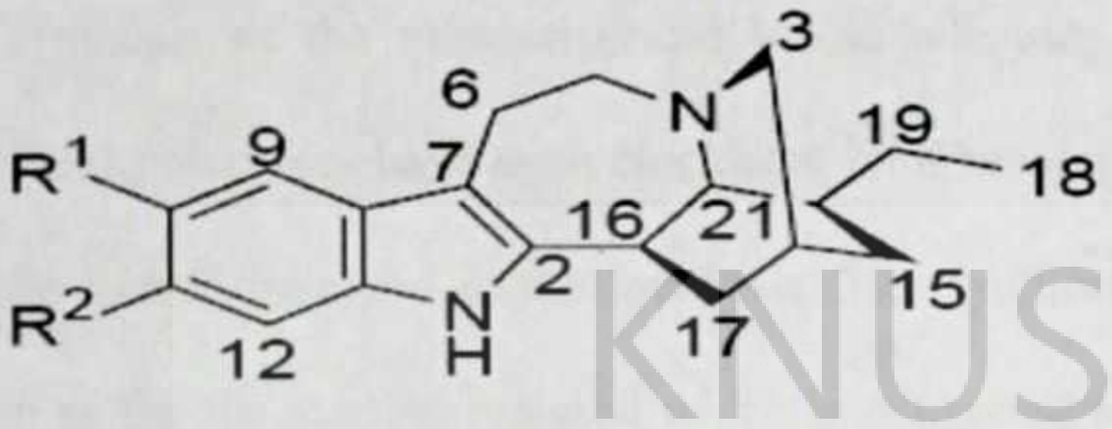

Figure 2-3 Iboga Alkaloid

Table 2-2 Indole Alkaloids of Voacanga Africana

INDOLE ALKALOID	R^1	R^2
Ibogaine	OCH_3	H
Ibogamine	H	H
Ibogaline	OCH_3	OCH_3

2.2.2 BIOSYNTHETIC FORMATION OF INDOLE ALKALOIDS IN VOA CANGA SPECIES

The monoterpenoid indole alkaloids are biosynthesized from basic biological components, tryptophan and secologanin which is a terpene iridoid. It was prostulated that the synthesis of the monoterpenoid indole alkaloids involved an iridoid after several iridoid precursors have been elucidated until secologanin was later found to be the specific iridoid involved in the synthesis. The synthesis of strictosidine involves tryptophan as the the starting material which is converted to tryptamine by enzymatic action as shown below. The tryptamine is then condensed with secologanin to afford the synthesis of strictosidine.

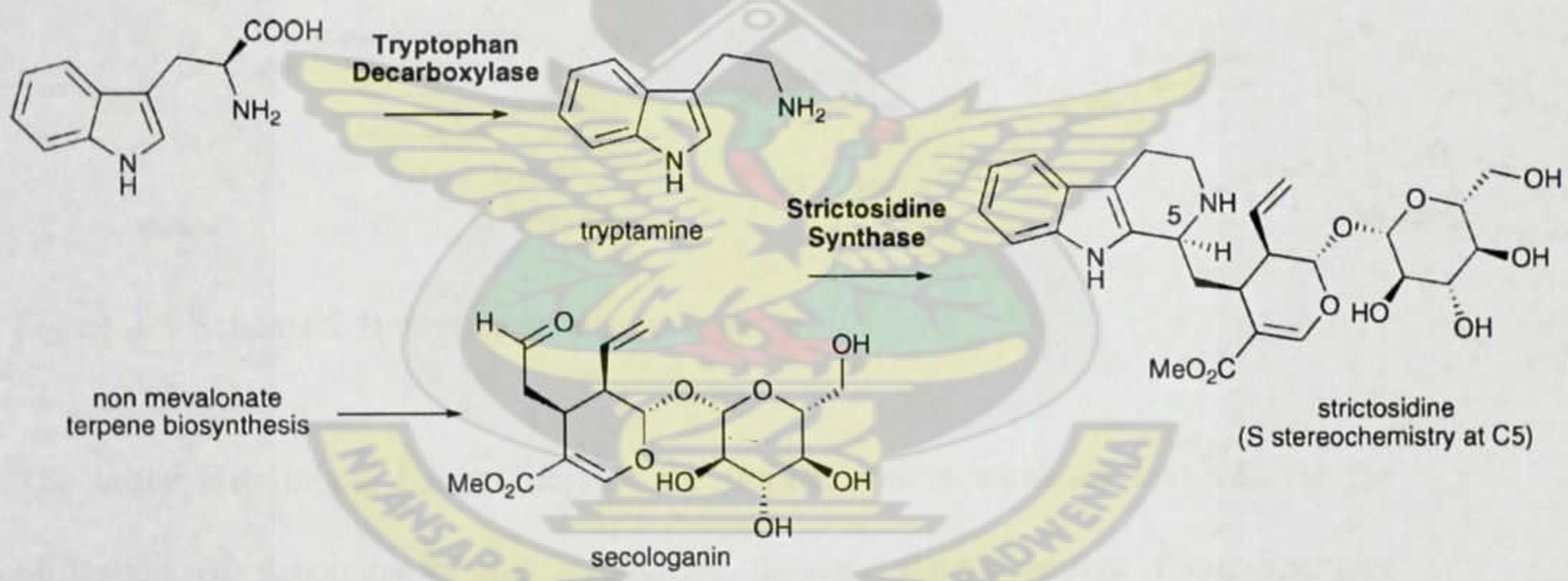

Figure 2-4 Scheme 1 Initial steps of terpene indole alkaloid biosynthesis

Secologenin occurs naturally in plants and its biosynthesis is not well elucidated but it has been suggested that secologanin is biosynthesized by the non-mevalonate or triose phosphate/pyruvate pathway as shown belowsecologenin occurs naturally in plants and its biosynthesis is not well elucidated but it has been suggested that secologanin is

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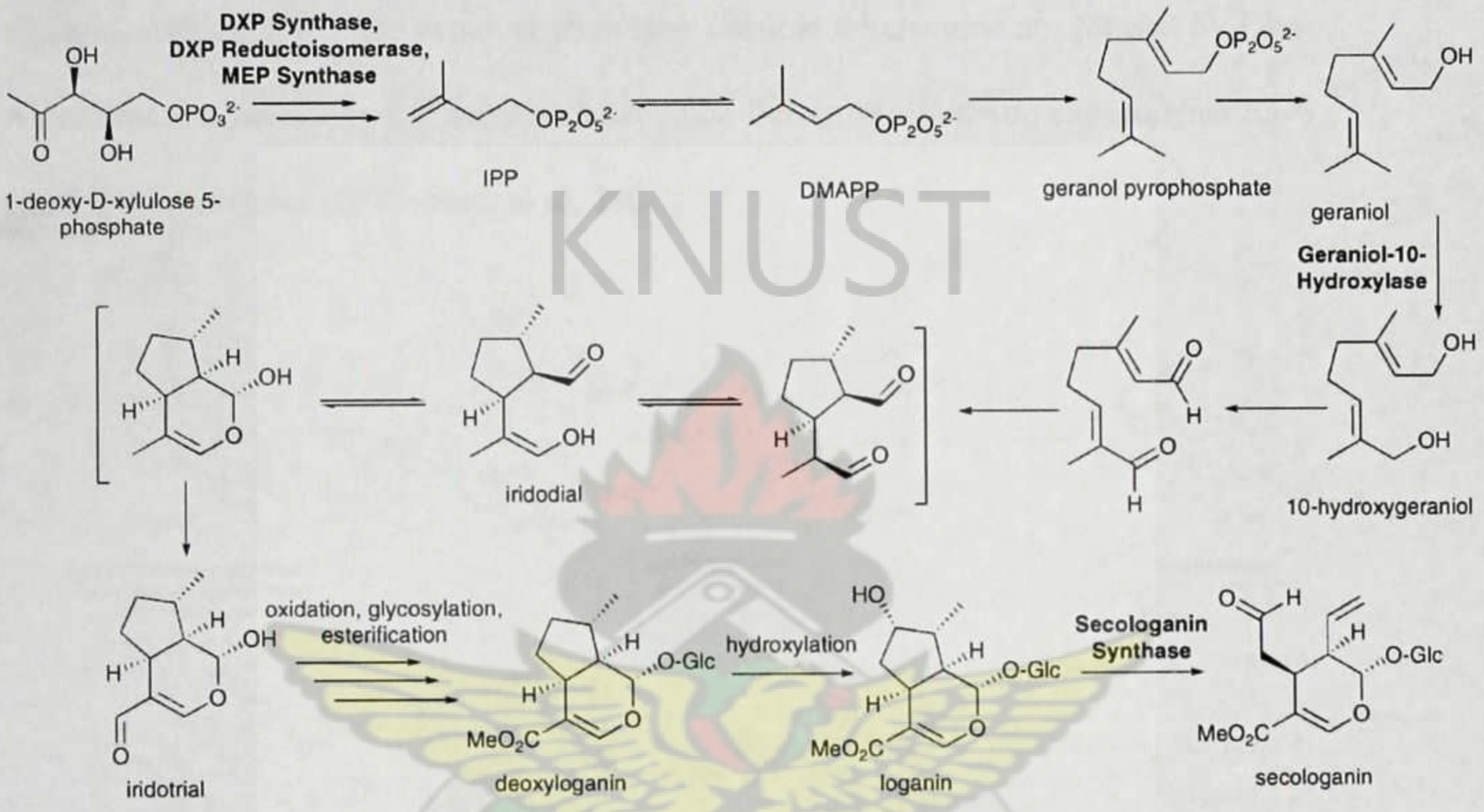

Figure 2-5 Scheme 2 Biosynthesis of secologanin

The initial step in the biosynthesis of the terpene indole alkaloids involves the the utilization of secologanin and tryptamine through stereoselective Pictet-Spengler condensation reaction. This leads to the formation of strictosidine. The reaction is catalyzed by the enzyme strictosidine synthase. It is quite intriguing in secondary metabolism how strictosidine rearranges itself into dramatically diverse structures of indole alkaloids found in the Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae families. Enzyme catalyzed deglycosylation of strictosidine yields a reactive intermediate; 4,21-dihydrogeissoschizine (deglycosylated strictosidine). The

geissoschine is converted to preakuammicine. The actual mechanism involved in this transformation remains unknown though several mechanisms have proposed. The preakuammicine is reduced to yield stemmadenine. Stemmadenine further rearranges itself the acrylic ester dehydrosecodine which is a common precursor for the synthesis of the aspidosperma and iboga alkaloids. It is believed that biosynthesis of iboga type alkaloid, catharanthine and aspidosperma type alkaloid tabersonine are formed by Diel-Alder reaction involving the dehydrodecodenine but no biosynthetic experiments have confirmed this claim **(O'Connor et al, 2006).**

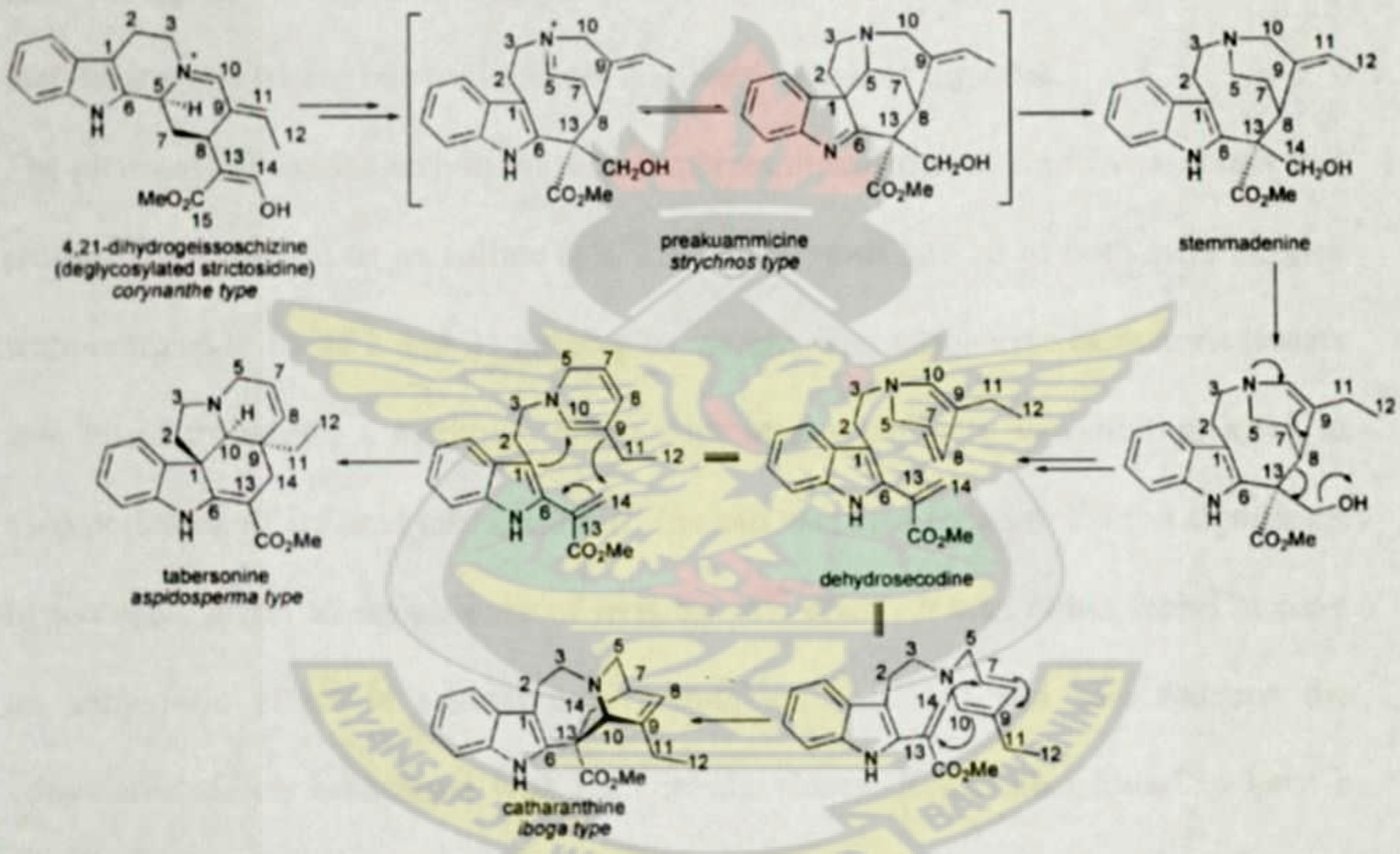

Figure 2-6 Scheme 3 Proposed biosynthesis of iboga and aspidosperma type alkaloids

2.2.3 PHARMACOLOGICAL PROPERTIES OF ALKALOIDS FOUND IN VOACANGA AFRICANA

The indole alkaloids have been the major secondary metabolites or active phytoconstituents and its pharmacological effects in various body systems has well been researched.

2.2.3.1 Efects on the Cardiovascular System

The pharmacological activity voacangine and voacamine isolated from of V. Africana started in early 1950s. It was realized that the isolates had similar mechanism of action compared to digitalin but a previous study using isolated rabbit auricle revealed that the isolates where less toxic; about one-third of that of digitalin.

The pharmacodynamics activity of the camphorsulfonate derivative of voacamine was studied and compard to its sulfate salt. The intravenous LD 50 of both salts in mice were realized to be 46.2 and 21.5mg/kg by weight. The voacamine camphorsulfonate was found to exhibit a myotonic effect without any coronary vasodilation in an in vivo perfusion of isolated rabbit auricle. The salt was also found to exhibit significant hypotensive effect in the absence of hypothermic effect. It was rather found to have an antipyretic effect in several experimental animals and and also reduced the convulsive effects associated with high insulin doses. It was also found to have a parasympathomimetic and sympatholytic effect (**Macebeo et.al, 2009).**

2.2.3.2 Effects on the Central Nervous System

Ibogaine, a powerful hallucinogenic compound found in voacanga African has been reported to block withdrawal syndromes and cravings in drug addicts by Kombian and co-workers. The nystatin perforated patch technique was employed and the mode of

action was linked to the modulation of the neuronal excitability and synaptic transmission in the parabrachial nucleus. The modulation is by the depolarizing parochial neurons with excitabilityand firing rate; by the depression of the non-NMDA receptor-mediator fast synaptic transmission; and lastly, by involvement of dopamine receptor activayon for its actions (**Macebeo et.al, 2009)**

2.2.3.3 Gastro-intestinal protective activity

The aqueous extract from the bark of V. Africana has been found to contain a number of polar alkaloids for which they have been found to exhibit gastro-protective effects when compared to its HCl/ethanol extract. It has also been found to have gastric mucosal protection property without significant reduction is the acidity of gastric juices. It was proposed that the mechanism of action was not through the neutralization of the HCl produced in solution but rather through increased mucous defenses or through the reduction of the proteolytic activity of the pepsin in the gastric juice. Another study has also reported that extracts from V. Africana can be used to treat intestinal disease caused by Entamoeba histolytica with an MIC = 62.5 ug/mL it was. In addition, organisms such as Campylobacter jejuni and C. coli have been found to be susceptible to V. africana extract.

TN, an alkaloid from V.africana whose structure remains unknown was assayed to investigate its anti- secretory, cytpprotective and ulcer healing properties. It was orally administered and the alkaloid was found to prevent ulcers induced by HCl/ethanol at 36-75%, absolute ethanol at 43-75%, indomethacin (NSAIDS) at 58-84%, pylorus ligation at 31-100%, cold restraint stress at 68- 100%, and lastly histamine at 49-100%. All these were dose dependent assay. TN was also found to

decrease the Shay-ligated acid secretion. Its cytoprotective and ulcer healing property have been foud to be related to its ability to increase mucus production which enhances mucosal protection and defense. A synergistic anti-secretory was observed when TN was combined with ranitidine, a histamine receptor antagonist used in treating ulcer (**Macebeo et.al, 2009**).

2.2.3.4 Anti-cancer activity

The alkaloid amataine isolated from V. Africana has been found to exhibit marked cytotoxic activity when tested against VERO cells. In the Cytotoxicity measurements, the Frame Kenacid Blue method was used for the proteins, Neutral Red for the lysosomes and Crystal Violet method for the nucleus and amataine exhibited an ID50 of 5 μg/mL, 3 μg/mL and 3μg/mL, respectively, based on the bioassay results of the three cytotoxicity methods. Using optical microscopy tests, the inhibition of the cell culture proliferation with pycnotic cells and clear vacuolization were qualitatively observed (**Macebeo et.al, 2009**).

2.2.3.5 Antiplasmodial Property

Voacamine, an ibogavobasine type alkaloid, has been reported to exhibit anti-plasmodial activity an in vitro assay. Further work on its in vivo anti-malarial activity showed that voacamine had significant anti-malarial activity but less pronounced compared to the reference compound chloroquine. Using a synchronized culture, voacamine was reported to act specifically on the trophozoite and schizont stages of the Plasmodium falciparum, suggesting a potential effect on the nuclear division of the parasite, possibly on the DNA or the protein synthesis (**Macebeo et.al, 2009**)

2.2.3.6 Anti-inflammatory activity

The total alkaloids from V.africana (HCl salts), reserpine and chlorpromazine were investigated for their ability to inhibit the edema in rat paw due to egg albumin and dextran. The results showed these compounds had the ability to reduce the edema through the increasing capillary resistance (**Macebeo et.al, 2009**).

2.2.4 ALKALOID VOACANGINE

Voacangine, the principle alkaloid is an ester indole alkaloid which is concentrated in the rootbark of the plant voacanga Africana. It is also found in the seeds, stem bark and the leaves of the plant. It has been reported to give prismatic needles when recrystallized from ethanol. Its major wavelength of absorption has been reported to be 225nm, 287nm and 300nm (**www.druglead.com**).

2.2.4.1 Pharmacological Actions of the Alkaloid Voacangine

2.2.4.1.1 Cardiovascular Effects

Using the spontaneously beating isolated guinea pig atrium and the electrically driven iolated guinea pig left atrium, voacangine has been found to cause a negative chronotropic and inotropic effect. Another study also showed that voacangine was able to block the chronotropic and ionotropic effects of noradrenaline on the heart which buttresses the previous study. Another study has also seek to discredit the antagonistic effect of voacangine on adrenaline by showing that voacangine has no effect on the heart (**chronotropic**). The discrepancies in the results have suggested

further research on the cardiovascular effect of voacangine and also to define its pharmacological properties. Voacangine has also proved to be able to inhibit acetylcholine in an invitro study. This could be a plausible reason why there were variations in the **chronotropic** effect of voacangine on the heart in the previous studies (**Pratchayasakul et al, 2008).**

2.2.4.1.2 CNS Effects

Voacangine has shown to possess an analgesic as well as a local anaestheic activity in mouse models. It has also been reported to potentiate the hypnotic effect associated with barbiturates **(Pratchayasakul et al, 2008)**.

2.2.4.1.3 Anti-microbial effects

Voacangine has been reported to show bacteriostatic activity against staph. Aureaus. It is able to kill up to 87% of bacterial cells at a concentration of 1.0 mg/ml. another study has showed that voacangine has a significant effect against mycobacterium species mainly tuberculosis, avium and kanasasii**(Macebeo et.al, 2009)** .

2.2.4.1.4 Voacangine as a novel anti-angiogenic agent both in vivo and in vitro

Voacangine has been reported to as a novel anti-angiogenic agent which acts by inhibiting the proliferation of HUVEs at an ICs of 18μM with any cytotoxic effects. It does this by both in vivo and in vitro. The in-vitro mechanism involves the suppression of the VEGF-induced tube formation and chemoinvasion. The in vivo mechanism is by the suppression of the chorioallantoic membrane at a non-toxic dose.

It has also suppresses the expression of the factor-1α and its target gene induced by hypoxia (Kim et al, 2012).

2.3 JUSTIFICATION

Though data that exist on the revenues collected from the exportation of medicinal products is scanty, trade in the seeds of voacanga is of economic benefit and this warrants the development of a simple RP-HPLC UV method to access its quality which can be adopted by the Export council to monitor the content of seeds supplied by collectors for export. The proposed method can be therefore used as a parameter for quality assessment of the seeds as well as the quantification of the biomarker in all parts of the plants suspected to contain the isolated biomarker since the method has proved to be relatively cheap, simple, sensitive, selective and specific and validated as required by the ICH for the method development.

AIMS AND OBJECTIVES

***Aim*: Identification of a bio marker and development of HPLC method for quality control of seeds of Voacanga africana (fam: apocynaceae).**

Objectives:

1. **To isolate and characterize alkaloid as a biomarker from the seeds of Voacanga Africana (fam: apocynaceae).**
2. **To develop HPLC method for its quantification.**
3. **To validate the developed HPLC method.**

2.4 THEORY OF WORK

2.4.1 SOLVENT EXTRACTION

2.4.1.1 General Methods of Extraction of Medicinal Plant

Extraction as used pharmaceutically is basically the isolation active therapeutic agents from parts of plants or animal tissues by use of selective solvents in standard extraction procedures, the products obtained are relatively Impure liquids, semi solids or powders which are employed for oral and and external use. Preparations can be classified as decoctions, infusions, fluid extracts, tinctures, pilular (semilsolid) extracts and powderd extracts. They are commonly called galenicals after the Greek phsycian, Galen.

The standardized extraction procedures for crude drugs seek to help obtain therapeutically desired components as well as to eliminate inert materials through the use of solvents selective for these components known as menstrum. These extracts may be used in the form of tinctures or fluid extracts in the treatment of diseases or may further be processed to be incorporated in dosage forms such as tablets or capsules. It may further be separated to obtain the secondary metabolites such as hyoscine, vincristine etc which have been adopted into orthodox medicine. The standardization of extraction methods contributes to final quality of a herbal drug.

2.4.1.2 Maceration

In this method of extraction, the powdered drug either whole or coarse is placed in stoppered container containing a suitable solvent and allowed to stand for a minimum of 3 days with frequent agitation. This is carried out at room temperature. The mixture is then strained, the marc is pressed, and the fluids combined. The liquid obtained is

clarified by simple filtration or decantation after allowing to stand for all debris to settle.

2.4.1.3 Infusion

Infusions are prepared by macerating the crude drugs for short period using cold or boiling water. The difference between the maceration and infusion is the period of extraction. They are fresh and diluted solutions which contain the readily soluble components of the crude drugs.

2.4.1.4 Digestion

This is a form of maceration where gentle heat is employed throught the extraction process. It is used when the stability of the components is not affected by elevated temperature. This enhances the solvent efficiency of the menstrum

2.4.1.5 Decoction

This process involves the boiling of the crude drug in a specified volume of water for a defined time. After, it is cooled and strained or filtered. It is most appropriate method for extracting water-soluble as well as heat stable constituents

2.4.1.6 Percolation

It is a procedure frequently used to extract active ingredients for tinctures and fluid extracts. A percolator which is a narrow, cone shaped vessel open at both ends is generally used. The material is moistened with an appropriate amount of the specified menstrum and allowed to stand for 4 hrs in a well closed container. The mass is packed in and the top of the percolator covered. Additional menstrum is added to

form a shallow layer above the mass and the mixture allowed to stand for 24hrs. The liquid is then collected by opening the outlet and allowing it to drip slowly. Additional menstrum is added, marc pressed and added to the percolate. Sufficient menstrum is added and the mixture clarified by filtration or decantation **(Handu et al, 2008)**.

2.4.2 EXTRACTION TECHNIQUES FOR MEDICINAL PLANTS

Extraction techniques seek to exploit the relative solubility differences in two immiscible liquids for their affinities for a solid solvent. Substances interact with the two solvents through equilibrium distribution which are physically separated and the species in each phase recovered. This can be accomplished by dissolving the substance and shaking the solution with a second immiscible solvent or by passing it through a sorbent or disk. Separation is only possible if equilibrium of the two solutes differ. The principal factor that determines distribution between the two phases is the polarities of the solute and the phases involved. Degree of ionization, hydrogen bonding and electrostatic interactions also play a role. Most solvent extraction methods involve the extraction of solutes from an aqueous phase into a non-polar such as hexane or slightly polar organic solvent such as trichloromethane etc though the reverse is possible. Solutes which can be easily extracted into organic solvents include neutral molecules with few ionized substituents and non ionizable substituents. Polar, ionized or ionic species remain in the aqueous phase. Solid phase extraction is a versatile technique which employs solid sorbents but the principle is the same as solvent extraction

The solvent extraction is governed by the Nernst distribution or the Partition law which states that at constant temperature and pressure, a solute, S, will always be distributes in the same proportions between two particular immiscible solvents. The distribution or partition coefficient, K_D, is given by the expression

$$K_D = \frac{[S]_{org}}{[S]_{aq}}$$

Where [ ] represents the concentration terms (activities) of the solute distributing between the two solvents. The K_D is independent of the total solute concentration. A more appropriate expression for the solute is the distribution or partition ratio since the solute species usually exist in ionized form, protonated, complex or in the form of polymerization. The expression now becomes;

$$D = \frac{(Cs)org}{(Cs)aq}$$

Cs represents the concentration of distributing solute S in each phase. If there is no interaction of S in either phase, it can be said that D and KD would be identical. The D is determined experimentally and can be adjusted over a wide range to suit the requirements of the analytical procedure

The value of the distribution ratio, D determines how efficient an extraction method is and also depends on the volumes of the two liquid phases. The **efficiency of an extraction method, E** can be given as

$$E = \frac{100D}{[D + (Vaq / Vorg)]}$$

Solvent extraction is a means of pre-treatment method to clean "dirty "samples or to separate analyte from a complex matrix components that might cause interference in the detection and quantification of the analytes. For analytes which are found in low concentrations or trace analytes, it is used to pre-concentrate analytes which might otherwise be difficult to detect and quantify.

In order to carry out an efficient extraction, the extraction is carried out in batchwise in a separating funnel and the sample is continuously exposed to fresh solvent that is recovered by refluxing in a specially designed apparatus.

2.4.2.1 Extraction of organic acids and bases

Organic compounds either acidic or basic can dissociate or protonate depending on the pH of the solution. Extraction can be achieved through the adjustment of the pH of the solution. The distribution ratio and the pH can be derived from the equation below

A weak acid dissociates in water according to the equation below

$HA = H^+ + A^-$

The acid dissociation constant can be given as

$$Ka = \frac{[H^+]aq\ [A^-]aq}{[HA]aq}$$

The undissociated form, [HA] gets extracted into the non-polar or slightly polar solvent eg.ether. The **distribution or partition coefficient, KD**, can be given as

$$KD = \frac{[HA]org}{[HA]aq}$$

The distribution ratio takes into consideration the dissociated and undissociated forms of the organic acid in the aqueous phase hence the equation becomes

$$D = \frac{[HA]org}{([HA]aq + [A^-]aq)}$$

Rearrangement of equation and substitution of [A-] into equation gives

$$D = \frac{[HA]org}{[HA]aq\ (1 + Ka/[H^+]aq}$$

Since KD = [HA]org/[HA]aq, substituting into equation gives

$$D = \frac{KD}{1 + Ka[H+]}$$

The equation above shows that at low pH extraction is at its greatest efficiency when the acid is in the undissociated form as $D \sim K_D$ whereas the efficiency decreases as the pH increases since the D decreases until a hight pH is attained where the acid is completely dissociated into the anion A- and none will be extracted.

Ionization of a weak organic base such as amines occur at low pH as shown below

$$RNH_2 + H^+ \longleftrightarrow RN^+H_3$$

The relationship between pH and distribution ratio for a weak base is the opposite of a weak acid. This makes it practically possible to separate acids from bases through pH adjustments but the dissociation constant should differ by several pk units **(Kealey et al, 2005)**.

2.4.3 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography is a form of planar chromatography which has been adopted in separation technique because of its simplicity, cost effectiveness and

versatility. It can be employed in both qualitative and quantitative or preparative separation. Despite its great variety and complete automation it lags behind as an analytical tool. However, it has become an irreplaceable tool in the qualitative analysis of plant extracts. The advantages of this technique are that it is less time consuming and a simple technique.

It has found prominent use in the standardization of plant materials including fingerprint profiling for the assessment of chemical constituents of drug and quantitative analysis of marker in plant drugs. A simple TLC procedure will involve sample preparation, selection of chromatographic layer, a suitable mobile phase, sample application, development and drying of plate and chromatogram evaluation.

2.4.3.1 Chromatographic Layer

The adsorbent layer can be made from a wide array of materials. Pre coated plates have come to replace laboratory-made plates which is marketed by several manufacturers. The precoated plates are machine made of glass, aluminium or plastic base coated different adsorbents. The adsorbent material that can be used include normal phase silica gel which is the commonest, reverse phase gel (RP_2, RP_8,RP_{18}, cyano, diol and amino plates), aluminium oxide, cellulose, kieselguhr, hybrid (can be used both as reverse and normal) and derivatized adsorbent layers. They are sold in different sizes from small strips to continuous rolls (20×20 cm^2 is the most common) The nature of the compounds defines the type of adsorbent layer to be used. Aluminium oxide which is a strong adsorbent is used for weakly adsorbed material since there will less interaction between the adsorbent and the compounds. Weak adsorbents such as cellulose are used for strongly adsorbed compounds. Normal phase silica is suited for non-polar compounds while reverse phase silica gel is suited for

polar constituents which are eluted first on a rverse phase TLC. Silica gel plates containing fluorescent dye (F_{254}) of aluminium base are widely used. About 80% of analyses are carried out using these plates as they are optimally efficient and cost effective.

2.4.3.2 Selection of Mobile Phase in TLC Analysis

Mobile phase for TLC analysis provides no or few restrictions on the choice of solvents for TLC development unlike HPLC where choice is limited. Usually, mobile phase components of 1-3 are preferred over a multicomponent mobile phase. Personal experience coupled with existing knowledge and trial and error method is used to select the composition of the mobile phase. The mobile is freshly prepared for each run and the solvents are mixed outside before transferring to the developing tanks. The tanks are allowed to saturate which can be quickened by lining the inside walls with a filter paper and pouring the mobile phase over it. The chamber is closed and allowed to stand for some time at room temperature to allow saturation.

TLC analysis is sensitive to temperature and humidity variations. It is therefore necessary that analysis carried during which the plate is exposed to air should be done at a relative humidity of 50%-60% at a controlled temperature of 20-30^{o} C

2.4.3.3 Application of the Sample

The application of the sample solution onto the TLC plates can be done in three ways. It can be done manually, semi-automatic and automatic application. A capillary tube is used in the manual application and the solution is applied by a touch and deliver. The semi-automatic involves the use of devices such as linomat 5 from Camag or Applicator AS 30 from Desaga which uses a syringe which has to be manually

cleaned and filled. The remaining part of the application is automated which involves computer commands. The solution is applied as a spot or band of predetermined size at predetermined points by touch and delivery or spray-on technique. The fully automated method involves computer controlled commands including washing of the delivery line.

2.4.3.4 Developing the Chromatogram

Plates are developed in special purpose jars or simple containers capable of holding the solvent in an air-tight environment. The special purpose jars help to produce better chromatograms. Twin trough chambers allow use of another mobile phase for the purpose of saturation. Saturation before development reduces Rf values and helps to correct side distortions of the solvent front. The plate should be place in a nearly vertical manner in the tank and the solvent should be below the point of application. The side of the plates should not touch the sides of the inner wall. The chamber should be protected from sunlight and ordinary light

2.4.3.5 Drying of Plates and Derivitization

The plates are dried after development which can be accomplished by drying in air at room temperature, in a vacuum dessicator or by heating or blowing hot-air over the surface of the pates. In all instances, it should be ensured that the mobile phase has been driven off before derivitization is done.

Derivitization helps in locating the position of the constituents in qualitative analysis or quantification of ultraviolet insensitive markers and it is done by spraying the developed plates with suitable spray reagents. Derivitization can be done in two ways

either spraying with a fine mist of reagent (traditional method) or dipping in the reagent which is gaining popularity. One disadvantage of the spray method is the non-uniform wetting of the plate producing areas of high wetting and deficient spray which affects the precision and accuracy especially in quantitative determination. The dip in method produces a uniform wetting on the plates. Heating of the plates is most often necessary after spraying. It is most suitable to heat the plates in open air since they give better results than heating in an oven. Heating is usually done at a temperature of 110o C for 10 min or until the spots are visible.

2.4.3.6 Evaluation of Chromatograms

TLC plates may be observed in daylight, under short wavelength or long wave ultraviolet light. R_f values are usually calculated helps in identification when a standard and test samples are run on the same plate. The centre of the plate is marked with a needle and the distance the compound travels from the point of application to the centre of the spot to that travelled by the solvent front gives a ratio known as the retardation factor, R_f. R_f values depend on temperature, degree of saturation, mobile phase composition and the activity of the adsorbent layer

$$R_f = \frac{\text{Distance travelled by analyte}}{\text{Distance travelled by the solvent front}}$$

Quantitative evaluation can be achieved by scanning the plate in a TLC densitometer or scanner. The densitometer uses two modes of transmittance and reflectance depending on available optics. For quantification, it employs fluorescence mode,

ultraviolet absorption or visible light depending on the optioned exercised **(Handu et al 2008)**.

2.4.4 COLUMN CHROMATOGRAPHY

Column or gravity chromatography is a simple technique for the separation of components of a mixture. This basic principle is the differential interaction of components of a mixture with an an adsorbent (stationary phase). The basic principles behind the separation include adsorption, partition, size exclusion and ion exchange. The stationary phase is an inert material supported in a glass column. Stationary phases usually employed include silica gel, alumina, cellulose, magnesia.

Silica and alumina are most commonly used but their adsorbing properties are reduced if they absorb water which can be reversed by heating to 200-400 ^{o}C. Silica is slightly acidic hence good choice for basic solutes. Alumina is slightly basic and therefore strongly adsorbs acidic solutes.

A wide range of solvents are used in this technique including hydrocarbons, aromatic compounds, alcohols, ketones, esters and halogenated hydrocarbons. A mixture of solvent is usually employed.

In setting up a liquid chromatography, it is prudent to saturate the stationary phase with solvent because the presence of air can lead to incomplete separation and disrupt the smooth flow. The solutes are allowed to descent the column under gravity. It is easier to collect coloured compounds. If the components are colourless, small volumes of eluate are collected in tubes and the components identified by either by TLC or fluorescence under ultraviolet light.

2.4.5 HPLC PHENOMENON

HPLC or liquid chromatography (LC) is a physical separation technique carried out in a liquid phase. A sample made of a few to many components is distributed between a mobile phase (a flowing solvent) and a stationary phase (adsorbent) held in a column. The mobile phase can be an organic solvent such as hexane and the stationary phase can be porous silica particles packed in a long column. HPLC is a modern form of liquid chromatography where the mobile phase is passed through the column containing small particles at a high pressure hence the name high pressure liquid chromatography.

2.4.5.1 Brief History

The word "chromatography" means "colour writing" was discovered by a Russian botanist called Mikhail Tswett and was able to separate plant pigments on chalk (CaCO3) which was packed in glass columns in 1903. Chemists have used gravity fed silica column to purify organic materials and ion-exchange resins to separate ionic compounds and radionuclides. The invention of the gas chromatography was the driving force for the development of LC. Gas chromatography was invented by British chemists A.J.P. Martin and co-workers in 1952 with successful application. Small particle columns came into existence in the 1960s which required high pressure pumps. The first generation high performance liquid chromatograph was developed in 1960s by researchers including Horvath Kirkland, and Huber. The inclusion of in-line detectors and reliable injectors made HPLC a sensitive and quantitative technique leading to its wide application. It became an indispensible technique in the 1980s because of its high precision and versatility in pharmaceuticals and in other diverse

industries. HPLC continues to evolve rapidly towards higher speed, efficiency and sensitivity which has been driven by emerging needs of life science and pharmaceutical application and has become more sophisticated.

2.4.5.2 Advantages and Limitations of Hplc

HPLC is a technique which is a capable of separating a complex mixture of components with high versatility and precision of < 0.5% relative standard deviation

HPLC is highly automated in modern times with sophisticated autosamplers and data systems which requires unattended analysis and data generation.

Limit of detection can be extended to nanograms, pictogram and even femtogram levels which require an array of highly sensitive and specific detectors.

It can be used as a preparative technique to separate liable compounds in milligrammes to kilogramme quantities.

One major limitation to its use the unavailability of a universal detector such as the equivalence or flame ionization detector which is the detector used in GC hence making detection problemtic if the compound does not absorb UV radiation or cannot be easily ionized for mass spectroscopic determination. Secondly, it has less efficiency than GC thus the analysis of complex mixtures is more difficult. Finally, HPLC is quite sophisticated with many operating parameters which make it difficult for a novice.

2.4.5.3 Modes of Hplc

2.4.5.3.1 Normal-Phase Chromatography

It is also known as liquid-solid chromatography or adsorption chromatography. It is the traditional separation method which is based on adsorption/desorption or analytes onto a polar stationary phase. Here, the polar stationary phase commonly used is silica or alumina and a non-polar mobile phase. The porous silica particles have silanols (Si-OH) sitting on their surface and inside its pores. Polar compounds migrate slowly through the column due to the strong interaction with silanol groups. Surface layer of water is believed to reduce the activity of the silanol groups and yields more symmetrical peaks. It is employed when non polar compounds are to be separated as well as fractionation of complex samples since they will elute early due to the weak interaction with the silanol groups. One major disadvantage is easy contamination of the polar surface by the sample components. This problem can be circumvented by bonding the silanol groups with polar functional groups such as amino or cyano moiety.

2.4.5.3.2 Reverse-Phase Chromatography

It is the most common and this mode of separation is used when polar compounds are to be separated. The separation is based on partitioning of analyte between a polar mobile phase and a non-polar stationary phase. The first generation stationary phases were solid particles coated with non-polar liquids but they were quickly replaced by more permanent bonding hydrophobic groups such as octadecyl (C18) bonded groups on a silica support. The name "reverse phase chromatography" evolved from the

elution of "polar first and non-polar last" and the reverse of what happens in normal phase chromatography. The most commonly used polar solvent is RPC are methanol, acetonitrile and water. Water is mixed with methanol or acetonitrile to give a polar mobile phase. The interaction here is predominantly a hydrophobic interaction. It is most appropriate for polar, semi polar and some non-polar molecules. Ionic analytes can be separated using ion-suppression or ion-pairing techniques.

2.4.5.3.3 Ion Exchange Chromatography

This mode of separation is based on the exchange ionic analytes with counter ionic group attached to solid support. Stationary phases used here are cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials. Mobile phases consist of buffers often with increasing ionic strength to force migration of the analytes. This method of separation is used when amino acids, proteins/peptides and polynucleotides

2.4.5.3.4 Size Exclusion Chromatography

This mode of separation is based on the molecular size of the molecules to be separated. Large molecules which cannot penetrate the pores of the gel migrate quickly through the column while small molecules pass slowly through the pores. It is called gel permeation chromatography (GPC) when used to determine the molecular weights of organic molecules and gel filtration when used in the separation of water soluble biological molecules. In GPC, the stationary phase is a cross-linked polystyrene beads of controlled pore sizes and eluted with common solvents such as toluene and tetrahydrofuran. Detection with refractive index detector is common.

2.4.6 BASIC CONCEPTS IN HPLC

Retention time (t_R) is the time between sample injection and the peak maximum as shown in the diagram below.

Void time or hold-up time (tM) is the time of an unretained component or a the first baseline disturbance by sample solvent. tM is also the total time spent by a component in the mobile phase

Adjusted retention time (tR') is given as (tR – tM) thus it represents the time the solute resides in the stationary phase. Therefore, tR = tR' + tM which is the retention time or the total time the compound spends in the stationary phase and mobile phase.

Retention volume (VR) is the volume of mobile phase required to elute the analyte at specified flow rate (F) and it is expressed as retention volume, VR = tRF.

Void volume is the volume of mobile phase contained in the column. It actually represents the volume of empty column (Vc) minus the packing material. Void volume also is expressed as void volume, VM = tMF

Retention factor (k) is the degree of retention of sample components in the column and it represents the time the sample stays in the stationary phase (tR') relative to the time it stays in the mobile phase (tM'). It also referred to as **k'** or the capacity factor and it is represented as

$$K = \frac{tR - tM}{tM}$$

Separation factor (α) is the measure of relative retention k_2/k_1 of two sample components. For good separation selectivity must be > 1.0. Factors which affect selectivity include mobile phase composition, properties of the solute and nature of stationary phase.

Column efficiency and Plate number (N)

The efficiency of a column depends on the number if samples it can separate within a short period and how sharp the peaks are. Peaks attained assume a Gaussian shape and broaden with time. The number of theoretical plates or plate number (N) is the measure of the efficiency the column where $N = (tR/\sigma)^2$ or $16\ (tR/Wb)^2$ since wb is equal 4σ for a Gaussian peak where σ is the standard deviation of the peak and wb is the width of the peak. Since there is difficulty in measuring σ or wb, using the width at half height (w1/2) is most appropriate for calculating N. the expression becomes $N = 5.546(tR/w_{1/2})$.

Height Equivalent to Theoretical Plate (HETP) is given as the length of the column (L) divided by the plate number (N). Although there are no plates in the the column, this concept can plate number can be applied. HEPT = L/N

Resolution is the most important parameter to a pharmaceutical analyst since the goal of every analysis is separation of a mixture to one or more analytes. Resolution is the measure of the degree of separation of two adjacent peaks. It is given as the difference in the retention times of two peaks divided by the average peak width

Resolution, $R = tR2 - tR1 = \Delta tR$

$$\frac{wb1 + wb2}{2} \quad wb$$

R = 0 means no separation, R = 1 means partial separation and R = 1.5 means baseline separation. The goal of most HPLC methods is to achieve a separation of Rs = (1.5 – 2.0)

Asymmetrical Factor (A_s) and Tailing factor (T_f)

Most peaks observed in HPLC analysis are not perfectly symmetrical and they usually suffer from tailing or fronting. The asymmetrical factor helps to calculate the degree of peak symmetry and is defined as peak width of 10% of peak height ($W_{0.1}$).

Asymmetrical factor, $A_s = B/A$

Tailing factor, $T_f = W_{0.05}/2f$

Tf = 1.0 is an indication of a perfectly symmetrical peak, Tf > 2 is an indication of a tailing peak which is unacceptable due to difficulty in integrating the peak area. The most acceptable range is 0.5< Tf < 2.0. Usually the A_s and T_f are fairly similar. A severely tailing peak usually has larger A_s than T_f.

Peak tailing is usually caused by adsorption process or due to extra column band broadening while peak fronting is typically caused caused by column overload or chemical reaction of analyte during chromatography. A typical example of tailing is usually observed in basic compounds where there is polar interaction with residual acidic silanol groups in silica based columns

2.4.7 MOBILE PHASE

The mobile phase is the solvent that carries the analyte through the column. In HPLC, the mobile phase interacts with both the solute and the stationary phase and actually determines the solute retention and separation.

2.4.7.1 Requirements of an Ideal Solvent for Use as Mobile Phase

- Should have high solubility for the components
- Should be non-corrosive to the HPLC components
- High purity, low cost and UV transparent
- Low viscosity, low toxicity and non-flammability

2.4.7.2 Solvent Strenght and Selectivity

Solvent strength refers to the ability of the solvent to elute the solutes from a column. Solvent strength depends on its polarity. In normal phase chromatography, non-polar hexane is a classified as a weak solvent whereas water is a strong solvent but in reverse phase chromatography water is a weak solvent while organic solvents are strong solvents since a non-polar stationary phase is used here. Solvents are usually classified in normal phase by Hildebrand's scale. For reverse chromatography, the order of solvents are THF> ACN>MeOH>water (modern HPLC).

The polarity and selectivity of a mobile phase solvent can affect the separation. A more polar solvent in RPC increase the retention while retention is reduced in NPC. A rule of thumb is that a 10 % decrease in the organic solvent in the mobile phase in RPC causes a 3-fold increase in the retention time

2.4.8 BUFFERS

The pH of the mobile phase can have a dramatic effect on the retention of ionizable compounds either acidic or basic. Since the principle mechanism in reverse chromatography is partition coefficient, ionized solutes are not able well partition well into the hydrophibic stationary phase which leads to a low k value than the unionized form. There is therefore the need to suppress the ionization of these solutes leading to higher retention. Buffers are therefore used to control the pH of the mobile phase. It is worth noting that buffers are only effective within ± 1.5 pH units from the pKa. Commonly employed buffers and their respective pKa and UV cut-off has been summarized below.

In RPC, acidic pH of 2.5 – 3 is usually encountered in many applications. The low pH suppress the ionization of weakly acidic and the surface silanol groups are not ionized lessening the incidence of tailing with basic compounds commonly used acids in the mobile phase preparations include phosphoric acid, trifluoroacetic acid (TFA), formic acid and acetic. Basic compounds are. Basic compounds are ionized at low pH and are not retained significantly.

Ion- paring additives are detergent like molecules which aid in the retention of acidic or basic compounds. Long-chain alkyl sulfonates when added to mobile phase combine with basic solutes under acidic conditions to form neutral "ion-pairs" that enhance retention. TFA has some ion – pairing properties **(Dolan, 2006)**

2.4.9 HPLC COLUMN

The column containing the packed stationary phase functions as the heart of every separation process. It is therefore prudent to obtain a table, high- performance column which will be essential in developing a a rugged and reproducible method. Commercial columns available differ widely among suppliers, and even between supposedly identical columns from the same source. Column specifically differ in plate number, band symmetry, retention, band spacing and lifetime.

In the selection of a column, column to column reproducibility is very essential in method development. Most chromatographers dislike having to reproduce a new method on different column after standardization of the method on a particular system. Manufacturers of columns guarantee reproducibility of certain column performance such as plate number, retention, back pressure etc. Ruggedness of an HPLC method is of great importance to an analyst.

2.4.10 COLUMN PACKING PARTICLES

Silica particles are the most common packing material though columns based on porous polymer are occasionally used in certain separations. These particles come in varied pore sizes, diameters and surface areas so that all types of HPLC methods can be developed with these materials.

2.4.10.1 Silica Packing Material

Silica packing material is the the most popular HPLC column packing material due to its favorable physical characteristics. Totally porous silica particles can be prepared with a narrow pore-size distribution in a wide choice of pore sizes eg. 8, 30,100 nm. Packing material of appropriate particle size exist for both preparative and analytical application. The advantages of silica particles as a packing materials include;

- High mechanical strength which permits formation of stable beds which can withstand high pressure
- Highest column efficiency
- Rigid and high strength particles produce column of lower back pressure and longer life
- A desired property of silica particles is the ability to chemically modify the surfaces with varied bonded phases which can broaden its functionality.
- Silica based packing are stable to water and all organic solvents

Silica particles however, are not the perfect support for HPLC column since they hsuffer from undesired characteristics summarized below;

- Solubility at high pH thus pH>8
- Acidity of the surface groups (silanol) which causes problems in the separation of basic compounds

2.4.11STATIONARY PHASES

2.4.11.1 Bonded Silica

Silica used in reverse phase chromatography are made by covalently bonding the organosilane or by depositing a polymeric organic layer on the support surface. Most bonded silica are made using a monofunctional reagent due to reproducibility of packing material made by this method. Chlorodimethylsilanes is a common monofunctional reagent used in this reaction. Various alkyl and substituted alkul silicas are made by this reaction eg. ODS or C_{18}.

It is quite logical that one silanol group reacts with silane molecule but an attempt by manufactures to completely react the silica surface proves futile because of the steric bulk of the bonded phase ligands thus all of the silanol groups cannot reacted. Some manufacturers to overcome this problem use a process known as endcapping. Encapping consist of a subsequent reaction of the bonded packing with a small silane such as trimethylchlorosilane. This approach increases the number of bonded silane but does not completely overcome the problem. This results in reaction of some of the residual silanol groups to minimize unwanted interaction with solutes. Endcapping

does not overcome the problem of acidic slilica support. Small endcapping group can be hydrolyzed at low pH making this method a questionable merit for ruggedness and long term application. Endcapped column are most stable at intermediate pH and higher pH because of better protection of silica support against dissolution **(Synder et al, 1997).**

2.4.11.2 Isocratic and Gradient Elution

Isocratic elution is most common form of analysis and in this method the same mobile phase composition is used throughout the elution of the sample. It is preferred method for simple mixtures.

In gradient elution, the strength of the mobile phase is increased with time during the elution of the sample is the preferred method for complex mixtures containing analyte of diverse polarities.

Advantages of gradient elution include;

- Suitable for complex mixtures of diverse polarities which require simultaneous quantification of all peaks
- Better resolution of early and late peaks
- Higher peak capacity

Disadvantages include;

- More complex HPLC system required
- Method development, implementation and transfer are more difficult

- Longer assay times are required since the column has to be equilibrated with the initial mobile phase

2.4.12 DETECTION

An HPLC detector measures the concentration of eluting analytes by monitoring some of the inherent properties of the compound such as UV absorbance. UV detector is the most commonly used since it can provide adequate response for most samples. Other forms of detection include fluorescence detection, refractive index detection, photodiode array detection etc

2.4.12.1 UV Detector

The UV detector measures the absorbance which is proportional to the concentration of the analyte being eluted. They are the most commonly used detectors. The principle behind UV detection is the Beer's law where

Absorbance = molar absorptivity × pathlenght × concentration

Compounds with chromophores (light absorbing systems) are capable of being monitored using UV detectors.

The UV/VIS detector consists of a deuterium lamp, monochromator and a small flow cell. The monochromator allows light of specified wavelength to pass through the exit slit. The light is slit into sample and reference bean and the sample beam passes the sample flow cell. The intensity of the beam is monitored by a photodiode.

2.4.12.2 Photodiode Array Detector

It provides UV spectra of eluting peaks as well as functioning as multi wavelength UV/Vis absorbance detector. It is very important in peak identification and method development.

In DAD, the deuterium lamp passes through the flow cell onto the diode array which measures the intensity of light at each wavelength

2.4.12.3 Flourescence Detector

It is selective and highly sensitive (picogrammes to femtograms) and its suitable for trace analysis but its use is limited to compounds that have inherent fluorescent properties.

The detector monitors the fluorescence emitted by the eluent in the flow cell with an irradiation of an excitation light at right angle **(Dolan, 2006)**

2.4.13 QUANTIFICATION IN HPLC ANALYSIS

One major importance of HPLC is that it is has excellent analytical properties with high precision. HPLC can be used in the quantification of major components in a sample or complex mixtures. It can even be used in the assessment of trace impurities in a sample. A well designed and validated method should show high accuracy and precision for the analysis of a particular component.

2.4.13.1 Peak Height

The response of the detector can be measured by measuring the peak height of the signal which is the simplest way of measuring the detector response. The peak measurement is a preferred approach in trace analysis. The distance between the baseline and the peak apex is the measure of the peak height. The baseline value should an average of many data before the start of the peak and after the end of the peak. Peak height measurement is a simple manual procedure but most data systems are able to calculate the peak heights

2.4.13.2 Peak Area

The peak area is directly proportional to the concentration of the analyte hence it is the most widely used parameter for quantification. A peak should be well resolved for its area to be calculated accurately. The peak area is defined as the integral of the signal response over time from the beginning to the end of the peak. The accuracy and precision of peak area measurements depends on factors such as;

- Establishment of correct baseline especially in the presence of short or long term noise
- Accurate definition of beginning and end of peak which is difficult in non-symmetrical or tailing peak
- Large number of data points to collect to accurately assess the peak area

Peak area calculation can be done using an intergrator or a computerized system. In the absence of these, it can also be done manually.

2.4.13.3 Peak Height vs Peak Area

Peak height or peak area is a parameter for quantification provided a good calibration is obtained. The use of peak area for quantification is a common method though it not always the best. For a nearly symmetrical peak, peak height is more accurate and precise than peak area but a small change in operating factors can affect peak height or the peak area. For an example, a small change in flow rate will have a minimal effect on the peak height than the peak area while a change in column condition which affects plate number will affect peak height more than the peak area. Peak height is very useful in trace analysis and there is less potential interference in determining peak height.

2.4.13.4 Noise

Noise is the uncertainty in the baseline in the absence of an analyte. Any signal is measured relative to the baseline. Noise can be classified into short term, long term and baseline drift.

Short term noise is very important in the measurement of the signal – noise (S/N'). Factors which can affect the noise are detector response, pulsation due to pump and electronic noise. It can be realized that short term noise can be internally generated or is due to external factors.

Long term noise are usually due to external factors such or system problem such as temperature fluctuations, poor mixing of solvent bring variation in the mobile phase, bleed of stationary, late eluting compounds from previous injection. It can be corrected when identified in the system.

Baseline drift is special type of noise which can occur even in a well-developed and validated method. This type of signal is well noticed in gradient elution where the mobile phase composition is changed with time. This results in a change in the detector response due to the variation it the solvent composition (**Synder et al, 1997**).

2.4.14 QUALITATIVE ANALYSIS IN HPLC

2.4.14.1 Comparison of Retention Times

The retention time or volume as be used as a qualitative tool in HPLC analysis. This requires the injection of standard and unknown compounds sequentially under stable and identical conditions with little between runs. The comparison of the retention times or volume on the chromatograms obtained can help identify unknown compounds.

2.4.14.2 Spiking Samples with Unknown

It is possible to add a known compound to a sample suspected to contain the known compound and chromatographing. The original sample is first injected and subsequent ones are obtained by spiking the sample with the known compound. Any peak in the original chromatogram which has the peak height increased significantly in the spiked sample can be concluded to be the compound of interest.

2.4.14.3 Interfacing

Interfacing the chromatograph with a spectrometer which provides spectral information for each compound separated as well as retention data. The spectra of the

unknown can be compared to those in computerized library database or those interpreted manually (**Kealey et al, 2005**).

2.4.15METHOD VALIDATION

For a developed method to be accepted and become a standard method for quatification, it has be validated. Method validation aims to challenge the method in order to investigate the limits of the variations which can affect the method. The validation method should be well detailed and should describe the entire procedure of validation.

Parameters for method validation include accuracy, precision, linearity, range, limit of detection, limit of quantification, specificity, ruggedness and robustness.

Accuracy

The accuracy of a measurement is the closeness of a measured value to a true value. A method with high accuracy should ideally the measured value should be the same as the actual value. Recovery studies can help monitor the the accuracy of a method. Accuracy can be determined by comparison with a reference standard, recovery of an analyte spiked into a blank matrix and lastly by standard addition of analyte.

Precision

Precision measures the degree of agreement of bwtween individual test when the procedure is carried out repeatedly to multiple sampling of homogenous samples.

Precision has further been divided by the international conference on harmonization (ICH) into repeatability, reproducibility and intermediate precision.

Repeatability is the precision of a method over a short period of time under the same operating conditions. It is done by repeatedly injecting the same concentration (typically 10 or more times) and averaging the peak area or peak height and determining the relative standard deviation (RSD). Different concentrations can also be prepared and injected several times at same operating conditions and this is terned as intra day precision.

Intermediate precision is the agreement of the same method under the same operating conditions when the method is applied several times within the same laboratory. This also includes analysis on different days, instrument and analyst.

Reproducibility measures the precision in different laboratories and is often determined in collaborative works or method transfer experiments.

Precision is expressed in terms of standard deviation or relative standard deviation.

Linearity

Linearity of a method measures how a calibration plot of detector response against concentration approximates a straight line. Different analyte concentrations are prepared and their response measured and their linearity determined by plotting a calibration curve. The slope, the y – intercept and **coefficient of regression** provides information of the linearity of a measurement. Correlation coefficient of 0.999 and above is usually acceptable for major components in a method.

Range

The range is the lower and the upper concentrations of the analyte for which the method is accurate, precise and linear.

Limit of Detection

The limit of detection is the smallest level of the analyte that gives a measured response. It usually based on a certain signal-to-noise ratio (S/N) of 2 or 3. Signal-to-noise ratio of 3 is usually preferred.

Limit of Quantification

It is the smallest level of analyte which gives a response which can be measured accurately. It can also be defined as the level of concentration at which the precision is poorer than a certain value typically RSD>3%. An arbitrary value of S/N of 10 can be set which can be used in the calculation of the LOQ.

Both LOQ and LOD are affected by factors such as reagents (presence of contaminants affects the S/N), column and more especially instrument. Detectors and pumping system can significantly affect the LOQ and LOD of an analyte.

Specificity

It is one of the most important parameters in method validation and it is defined as the ability to measure accurately the concentration of an analyte in the presence of other components. If a method is not specific for a particular analyte, precision and linearity are both compromised. Specificity can be achieved in two ways

- The effect of interfering components with peak of interest with specified resolution is tested
- The use of selective detectors which can only detect the analyte but not the interfering components (**Synder et al, 1997**).

2.4.16 NMR PHENOMENON

Nuclear magnetic resonance is a powerful analytical which has been employed in the elucidation of chemical structures. NMR relies on the magnetic properties of the nucleus to determine the proximity of electronegativity groups, double bonds and other magnetic nuclei in the chemical structure but NMR is sensitive when compared to other analytical tools like the mass spectroscopy. For structure elucidation, about a milligram of pure compound is required whiles a microgramme for mass spectroscopy. The sensitivity is proportional to the concentration of the compound.

When a nucleus is placed in a strong magnetic field, it resonates at a characteristic frequency in the radio frequency range of the electromagnetic spectrum. Since the nuclei find themselves in different environments, there are slight variations in the resonant frequency and this is useful in the determination of the molecular structure in which the atom resides.

Atoms bear protons and neutrons in their nucleus. Because of the protons in the nucleus, they behave like spinning charge entities which creates a small magnetic field. The nucleus when placed in an external magnetic field, the nucleus tries to align itself with it like a compass needle in the earth's magnetic field. Because th nucleus is spinning, the torque exerted by the external magnetic field causes the nucleus to undergo a precessional motion which is proportional to strength of the magnetic field

$$\nu o = \gamma Bo/2\pi$$

Where νo is the precessional frequency or Lamor frequency in hertz, γ is the magnetogyric ratio or the strength of the nuclear magnet and Bo is the strength of the

external magnetic field. The resonant frequency is measured by applying a radio frequency signal and varying the frequency until there is the absorbance of energy which is then detected.

2.4.16.1 The Quantum Model

Considering the quantum model of the nucleus in magnetic field, useful nuclei with also known as "spin ½" have two quantum or energy states that can be visualized as having spin axis pointing up or down. in the absence of a external magnetic field, the two states have the same energy level and at thermal equilibrium exactly one half of the nuclei will be in the upstate and half will be in the down state. In the presence of a magnetic field, the up state is aligned with the magnetic field and the down state is opposed to it. The energy difference between the two states id proportional to the strength of the magnetic field applied. In large population of nuclei, slightly more than half reside in the up sate slightly less than half reside in the down state. Nuclei in the lower energy state can absorb energy and be promoted to the higher energy state. The energy of the photon absorbed must be equal to the energy difference between the two states of and must correspond to a specific frequency of the electromagnetic radiation.

$$\Delta E = h\nu o = h\gamma Bo/2\pi$$

Where h is plank's constant and νo is the resonant frequency or lamor's frequency

2.4.16.2 Useful Nuclei for NMR

The resonant frequencies of some important nuclei and their sensitivity are shown below for the magnetic field strength of a typical NMR spectrometer (Varian Gemini-200):

Table 2-3 Useful Nuclei for NMR

NUCLEUS	ABUNDANCE (%)	SENSITIVITY	FREQUENCY (MHZ)
H	100	1.0	200
C	1.1	0.016	50
N	0.37	0.001	20
F	100	0.83	188
P	100	0.066	81
Fe	2.2	3.4×10^{-5}	6.5

Each nucleus has its characteristic frequency it resonates or comes to resonance hence the NMR spectrophotometer receives radio frequency signal which tunes in each nucleus at its resonant frequency. Since the resonant frequency is proportional to the external magnetic field, all the resonant frequency will be increased by the same factor with a stronger magnetic field. The relative sensitivity is a due to magnetic field strength. It is therefore obvious that sensitivity is reduced for those nuclei with low natural abundance. ^{13}C at natural abundance is 5700 times less sensitive (1/(0.011 × 0.016)) than ^{1}H due to factors explained above.

2.4.16.3 Chemical Shift

The resonant frequency of a nucleus is dependant on the position of the atom in the (chemical environment) and the type of nucleus. The bonding electrons creates their own small magnetic field which modifies the external magnetic field reaching the nucleus. The subtle variation measured in parts in a million is called the chemical shift which provides in depth information about the structure of the molecules. Different atoms in the molecules can be identified by their chemical shift. Chemical shift is affected by nearby electronegative atoms, unsaturated groups and molecular symmetry. Chemical shift is measured in parts per millon and is represented by the Greek letter delta (δ).

The spectrum is made up of resonant frequencies centred on fundamental resonant frequency of a nucleus of interest. Each peak represents a chemical environment within the molecule being studied (**Jacobson, 2007**).

2.4.16.4 Spin-Spin Spliting

The phenomenon of spin-spin splitting is very essential in structrure elucidation. Consider two protons (H_xC-CH_a) on two adjacent carbon atoms with different chemical shift. The magnetic field of the nucleus of Ha can align with the magnetic of H_x or oppose the magnetic field of H_x. This perturbs the external magnetic field experienced by H_x either by adding a small amout or subtracting from it. Since the resonant frequency depends on the magnetic field reaching the nucleus, it resonates twice to give two peaks of equal intensity (a doublet) with separation of J hz known as the coupling constant. This effect is transmitted through the bonds and occurs when the two nuclei are close. The splitting becomes more complicated when there are

more than one neighbouring proton. As realized from the above example, the number of peaks observed is one more than the number of neighbouring protons during the coupling. Two neighbouring protons will give a triplet in the ration 1:2:1 and three neighbouring protons will give quartet in the ratio 1:3:3:1. This lead to the general rule (n+1) with the intensity determined by the Pascal's triangle.

2.4.16.5 Spin Spin Decoupling

Spin Spin coupling is usually renders spectrum complex and difficult to intepreteth ere the technique of spin spin decoupling is used to reduce the amount of fine structure and clarify the spectrum but enabling interacting protons to be identified. During NMR experiment, sample is usually subjected to a single radiofrequency. If a second radio frequency is applied in a specific manner then a nuclear magnetic double resonance results. A simple AX system can be used to explain the spin spin decoupling. When a nucleus (H_x) adjacent to H_A is irradiated with a second radiofrequency signal of frequency (v_x) equal to the precessional frequency of H_x, then H_x will undergo a rapid changes of spin state. The nucleus H_A only "sees" one averaged spin state of H_x therefore resonance appears not as a doublet but as a sharp singlet. The irradiation of H_x effectively decouples the signal from Ha. This technique helps to simplify NMR spectrum (**Beckett and Stenlake, 1988**)

2.4.16.6 NMR Hardware

The instrument used in nmr spectroscopy consist a magnet which is made up solenoid (closed loop) of superconducting Nb/Ti alloy wire immersed in liquid helium for which current is passed through the loop to create a strong magnetic field, radio transmitter, a probe which is made up of coil of wire around that serves to transmit

and receive the radio frequency signals, radio receiver, an analog-to-digital converter (ADC), and computer which serves to transmit a high power and short duration pulse of radio frequency to the probe coil. A weak signal is received by the probe which is amplified and converted to an audio frequency signal sampled by the ADC at regular intervals to generate a digital FID signal. The digital information is processed by the computer supplied by ADC to give an NMR spectrum displayed on the computer monitor and plotted on a digital paper (**Jacobson, 2007**)

2.4.17 MASS SPECTROSCOPY

2.4.17.1 Introduction

Mass spectroscopy is highly sensitive and important analytical tool which gained recognition in 1960. Its popularity in analytical chemistry can be attributed to two features mainly its possibility to determine the molecular mass and the elemental composition of the compound. Also the fragmentation pattern helps to make deductions about the structure of the compound. These two have been essential in the development and application of mass spectroscopy.

A limitation to its use lies in the polarity of the substance since it is inversely proportional to its volatility. Also, the larger the relative molecular mass, the greater the number of functional groups hence the danger of thermal decomposition

An enormous amount of information can be gathered from a mass spectrum. Fast and dependable spectrometers have been developed which helps in the determination of the empirical formula of fragment ions. Additional equipment have been developed which can measure he metastable ions or record collision activation spectra.

Results from isotopically labeled derivatives have contributed to successful interpretation of the spectra (**Hesse et al, 2008**).

2.4.17.2 Principle of Mass Spectroscopy

The sample is first vapourized, ionized and then accelerated with the positively charged particles in the gas phase separated according to their mass-to-charge ratio their mass under the influence of a magnetic field in the magnetic analyzer and usually detected electrically or in some cases photographically. The signals of the m/z versus relative abundance of each ionic species present are recorded (**Olaniyi, 2010**).

2.4.17.3 INSTRUMENTATION

There are four major components of the mass spectrometer; the sample inlet system, ion source, an analyzer and detection scheme.

Sample inlet system allows vaporization of the sample to be presented to the ion source while the high vacuum pressure is maintained.

Ion source is used to ionize the compound and retain the ions for fragmentation.

Analyzer separates the ions into their characteristic mass components based on their mass-to-charge ratios.

Detection scheme detects the ions and records their relative abundances of the resolved ions on a chart recorder or in a computer to yield a spectrum (**Olaniyi, 2010**).

2.4.17.4 Spectra Presentation

The mass-to-charge ratio (m/z) is plotted against the percentage relative abundance of the ions. The highest peak is assigned 100% and the other signals are scaled accordingly as fractions of the 100%.

MOLECULAR ION

The ion is the peak with the greatest m/z ratio and it is formed by the loss of an electron from the neutral molecule. It is also known as the parent peak

METASTABLE ION

Fragmentation results in the generation of an ion and a neutral fragment and this occurs in the ion source before the positive charged ions are accelerated which results in distinct peaks for each fragment ion. If the M+1 further breaks down slowly during acceleration into M+2, the M+2 usually appears at a low m/z than the M+ and this peak is known as the metastable ion (M*). It is useful in the establishment of the relationship between the parent ion and daughter ion

BASE PEAK

The most intense peak is known as the base peak and it is used to normalize the intensities (**Olaniyi 2010**).

2.4.17.5 Fragmentation Reaction of Organic Compounds

The most frequently observed fragmentation reaction in organic compounds have been summarized below.

α-Cleavage

α-bonds adjacent to heteroatoms such as N,O,S can be cleaved because charge that develops can be stabilized by the heteroatom. α-cleavage can occur once in a

decomposition reaction this is due to the high energy required by the cation generated through a homolytic cleavage.

Retro Diels-Alder Reaction (RDA reaction)

This type of reaction usually generates the fragments, ene and the diene components. Usually a double bond in a six membered cyclic system fragments into these fragments through a concerted decyclisation reaction. The charge carrier is the diene component but the ene components is a times observed in the spectra. The RDA can occur in the molecular ion as well as the fragment ions which contains a double bond. RDA is helpful in structure elucidation of many organic natural compounds including indole alkaloids.

McLafferty Rearrangment

This reaction is also known as a β-cleavage with an H-atom migration. In this reaction, a H-atom is migrated through a six-membered transition state from the γ-postion to another atom which must be at least double bonded. There is also a simultaneous migration of the double with the formation of a neutral fragment containing the β and γ-position atoms. Groups that can undergo McLaffferty rearrangement are C=O (eg. carboxylic acids, ketones, aldehydes, amides, lactams, esters, lactones), C=N (eg. azomethines or schiffs bases, hydrazones, oximes, semicarbazones), S=O (eg. sulphonic acids, esters) and C=C (eg. alkyl arenes, alkyl heterocycles, benzyl ethers, olefins) (**Hesse et al, 2008**).

2.4.18 INFRA RED SPECTROSCOPY

2.4.18.1 INTRODUCTION

Molecules undergo vibrational transitions when they absorb infrared waves in the electromagnetic spectrum. These vibrational spectra give information about the functional groups in the molecules.

The vibrational levels of molecules are separated by energies in the infrared (IR) region of the electromagnetic spectrum. The wave number ranges from 13000 to 10cm-1. Bonds in molecules have specific spatial arrangement and energy. The bonds are not rigid but distorted , bent or stretched when supplied with energy. For a molecule to absorb infrared radiations;

- The dipole moments must change during the vibration

It is worth noting that as the molecule becomes more complex, the number of vibrations increase.

Some IR absorptions correspond to combinations of vibrations giving rise to lower than expected number of vibrations and also overtones.

The IR spectrum can be divided into regions. There are 3 main regions for the functional groups which are highly characteristic

- Stretching vibration between hydrogen and a heavier atom, H-X. The H atom has a low mass compared to the -X- and a strong connecting bond exists between. These account for the HX stretching vibrations which almost all occur between 2000-4000 cm-1. The atom attached to the H atom determines the exact frequency. Hydrogen bonding for example with electronegative atom being O or N makes the OH bond weak and more variable. Free OH stretch

will give a sharp absorption at 3600cm -1 and liquid –OH will give a broad band close 3200 cm-1.

- Double bond, triple bonds and aromatic systems have high bond energies which are less susceptible than single bonds to the structure they are attached to. The double bond region is usually observed between 1900 cm-1-1600cm-1. It is this region that -C=C-, C≡N and C=O are observed. C=O especially gives a sharp band at 1700cm-1.

- Bending vibrations of organic molecules give characteristic group frequencies. Bending vibrations occur at lower frequencies or wave number. This is the region below 1500 and is known as the fingerprint region. The fingerprint region helps to identify a substance by comparing with a pure sample (**Kealey et al, 2005**).

2.4.18.2 INSTRUMENTATION

There are two basic instruments in use thus, the traditional grafting or prism (scanning) instruments and the more powerful fourier transform (FT)- IR spectrometer. Both work on the same principle

An IR source emits the radiation whose strength is reduced as it passes through the sample. The reduction is frequency dependant which corresponds to the excited molecular vibration. The residual radiation is measured with a detector and electronically converted into a spectrum. The detector plays the role of collecting incoming radiation and then converts the optical signal into electrical signals

CLASSICAL (SCANNING) IR SPECTROMETERS

The double beam principle is employed here thus the use of a beam splitter (chopper) which splits the continuous radiation from the source into two beams of the same intensity. One beam passes through the sample, the other serves as a control beam which usually passes through the air in the case of solution measured in a cell with pure solvent. After optical comparison in the photometer, the light beams are combined. The monochromator (a prism or diffraction grating) spectrally analysis the resultant the resultant radiation. The spectrum is recorded as a function of the wavelength. A single frequency is recorded at any point in time. The signals are plotted on a chart recorder after amplification as a spectrum (abscissa: wavelength increasing from left, ordinate: % transmittance)

FOURIER TRANSFORM IR SPECTROMETERS

The fourier transform technique is an advancement of IR spectroscopy using the modern computer technology for the storage and the processing of large amounts of data. It is currently the standard method and supersedes the conventional spectrometers.

It offers the following advantages;

1. Time saving
2. Better signal to noise ratio compared to the conventional method where a single wavelength is recorded at a time
3. High wavenumber precision

Its basic principle is the simultaneous collection of data at all frequencies in the IR spectrum. It is time saving compared to the convention spectrometer where different frequencies have to be scanned. This is achieved by using an interferometer to convert the intense, multifrequency IR radiation into an interferogram, which is a function of time and not frequency. This radiation is passed through the sample and the interferogram is converted back to the spectrum

This method requires a totally different design of spectrometer (**Hesse et al, 2008**).

Chapter 3

MATERIALS AND METHODS

3.1 REAGENTS

Glacial acetic acid

Chloroform

Ammonium hydroxide

Dragendorff solution A and B

Ethylacetate

Petroleum ether

Acetone

Methanol

Cyclohexane

Kieselguhr silica gel

Silica 230/70

3.2 EQUIPMENTS

Buchi Rotavapour

Buchi recirculation chiller

Water bath

Oven

Glass column

Degasa spreader

Precoated TLC plate

Chromatank

Sonicator (Fisher scientific)

Glass funnel

5 litres separating funnel

Phenomenex Kromasil 5µm C_8 (250 × 4.60mm) column

Measuring cylinder

HPLC Chromatograph:

- LC 20AB Binary Pump-Schimadzu
- SPD-20AB UV Detector
- DGU-20A3 Degasser
- LC Solutions Software

Cecil CE 2041 single beam UV-VIS spectrophotometer

Analytical balance (Adams insruments)

3.3 METHODOLOGY

3.3.1 Extraction of crude alkaloid

2.5kg of the powdered seeds was percolated in 2% acetic acid in water solution at room temperature for 48 hours with frequent agitation. The aqueous extract was then decanted and filtered using a plug cotton and funnel. The filtrate was then basified with ammonium hydoxide to a pH 9 – 10 which afforded the observation of brownish precipitates. The aqueous phase was then extracted with repeatedly with 100ml portions of chloroform to obtain a yellow chloroformic extract. The chloroformic extracts were combined dried over anhydrous sodium sulphate. The above procedure was repeated several times to exhaust the powdered material of the alkaloids. The chloroformic extract was concentrated using the rota vapour and dried in a clean petri dish to yield **7.5014g** of deep brownish crude total alkaloidal extract

The extract was then screened for the presence of alkaloids. The Dragendorffs test was performed to detect the presence of alkaloids

3.3.2 TEST FOR THE PRESENCE OF ALKALOIDS

A portion of the chloroformic extract was vapourized on a petri dish and about 2ml 0f concentrated HCl added. 1ml of dragendorffs reagent was added to obtain a brick red precipitates which indicated the presence of alkaloids.

3.4 ISOLATION OF BIOMARKER

3.4.1 COLUMN CHROMATOGRAPHY

7.0g of the crude alkaloid was weighed and dissolved in 50 ml of chloroform. It was then mixed with a portion of column chromatography silica gel (mesh size 70/230), stirred continuously to dryness and formation of a uniform powder. A long glass

column with a tap at the end was plugged with cotton wool and filled with silica to a height of about half the column length. The side of the column was tampered to allow packing of the stationary phase and to avoid air bubbles. The dried material was packed into the column and side of the column tampered to allow packing of the material. A wad of cotton was placed on top of the dried material

A gradient elution was carried using the following mobile phases sequentially: 100% petroleum ether (100ml) and a colourless fraction was eluted. 5% ethyl acetate in petroleum ether was added to the column 30ml× 2 colourless fractions were collected into clean bottles. A deep blue fraction was collected 30ml×3 and light blue fraction was collected 30ml×2. The mobile phase was changed to 15% ethyl acetate and 30ml×2 colourless were collected. 30% ethyl acetate was then run through the stationary phase to afford the elution of very light yellow fraction (30ml), 30ml×2 yellowish fraction and a 30ml 0f light yellowish fraction. 40% ethyl acetate eluted a light greenish fraction (30ml×2), greenish fraction (30ml). 50% ethyl acetate in pet ether afforded the elution of light yellowish fraction (30ml×2), yellowish fraction (30ml×7) and greenish fraction (30ml×2). 45% ethyl acetate in pet ether eluted light yellowish fracttions (30ml×2) and greenish fractions (30ml×5). The polarity of the mobile phase was decreased to 40% ethyl acetate in pet ether and yellowish fractions (30ml×6) were collected. A total of 45 fractions were collected.

Using the chloroform and ethyl acetate (5:1) as the reference mobile phase, the elution of compounds was monitored using precoated TLC plates. Similar fractions in base on the TLC profile were bulked and labeled from F_1 to F_{13}. Bulked fractions F_3 to F_9 were suspected to contain vaocangine based on the R_f values obtained from them. These

fractions were tested for alkaloids as described above and each gave positive results for the presence of alkaloid.

3.4.2 PREPARATION OF TLC PLATES FOR PURIFICATION OF FRACTIONS

3.4.2.1 Preparation of Slurry

Ratio of silica gel to water; 1:2

The required amount of silica gel was weighed and the corresponding volume of water added. It was mixed well in mortar. Silica gel was added and mixed well to the required texture and consistency. The slurry produced was transferred to a spreader and was applied evenly over the surface of the plates in one direction. The applied slurry was allowed to set and air dried for 1 hour. The plates were activated in an oven over 1 hour at a temperature of 105 C. The plates were ready to be used after 1 hour. They were allowed to cool and the fractions were spotted in a continuous stream using clean disposable capillary tubes. The spotted plates were allowed to dry and ready for development.

Chromatographic tank of size 22cm×22cm×10cm was filled with 130ml of mobile phase. The lid was greased and replaced to make it air tight. The chromatographic tank was left on the bench for 1 hour to saturate with the solvent. The plates were developed in the saturated tanks by allowing the spots to travel a distance of about 16cm. The plates were removed and the solvent was allowed to dry off. The plates were then developed under the UV viewing system.

Mobile Phase: Chloroform: Ethyl acetate: Methanol: 10: 2: 1

The mobile above was used to purify fraction F_7 to F_9

The mobile phase was changed to Chloroform: Ethyl acetate; 5: 1

This mobile phase was used to purify fraction F_3 to F_6

The plates were viewed under 254nm and 366nm and the appropriate band on TLC plates suspected to be voacangine marked out and scraped in clean flask. The scraped silica gel was washed repeatedly with ethyl acetate to extract the bio marker and the filtrate poured into a petri dish. The petri dish was allowed to dry leaving behind a yellowish brown amorphous solid which was suspected to be a pure marker.

The isolate was dissolved in chloroform and spotted on a precoated TLC plate in the mobile phase Chloroform: Ethylacetate (5:1). A single spot was observed under 254nm with $R_f = 0.78$. Faint bands were observed below the spot of interest indicating that isolated solid was impure. The dissolved solid was spotted in chloroform (100%) and a major band which represented the biomarker was observed with faint bands above the bio marker. The dissolved solid was finally spotted in a 3rd mobile phase Cyclohexane: Ethylacetate (4:1) and a single visible spot was observed with an $R_f = 0.2631$. The faint bands were observed above the bio marker in the mobile phase above.

3.4.2.2 Purification of Biomarker

The biomarker was further purified by washing it repeatedly with methanol to ensure the possible removal of all the trace compounds not desired. The insoluble solid left on the filter paper was dissolved with acetone into a clean petri dish. The acetonic solution was spotted in the mobile phase Cyclohexane: Ethyl acetate (4:1) and only a single spot with $R_f = 0.23$ was observed. The acetone was driven off in open air to obtain an almost white amorphous solid which was transferred into a sample tube.

3.4.3 SPECTROSCOPIC ANALYSIS OF THE ISOLATED BIOMARKER USING THE SINGLE BEAM SPECTROPHOTOMER

A well calibrated UV spectrophotometer was employed to determine the major wavelengths of maximum absorptivity of the isolated biomarker.

Serial dilutions of the biomarker in methanol were made and run over 200-500nm on the uv spectrophotometer using methanol as the reference solution and the wavelength of maximum absorptivities obtained were; 202nm, 226.5nm and 287.5nm.

3.4.4 HPLC ANALYSIS

3.4.4.1 Sample Preparation

0.0035g of pure biomarker was weighed accurately and dissolved in about 10ml of ethyl acetate with aid of the ultra sound sonicator. It was quantitatively transferred into a 25mls volumetric flask and diluted with ethyl acetate to the mark. The stopper was replaced and labeled appropriately (0.014%). The stock solution was kept at a temperature of -18°C in a freezer.

3.4.4.2 Method Development

1ml stock solution was pipetted into a 10ml volumetric flask and diluted with distilled methanol for HPLC analysis and development. An HPLC chromatograh with SPD-20AB UV Detector was used for the method development.Column used was Phenomenex Kromasil 5µm C_8 (250 × 4.60mm) and particle size 5µ. An isocratic elution was carried out and the following mobile phase compositions were used for the method development to select appropriate mobile phase which gave the best resolution: methanol: water (80:20), methanol: water (90:10) and methanol: water (80:20) (2.0% acetic acid) were used for the elution of the marker at a flow rate of 0.5ml/min. A run

time of 30 minutes was allowed for each injection. 20µl of diluted solution of biomarker and was injected and monitored at wavelength of 257nm at ambient temperature. The chromatograms obtained were noted and the mobile phase methanol: water (90:10) was found to be appropriate for elution of the the biomarker. The retention time was recorded and the peak area estimated was used as a measure of the concentration.

3.4.4.3 Calibration Curve Plot (Linearity)

Various concentrations were prepared from the stock solution 0.014% by pipetting 1ml, 0.8ml, 0.5ml, 0.25ml and 0.1ml to give concentrations 0.0014%, 0.00112%, 0.0007%, 0.00035% and 0.00014% respectively. Each solution pipetted was diluted to 10mls in a volumetric flask using methanol. Each solution was injected five times and the respective peak areas were recorded.

A blank injection was carried to determine the source of the extraneous peaks eluting between 5min to 7.5mins. In the preparation of the blank solution, 1ml of ethyl acetate was pipetted into a 10 ml volumetric flask and diluted with methanol to the mark. The blank solution was injected five times and the retention times of the extraneous peaks noted.

3.4.4.4 Robustness

3.4.4.4.1 Effect of Wavelenght Variation on Chromatogram of Isolated Voacangine

A concentration of 0.0014% was prepared and injected at three different wavelengths. The wavelengths of maximum absorptivity were changed to 254nm, 257nm, 255 nm and the peak areas of the biomarker were recorded. All other parameters remained constant.

3.4.4.4.2 Effect of Flow Rate Variation on the Retention Time of the Isolated biomarker

A concentration of 0.0014% was prepared and was injected at flow rates of 0.8ml and 1.0ml. The change in the retention times and the nature of the peaks were noted and recorded. The wavelength of maximum absorptivity was maintained at 257nm

3.4.4.5 Effect of Change in Column on the Retention Time of Pure biomarker

Using a C_{18} column, a concentration of 0.0014% of the biomarker was injected using mobile phase consisting of methanol: water (90:10) at a flow rate of 0.5ml/min. the elution was monitored at a wavelength of 257nm and a run time of 30 minutes was allowed. **No peak** was observed within **30 minutes**.

3.4.4.6 Precision (Intraday and Interday Precision)

50.0g of grounded seeds was weighed and soaked in 450ml of ethyl acetate for 48 hrs. It was filtered using a glass funnel and a wad of cotton. The filtrate obtained was then filtered again using a funnel and a filter paper to remove all the fine particles. The extract was concentrated with the help of a rota vapour. The concentrate was diluted to 200ml in a volumetric flask with methanol. A diluted solution was prepared by pipetting 10mls of the stock solution and diluting to 100mls. The varied concentrations were prepared by pipetting 3ml, 4ml and 5ml of the diluted solution and diluting to 10ml in a volumetric flask ensuring that their concentrations were in the range of the calibration curve plot. The solutions were injected at a flow rate of 0.8ml/min and wavelength of 257nm and the chromatograms obtained were noted. The procedure

above was repeated for three consecutive days and the concentration of the biomarker calculated using the equation of the calibration curve of the pure marker.

3.4.4.7 Extraction of Seeds Prior to Spiking and Determination of % Recovery

15.0g of grounded seeds were weighed and soaked in 100mls of ethyl acetate. The soaked seeds were constantly agitated for 2 hours and filtered using a glass funnel and a wad of cotton wool. The extract was further filtered with a whatman filter paper to remove all fine debris present.

0.5ml of 0.014% of pure marker was pipetted and diluted with methanol to 10ml in a volumetric flask. The solution was injected repeatedly at a flow rate of 0.8ml/min and the peak areas recorded appropriately.

1ml of extract was pipetted and diluted to 10ml in a volumetric flask. It was then injected repeatedly at a flow rate of 0.8ml/min and the peak areas obtained were recorded. 1ml of extract was pipetted and spiked with 0.5ml of 0.014% of pure voacangine solution. The mixture was further diluted to 10ml. The spiked solution was also injected repeatedly and the corresponding peak areas recorded

1.5ml of extract was also pipetted and diluted with methanol to 10ml. the solution was injected repeatedly. 1.5ml of extract was pipetted again and spiked with 0.5ml 0f 0.014% of pure biomarker solution. The mixture was diluted with methanol to 10mls and injected repeatedly.

Chapter 4

RESULTS AND CALCULATIONS

4.1 ELUTION PROFILE IN COLUMN CHROMATOGRAPHY

SOLVENT PETROLEUM ETHER: ETHYLACETATE P : E	ELUATE	BULK
100% P	Colourless (1)	1
95 : 5 P : E	Colourless (2 – 3) Deep blue (4 – 6) Light blue (7 – 8)	2 – 8
85 : 15 P : E	Colourless (9 – 10)	9 – 10
70 :30 P : E	Very light yellow (11) Yellowish (12 – 14) Light yellowish (15 – 16) Greenish yellow (17)	11 – 17
60 : 40 P : E	Light green (18 – 19)	18 – 20
50 : 50 P : E	Light yellow (21 – 22) Yellowish (23 – 29) Yellowish green (30 -31)	21 – 31
45 : 55 P : E	Light yellow (32 – 33) Greenish (34 – 38)	32 – 38
40 : 60 P : E	Yellowish (39 – 45)	39 – 45

4.1.1 SUMMARY OF % YIELD OF CRUDE ALKALOID AND PURE BIOMARKER

Weight of grounded seeds	Weight of total alkaloids obtained	% weight of total alkaloids obtained	Weight of voacangine obtained	% weight of voacangine in total alkaloid
2.5 kg	7.5013 g	0.3001%w/w	20.6mg	0.2746%w/w

4.2 RETARDATION FACTOR OF PURE ISOLATE

R_f = Distance travelled by spot

Distance travelled by the solvent front

Mobile Phase: Chloroform: Ethyl acetate (**5:1**)

Table 4-1 Retardation Factor in Mobile Phase: Chloroform: Ethyl acetate 5:1

Distance travelled by solvent front	Distance travelled by spot	Retardation factor
6.40	4.90	0.77
6.40	4.90	0.77
6.40	5.00	0.78
6.40	5.00	0.78
		Average = 0.78±0.006

Mobile Phase: Cyclohexane: Ethyl acetate (**4:1**)

Table 4-2 Retardation Factor in Mobile Phase: Cyclohexane: Ethyl acetate 4:1

Distance travelled by solvent front	Distance travelled by spot	Retardation factor
6.0	1.5	0.25
6.0	1.6	0.27
6.0	1.5	0.25
6.0	1.6	0.27
		Average = 0.26±0.012

4.3 HPLC ANALYSIS AND METHOD DEVELOPMENT

Conditions

HPLC column; Phenomenex Kromasil 5µm C_8 (250 × 4.60mm)

Flow rate; 0.500ml/min

Injection volume: 20µl

Wavelength; 257nm

Temperature; ambient

4.3.1 CANDIDATE MOBILE PHASES

MOBILE PHASE	NATURE OF PEAK	RETENTION(R_t) / min
Methanol: Water; 80:20	Sharp at the apex and broad at the base without tailing.	20.227±0.02515

Methanol: Water; 90:10	Sharp peak observed without tailing	9.2312±0.01746
Methanol: Water(2.0% acetic acid); 80:20	Broad peak without tailing	27.7825±0.4435

4.3.2 CALIBRATION CURVE PLOT (LINEARITY)

% CONCENTRATION OF ISOLATED BIOMARKER	PEAK AREA (n = 5)
0.0014	1,012,033
0.00112	804,087.3
0.00035	271,977.6
0.0007	518,748
0.00014	163,745

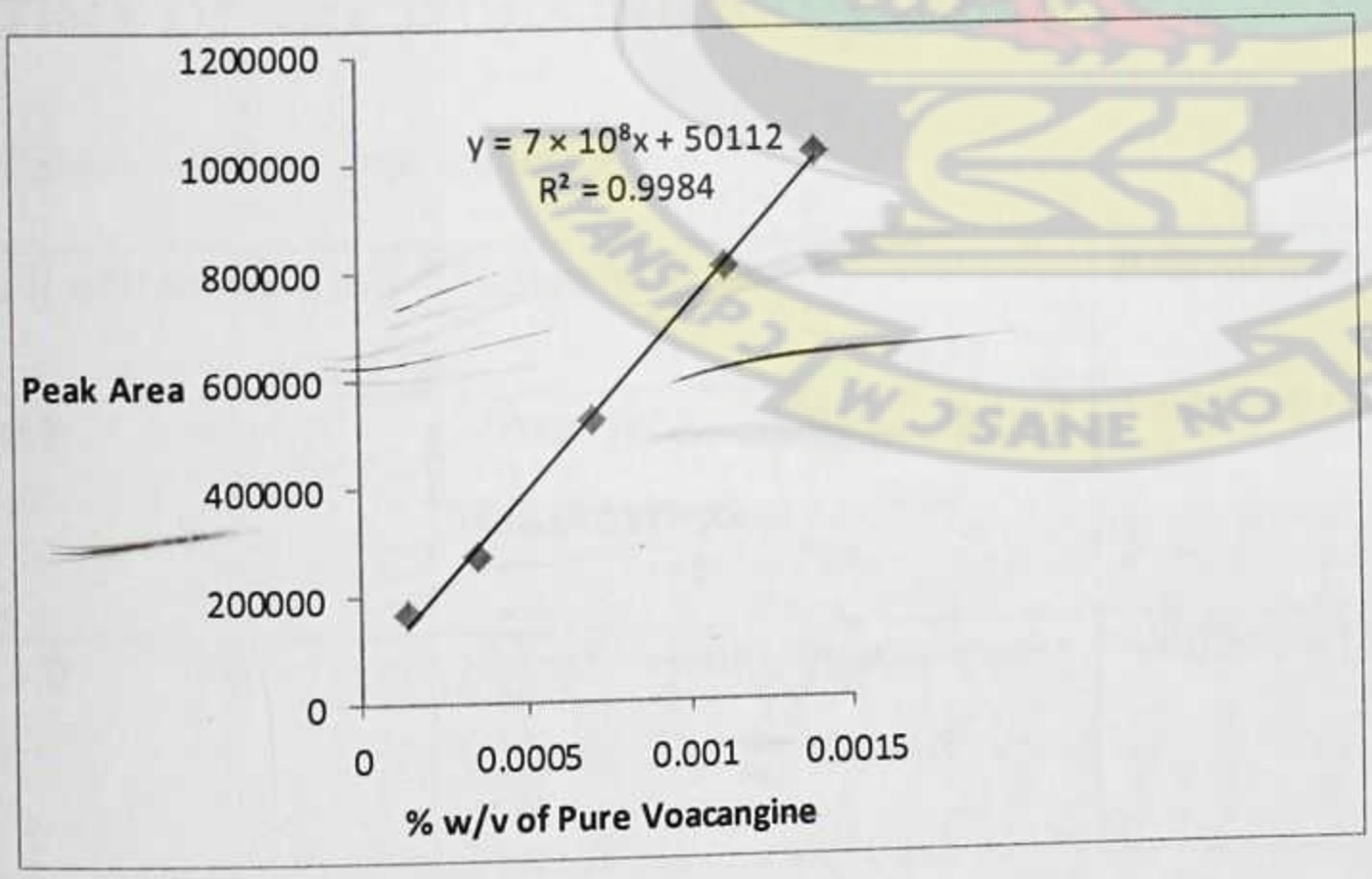

Figure 4-1 Calibration curve plot

Coefficient of regression, R^2 = **0.9984**, Range = **1.4µg/ml - 14µg/ml**

4.3.3 ROBUSTNESS

4.3.3.1 Effect of Wavelenght Variation On Chromatogram Of Isolated Biomarker

Table 4-3 Wavelenght variation

Wavelength of absorption	Nature of peak	Average Peak Area (n = 5)
255nm	Small broad peak observed without tailing	1,151,867
257nm	Long symmetrical sharp peaks without tailing observed	1,268,579
254nm	Long sharp peak observed without tailing	1,035,155

4.3.3.2 EFFECT OF FLOW RATE VARIATION ON THE RETENTION TIME OF THE ISOLATED BIOMARKER

Table 4-4 Flow rate variation

Flow Rate/ml/min	Nature of Peak	Retention time (Rt)/min
0.8	Sharp peaks without tailing was observed	5.9453±0.00611 (RSD = 0.027%)
1.0	Sharp symmetrical peaks without tailing obtained	4.669±0.0387 (RSD = 0.829%)

4.3.4 Determination of Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD = 3.3δ/s

Where δ = residual standard deviation of the regression line

S = slope of the calibration curve

The equation of the calibration curve is given as $\mathbf{y = 7 \times 10^8 x + 50112}$ where is y = peak area, x = concentration of biomarker (%w/v)

Slope = 7×10^8

Y – Intercept = 50112

Concentration of isolated biomarker (X% $^{w}/_{v}$)	Actual peak area (Y% $^{w}/_{v}$)	Estimated peak area (Y_{est}% $^{w}/_{v}$)	Y% - Y_{est}%
0.0014	1,012,033	1030112	-23134.4
0.00112	804,087.3	834112	-30024.7
0.0007	518,748	540112	-15633
0.00035	271,977.6	295112	-21364
0.00014	163,745	148112	15633
			Average = -14904.6
			Residual std, δ = 17826.57

LOD = $3.3 \times 17826.57/7 \times 10^8$

LOD = **0.00008403g/100ml or 0.8403µg/ml**

LIMIT OF QUANTIFICATION (LOQ)

LOQ = 10δ/s

LOQ = 10× 17826.57/7 × 10^8

LOQ = **0.000255g/100ml or 2.55µg/ml**

4.3.5 PRECISION

4.3.5.1 Intraday Precision (Day 1)

3ml of diluted extract pipetted

Peak Area (n = 5)	% w/w of biomarker in seeds in 50g of grounded seeds
151,827	0.01937
160634	0.02105
169410	0.02272
160775	0.02108
163341	0.02213
	Average = 0.0213% RSD = 6.01%

From the calibration curve, the equation of the line was obtained as **$y = 7\times10^8x + 50112$**

where "y" is the peak area and "x" is the concentration of biomarker in %w/v. **For the injection of solution prepared by pipetting 3ml of diluted extract and made up to 10ml, peak area obtained was 151,827.**

$151,827 = 7\times10^{8}x + 50112$

Hence $101,715 = 7\times10^{8}x$

$X = 0.0001453\%w/v$

But 0.0001453g is in 100ml of solution hence 10mls will contain 0.00001453g of biomarker

Since 3ml was pipetted and made up to 10ml, 0.00001453g is 3ml of solution

Weight of biomarker in 100ml = (100×0.00001453)/3 = 0.0004843g

0.0004843g should be in 10ml of stock that was pipetted to prepare 100ml above

Weight of biomarker in 200ml of stock solution = (0.0004843×200)/10 = 0.001969g

% weight of biomarker in 50g of gounded seeds = (0.001969×100%)/50 = **0.01937%w/w**

The same procedure above was used in the calculation of the % w/w content of the biomarker for the rest of the data.

4ml of extract

Peak Area (n = 5)	%w/w of biomarker in seeds in 50g of grounded seeds
264664	0.03065
260664	0.0307
250174	0.02858
251348	0.02875
262411	0.2901
	Average = 0.0295% RSD = 3.75%

5ml of diluted extract

Peak Area (n = 5)	% w/w of biomarker in seeds in 50g of grounded seeds
336203	0.03270
324707	0.03138
320918	0.03094
313852	0.03014
296664	0.02818
	Average = 0.0307% RSD =5.54 %

DAY 2

Volume of diluted extract	Average % w/w of biomarker in seeds in 50g of grounded seeds
3ml	0.02159% (RSD = 5.05%)
4ml	0.03120% (RSD = 1.16%)
5ml	0.03079% (RSD = 6.82%)

DAY 3

Volume of diluted extract	Average % w/w of biomarkerr in seeds in 50g of grounded seeds
3ml	0.02278 (RSD = 0.88%)
4ml	0.03135 (RSD = 3.5%)
5ml	0.03051 (RSD = 7.57%)

4.3.6 DETERMINATION OF % RECOVERY (SPIKING OF EXTRACT)

INJECTION OF 0.5ML 0F 0.014% PURE BIOMARKER (SPIKE)

INJECTION NUMBER	PEAK AREAS OBTAINED
1	498321
2	522042
3	502765
4	520768
5	511452
	AVERAGE = 511069.60 RSD = 2.066%

INJECTION OF 1ML OF EXTRACT AND SPIKED EXTRACT

INJECTION NUMBER	PEAK AREA BEFORE SPIKING	PEAK AREA AFTER SPIKING
1	128734	511142
2	111716	534038
3	107208	547793
4	108107	531004
5	122032	480112
	Average = 115559.4 RSD = 8.16%	Average = 520817.8 RSD = 5.04%

Amount Recovery = Average peak area after spiking – Average peak area before spiking

Amount Recovery = 520817.8 – 115559.4 = **405258**

% Recovery = $(A_{recov} / A_{std)} \times 100\%$

% Recovery = (405258 / 511069.6) × 100%

% Recovery =**79.29 %**

INJECTION OF 1.5 ML SOLUTIONS OF EXTRACT AND SPIKED EXTRACT

INJECTION NUMBER	PEAK AREA BEFORE SPIKING	PEAK AREA AFTER SPIKING
1	141003	493656
2	139983	663481
3	130324	545883
4	148432	523755
5	166411	553473
	Average = 145230.60 RSD = 9.28%	Average = 556049.60 RSD = 11.58%

Amount recovered = 556049.60 – 145230.60

Amount recovered = **410819**

% recovery = (410819 / 511069.6) ×100%

% recovery = **80.38%**

Chapter 5

DISCUSSION

UV spectrum of isolated biomarker

The biomarker showed λmax (methanol) **203.5nm**, **218.0nm**, **288nm** and **361.5nm**. Parent indole ring has been reported by Beckett to show λmax (cyclohexane) at 220nm, 280nm and 287nm. Indole alkaloids isolated from the leaves of voacanga foetida have shown λmax (chloroform) between 283nm-287nm being prominent as reported by Hadi et al. It is suspected that the biomarker contains an indole moiety or chromophore since it absorbs at λmax 288nm and 361nm. There is also the possibility of a substitution on the indole moiety. Substitutions on the parent indole ring can invariably cause a shift in the λmax. Voacanga Africana has been reported to contain a wide array of indole alkaloids.

Spectroscopic analysis of isolated biomarker

15.6mg of pure biomarker was sent to the Department of Pharmaceutical Chemistry, Agriculture and Mechanical University, Florida for spectral analysis including ^{1}HNMR, ^{13}CNMR, mass spectroscopy and IR spectrum. The ^{13}CNMR proved unsuccessful due to the low sample concentration. Only ^{1}HNMR was successful but data obtained was inadequate for complete structure elucidation.

NMR analysis of Biomarker

The ^{1}HNMR showed a singlet at 7.27 ppm and 8.20 ppm. The aromatic protons lies between 7-8 ppm hence signals for aromatic protons are observed in this region. The presence of an electron withdrawing group adjacent to aromatic protons at the ortho and para positions give signals between 8-9 ppm. It is likely that there is the presence of an electron withdrawing group attached to the aromatic ring. A quartet centered on signal of 1.257 ppm suggest a methyl group (- CH_3) which is likely to be attached to an

electronegative atom since methyl groups usually give signals around 0.85 ppm when attached to an sp^3 hybridized carbon far away from an electronegative atom.

The ^{13}CNMR was unsuccessful but signals were obtained at 129.7 ppm and 133.72 ppm which suggest an electron withdrawing group attached to the aromatic ring which shifts the carbons at the ortho and para position downfield. Electron withdrawing groups have been reported to shift ortho or para carbons by resonance to 130-140 ppm. This further supports the evidence of an electron withdrawing group as was observed in the ^{1}HNMR.

Mass Spectrospy of Biomarker

The mass spectroscopy data gave a base peak at 390.90 m/z which corresponds to 100% relative intensity and the highest m/z value obtained was 675.29. The data obtained was insufficient for complete structure elucidation

IR Spectrum of Biomarker

The IR spectrum showed significant bands between 650 cm^{-1} and 1253 cm^{-1}. A broad band medium band was observed at 1712.20 cm^{-1}.

The broad medium band obtained at 1712.20 cm-1 is due to - C=O stretch vibration which suggest that an ester group may be present. The broad stretching vibration occurring at 1250.23 cm-1 suggests a -C-O- linkage which further supports the evidence of an ester functional group.

Bands obtained at 1170 cm^{-1}, 1133.43 cm^{-1}, 1096.03 cm^{-1}, 1086.81cm^{-1}, 1063.77 cm^{-1}, 1034.75 cm^{-1} and 1045.16 cm^{-1} are due to C-H in plane bending or deformation vibrations in the fingerprint region.

Substitutions on the aromatic ring can be determined by the fingerprint region. Two strong bands were obtained at 669.69 $cm^{-1}$and 770.20 cm^{-1} which suggests a

monosubstitution on the aromatic ring. These bands are due to out of plane deformation vibrations (**Hesse et al, 2008**)

Retardation Factor

The purity of the biomarker was ascertained using thin layer chromatography (TLC). Two mobile phase systems were used to determine the purity of the isolate. The chromatograms showed single spots both under 254nm and 366nm. This confirmed the purity of the isolated biomarker .Chloroform: ethyl acetate (5:1) gave an R_f = **0.79** and Cyclohexane: ethyl acetate (4:1) gave an R_f = **0.26** (Achienbach et al, 1997). Factors such as humidity, distance of migration etc can affect retardation factor.

HPLC Method Development and Validation

Different mobile phase systems were investigated to for the isocratic elution of the biomarker. Methanol and water being readily available and relatively cheap was used in the trial phase. Methanol: water **(80:20)** at flow rate of 0.5ml/min gave a good chromatogram with retention time at approximately **20.0min** which was rejected due to cost benefit analysis. The proportion of the water in the former was replaced with 2.0% acetic acid and injected at the **0.5ml/min** to cause ionization of the alkaloid which is expected to result in a significant reduction in the retention time. A good chromatogram was obtained but with a retention time at approximately **28.0min** was obtained which was also rejected. As a rule of thumb, a 10% change in the organic phase should cause a three fold change in the retention time. The mobile phase was changed to **90:10** which gave a sharp symmetrical peak at approximately **10.0 min** at a flow rate of **0.5ml/min**.

The mobile phase methanol: water (**90:10 v/v**) was used to elute the biomarker to obtain a sharp symmetrical peak. The average retention times obtained were **9.2312±0.0175 min, 5.9452±0.0064 min, and 4.666±0.0387 min** at flow rates of **0.5ml/min, 0.8ml/min** and **1.0ml/min** respectively. The peaks were sharp at these retention times.

Minor peaks observed before the elution of the biomarker were due to the solvents used in the preparation of biomarker prior to injection and this was confirmed by injection of a blank solution. This further proved the purity of the isolate. A good resolution with a sharp symmetrical peak which was unperturbed by the matrix was obtained from the injection of the extract and the retention time was confirmed by the injection of a spiked solution of extract. This confirms the specificity and selectivity of the proposed method.

Spiking and % Recovery

The extract was spiked with 0.5ml of 0.014% of pure marker to determine the reproducibility of the method, the effect of the matrix on the peak of interest and also to confirm the retention time. The average percentage recoveries at two different concentration levels of the extract gave **79.29%** and **80.38%.** The high percentage recoveries indicate that other components in the extract have insignificant effects on the concentration of the biomarker. It is also a confirmation of the trueness of the peak.

Linearity and Range

A good linearity was observed with a coefficient of regression of **0.9984** over five calibration points. An R^2 greater than 0.998 is acceptable as stated by ICH guidelines for which the calibration plot satisfies this specification. The calibration plot was linear over the concentration range of **1.4µg/ml - 14µg/ml** with the **LOD** (S/N = 3) and **LOQ** (S/N = 10) of **0.84µg/ml** and **2.55µg/ml** respectively.

Robustness

The proposed method was found to be robust when the flow rate and wavelength were varied. The peaks obtained were unaffected with a high precision (RSD < 1.0%) when the above parameters were varied. It can therefore be inferred that the method is rugged.

Precision

Intra-day Precision (Repeatability)

Method precision is parameter which is critical for method development as stated by the ICH guidelines. For the volumes of 3ml, 4ml and 5ml of dilute extract pipetted for the intra-day assay, the precision was less than 6%. ICH guidelines stipulate a precision of ≤ 2% for an intra- day precision. The proposed method deviated slightly from the acceptable precision due to the complexity of the matrix injected which affects precision. The method is still useful for quantification of the biomarker. The employment of and HPLC system integrated with an auto sample would reduce the random error associated with precision.

Inter-day Precision (Reproducibility)

The ICH guidelines stipulate a precision of ≤ 2 for replicate determinations carried out over a period of three consecutive days. High precisions were achieved for all the determinations with the exception of 3ml and 5ml on the 2nd day which gave precisions of 5.05% and 6.82% respectively and 5ml dilution on the 3rd day which gave the lowest precision of 7.57% .The highest precision calculated during the replicate determinations was 0.88%. The diluted extract injected contains a wide array of components which possibly explains the slight random error observed in the method precision. The method can still be said to be precise.

Percentage Content of Biomarker in the Crude Extract Obtained from the Whole Seeds of Voacanga Africana Using the Isolated Pure Marker as a Secondary Reference Standard

Little data exist on the percentage content of the individual alkaloids concentrated in various parts of the plant. A calibration curve for the pure biomarker over 5 concentration levels was plotted and this was used as secondary standard to quantify the pure biomarker in the seeds. The average percentage content of the biomarker obtained

was **0.02775±0.00444% %w/w (n=9).** The seeds have been reported to contain 1.5-3.5% of total alkaloids with tabersonine being the major alkaloid in the seeds. This implies that every 100g of seeds contains between 1.5-3.5g of total alkaloids. Therefore, every 100g of seeds averagely contains 0.02775g of the biomarker. The concentration is subject to variation due to environmental conditions and stage of harvesting of the seeds.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSION

A pure biomarker was successfully isolated from the seeds of voacanga africana by column chromatography and preparative TLC. The isolate showed a single spot on analytical TLC plates in mobile phases chloroform: ethyl acetate (5:1) and cyclohexane: ethyl acetate (4:1) with Rf of 0.79 and 0.32 respectively. The isolate was subjected to spectroscopic analysis for which only ^{1}HNMR and IR spectroscopy were successful. All other spectroscopic methods proved futile. Complete structural elucidation was unsuccessful.

A reverse phase isocratic HPLC-UV method was developed for the quantification of the biomarker in the whole seeds using a Phenomenex Kromasil 5µm C_8 column of dimensions 250 × 4.60mm, mobile phase was methanol/water (**90:10 v/v**), injection volume was **20µl**, flow rate of **0.8ml/min** and temperature was **ambient**. A calibration curve of the pure marker over five concentration levels was plotted and detection was at a wavelength of 257nm. The calibration curve plotted was linear over the concentration range **1.4µg/ml - 14µg/ml** with a coefficient of regression, R^2 = **0.9984.** The **LOD** and **LOQ** were determined to be **0.84µg/ml** and **2.55µg/ml** respectively. The developed method was well validated and was found to be specific, selective, precise and robust. The method is therefore suitable for the quality control of the seeds of voacanga aAfricana.

The whole seeds were quantified to determine the amount of the biomarker. The average concentration of the biomarker in the whole seeds was determined to be **0.02775±0.00444% %w/w.** The average percentage recoveries were found to be **79.29%** and **80.38%** at two different concentration levels.

RECOMMENDATION

- Complete structure elucidation since the protocol for the isolation and purification has been established.

- Stability of the biomarker to variation in temperature and storage conditions must be investigated to help establish the appropriate temperature and conditions suitable for the storage of the seeds. This will further inform buyers on the appropriate temperature to employ during pre-treatment of the whole seeds prior to storage.

- Seasonal quantification of the amount of the biomarker in the voacanga seeds to establish the suitable period of harvesting where the amount of the biomarker is at its highest levels.

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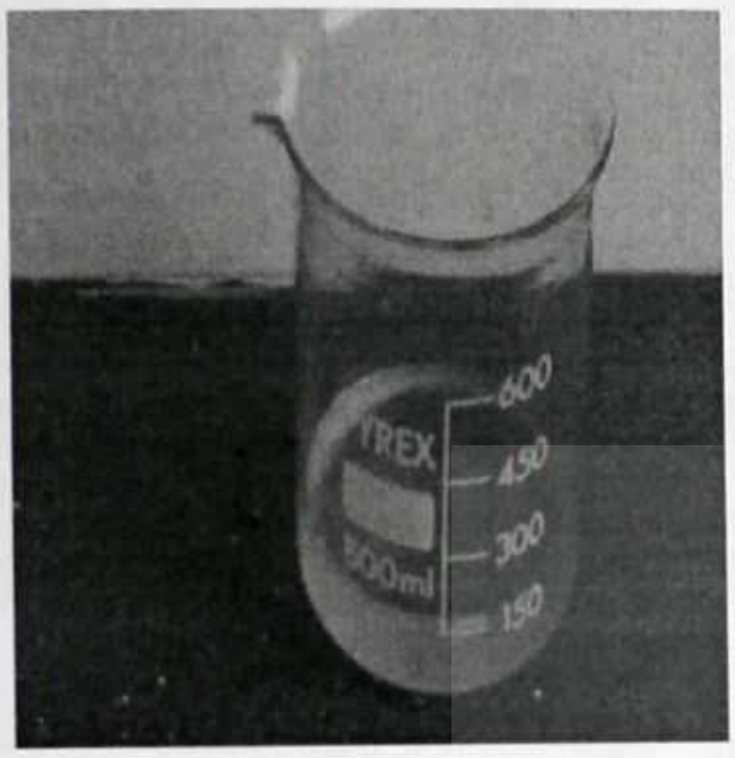
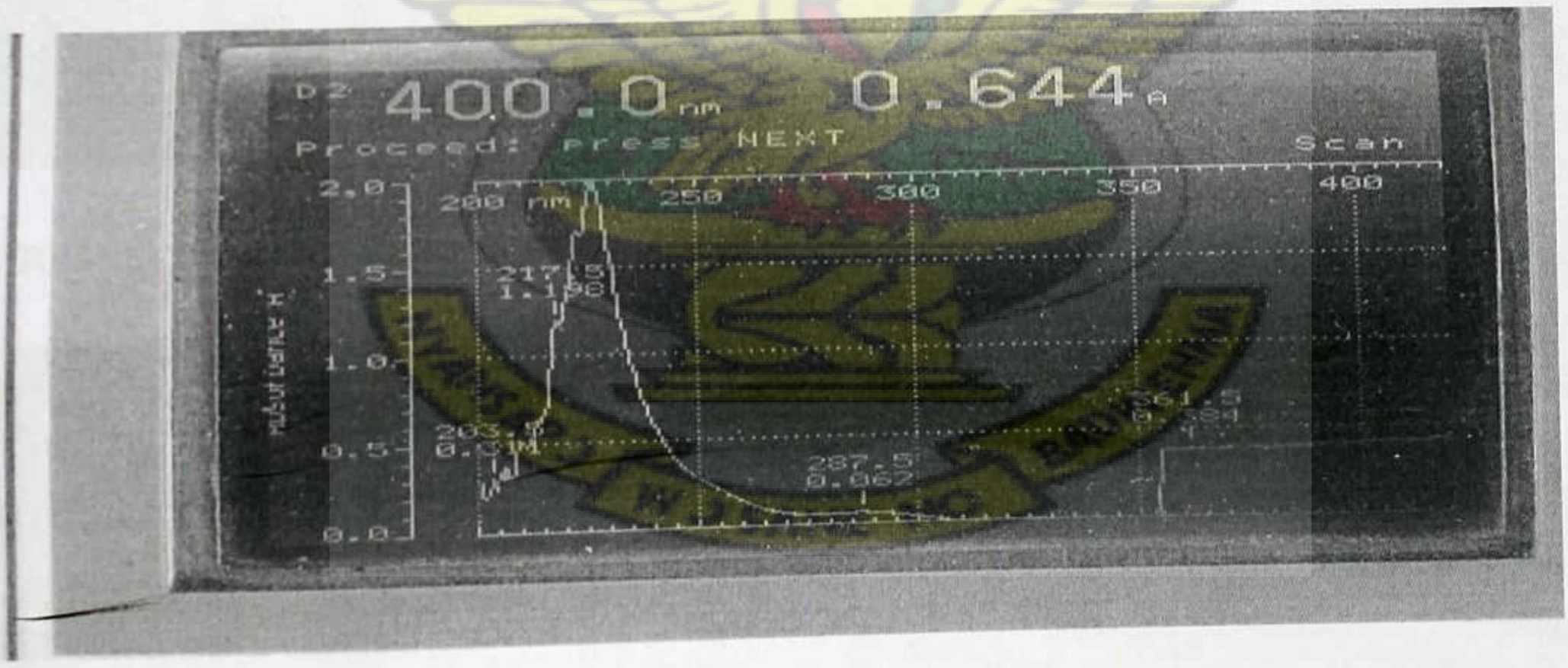
APPENDIX



Chloroformic extract of crude alkaloid

Typical fraction from column chromatography

UV ANALYSIS OF PURE BIOMARKER

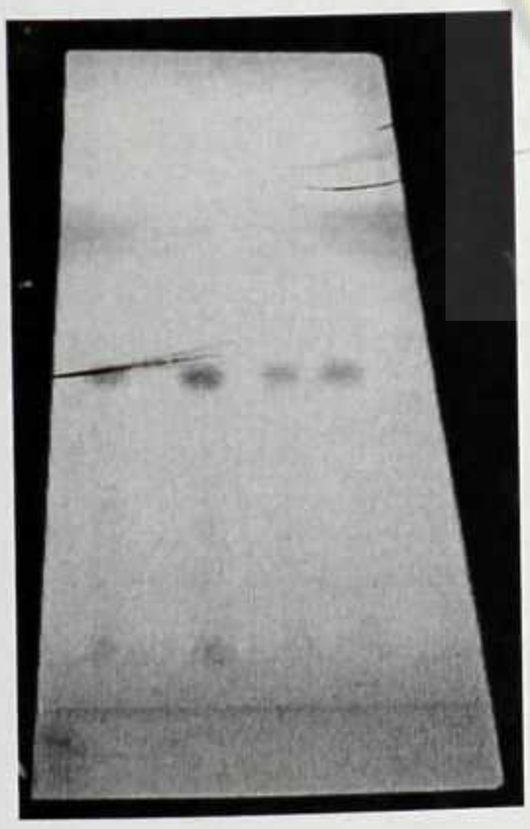


TLC CHROMATOGRAMS

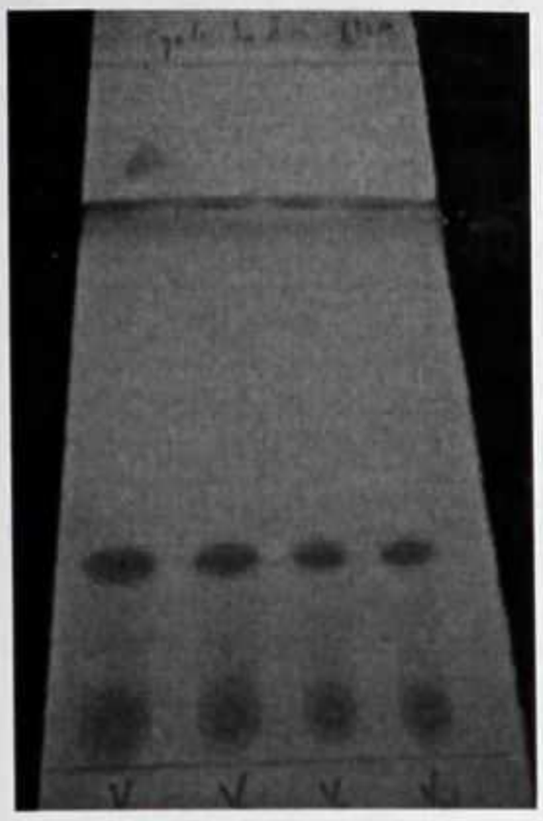
PRECOATED TLC CHROMATOGRAMS OF BULKED FRACTIONS

PREPARATIVE TLC ANALYSIS FOR PURE COMPOUND

PRECOATED TLC CHROMATOGRAMS 0F PURE BIOMARKER

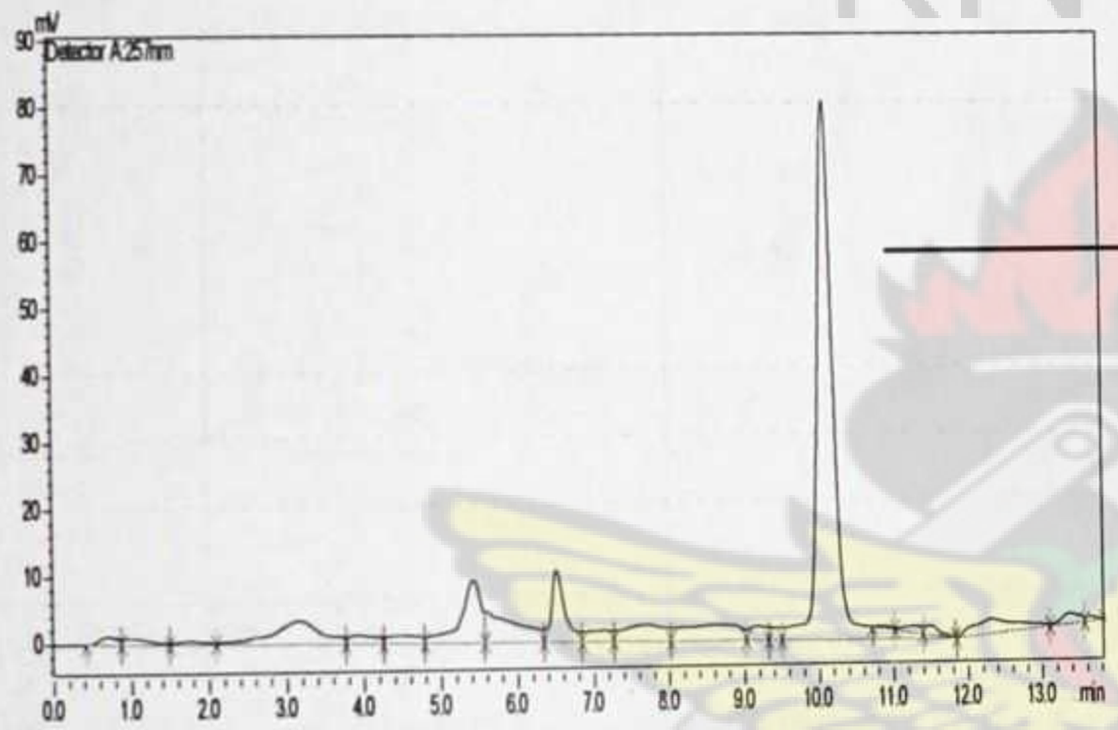


Mobile phase: Chloroform: Ethyl acetate (5:1)



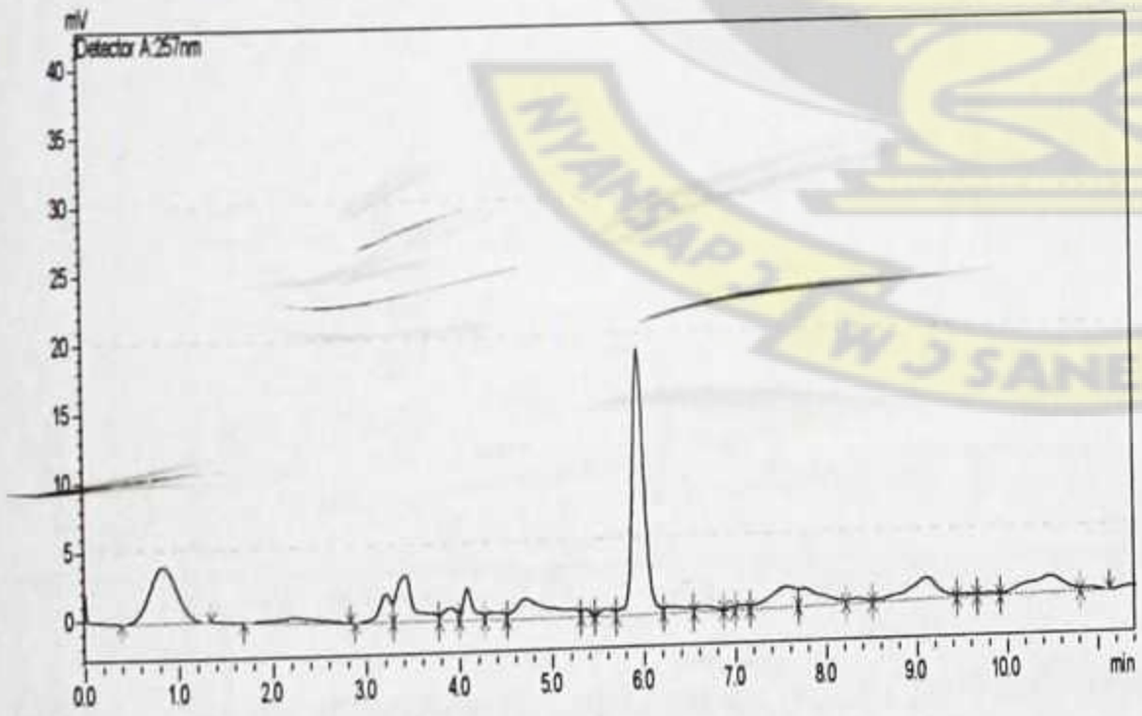
Mobile phase :Cyclohexane: Ethyl acetate (4:1)

HPLC CHROMATOGRAMS

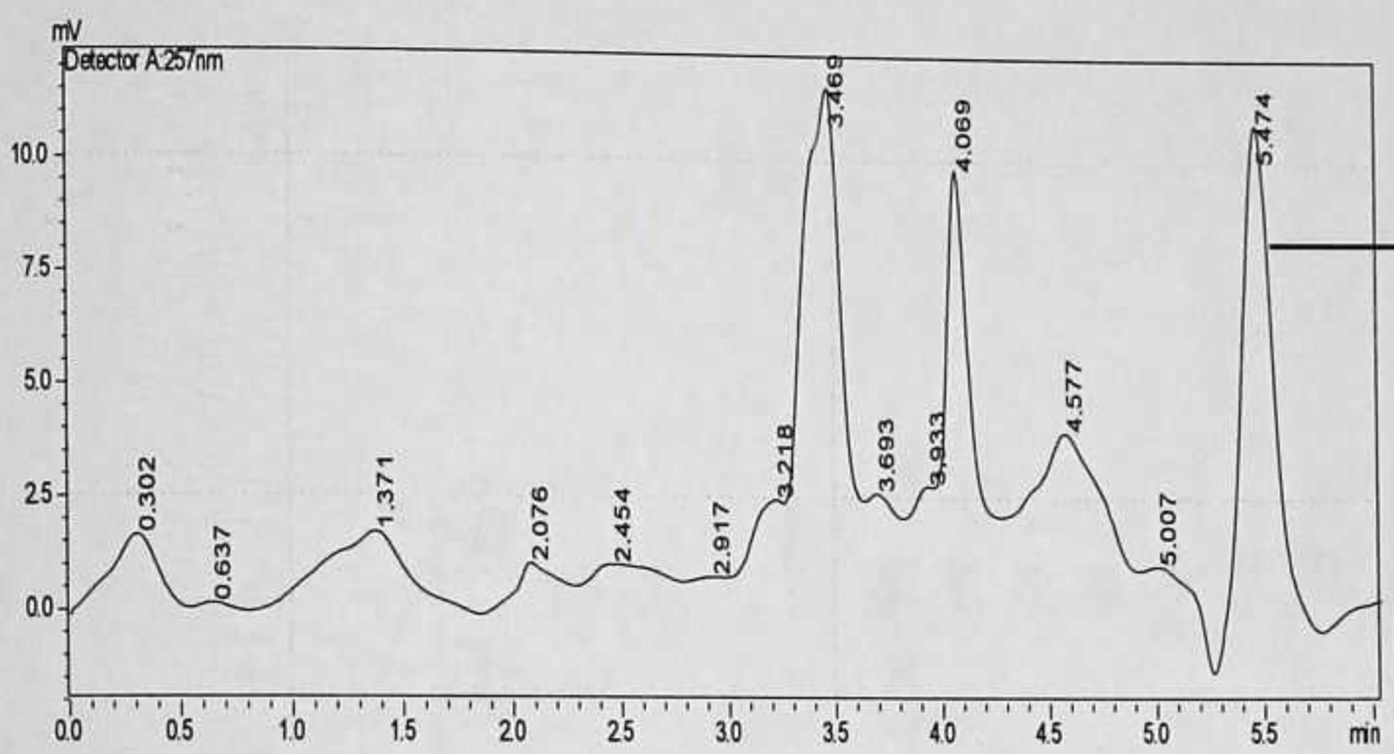


Pure biomarker

HPLC chromatogram of pure biomarker at flow rate of 0.5ml/min



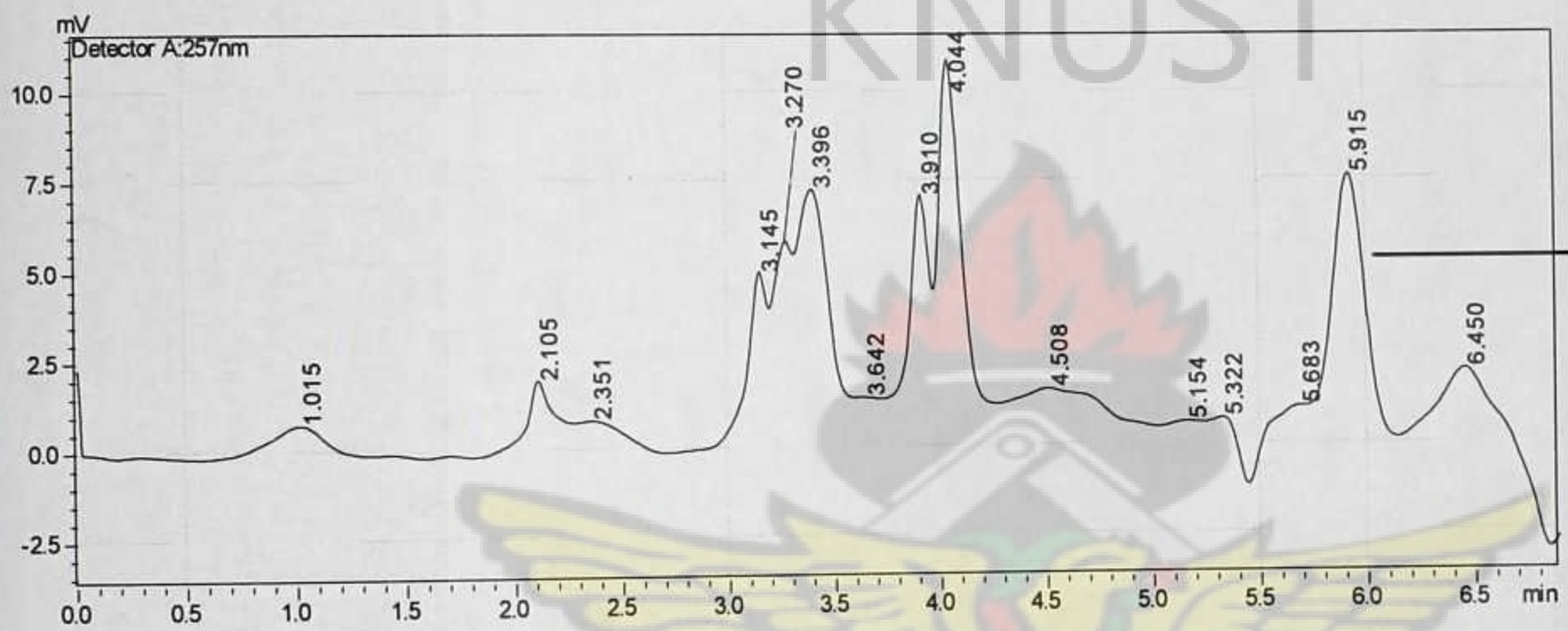
HPLC chromatogram at flow rate 0.8ml/min



Biomarker in diluted extract

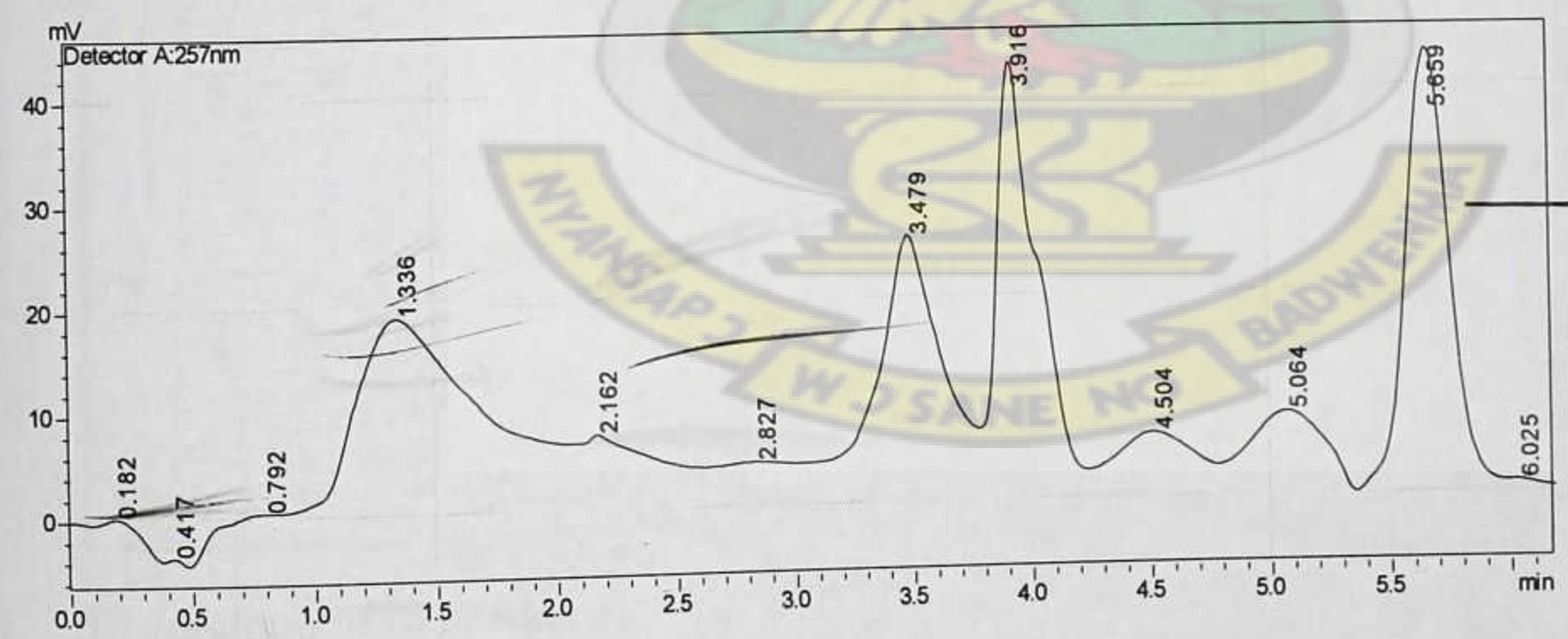
HPLC chromatogram of diluted extract at flow rat of 0.8ml/min

HPLC CHROMATOGRAMS FOR UNSPIKED AND SPIKED DILUTED EXTRACT



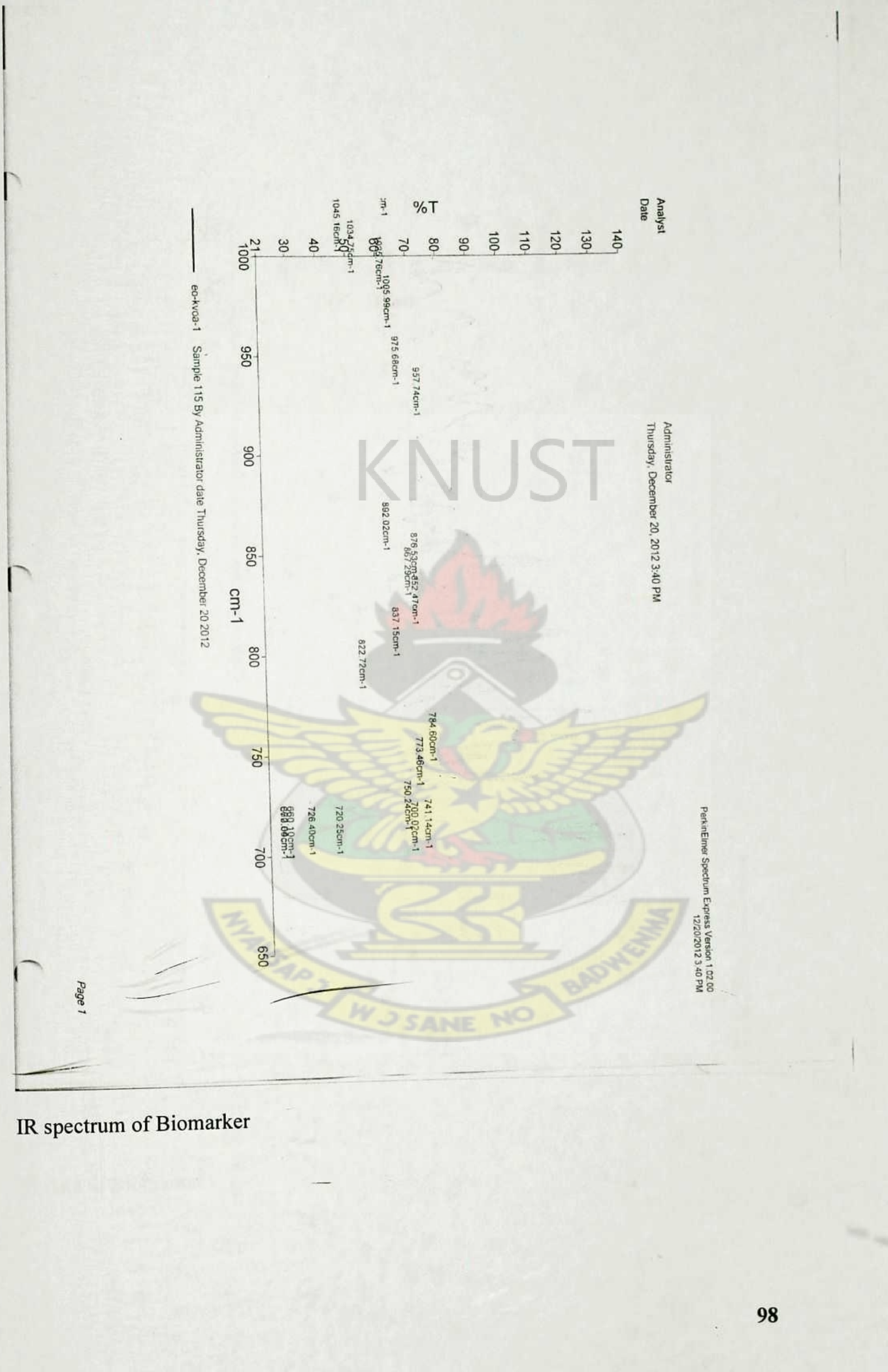
Biomarker in unspiked extract

HPLC chromatogram for unspiked extract (1ml of extract)



Biomarker spiked extract

HPLC chromatogram for spiked extract (1ml of extract)

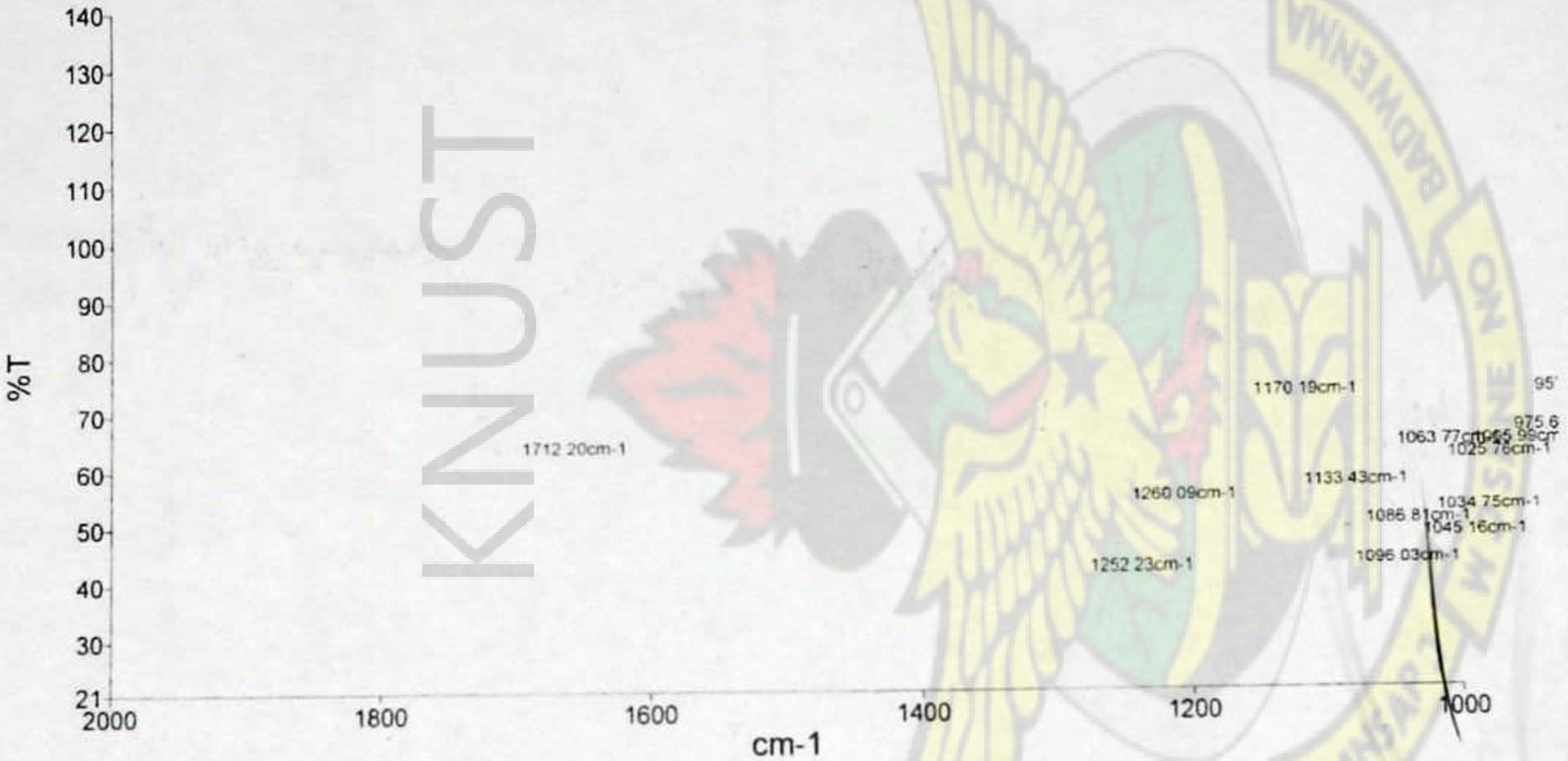


IR spectrum of Biomarker

PerkinElmer Spectrum Express Version 1.02.00
12/20/2012 3:39 PM

Analyst
Date

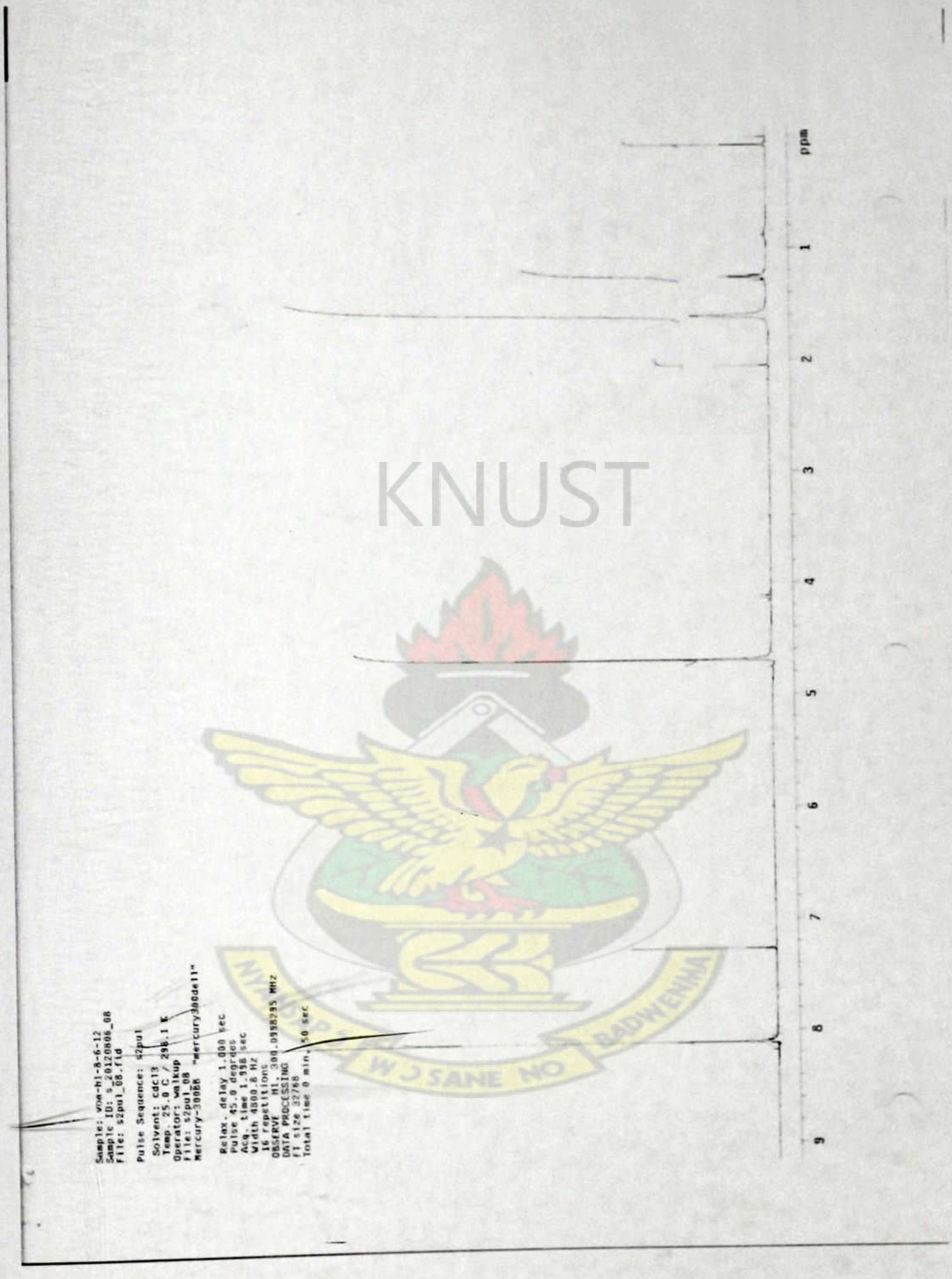
Administrator
Thursday, December 20, 2012 3:39 PM



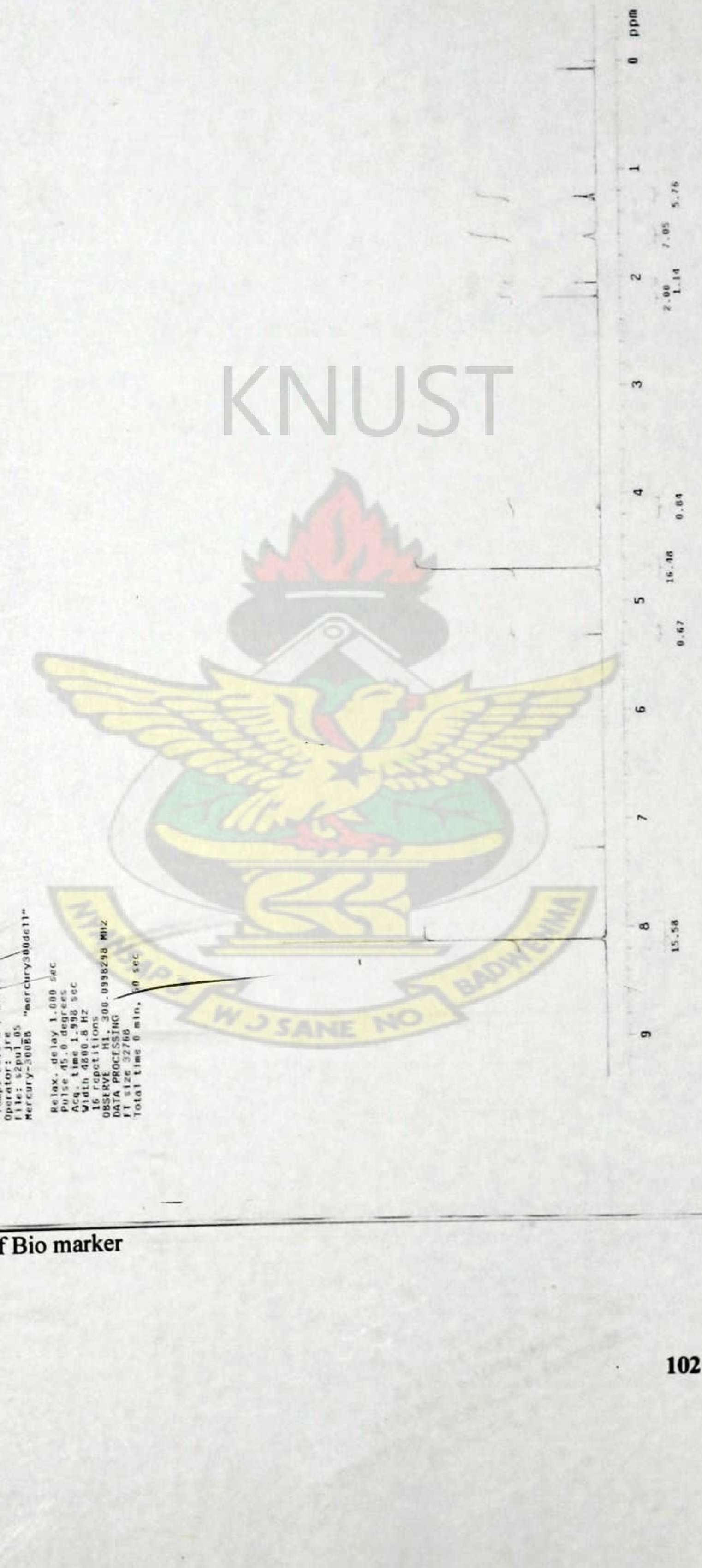
eo-kvoa-1 Sample 115 By Administrator date Thursday, December 20 2012

Page 1

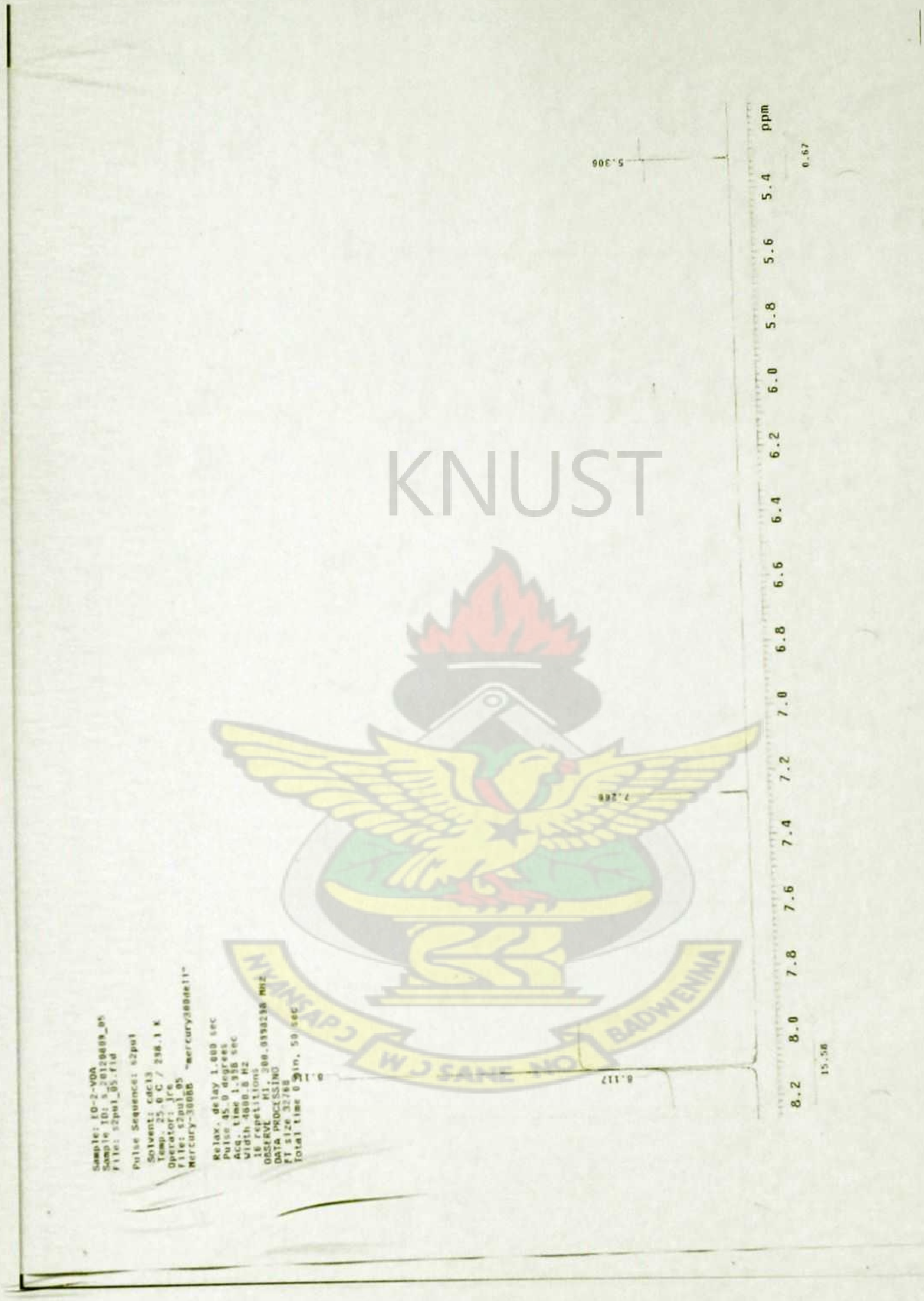
IR data of Biomarker



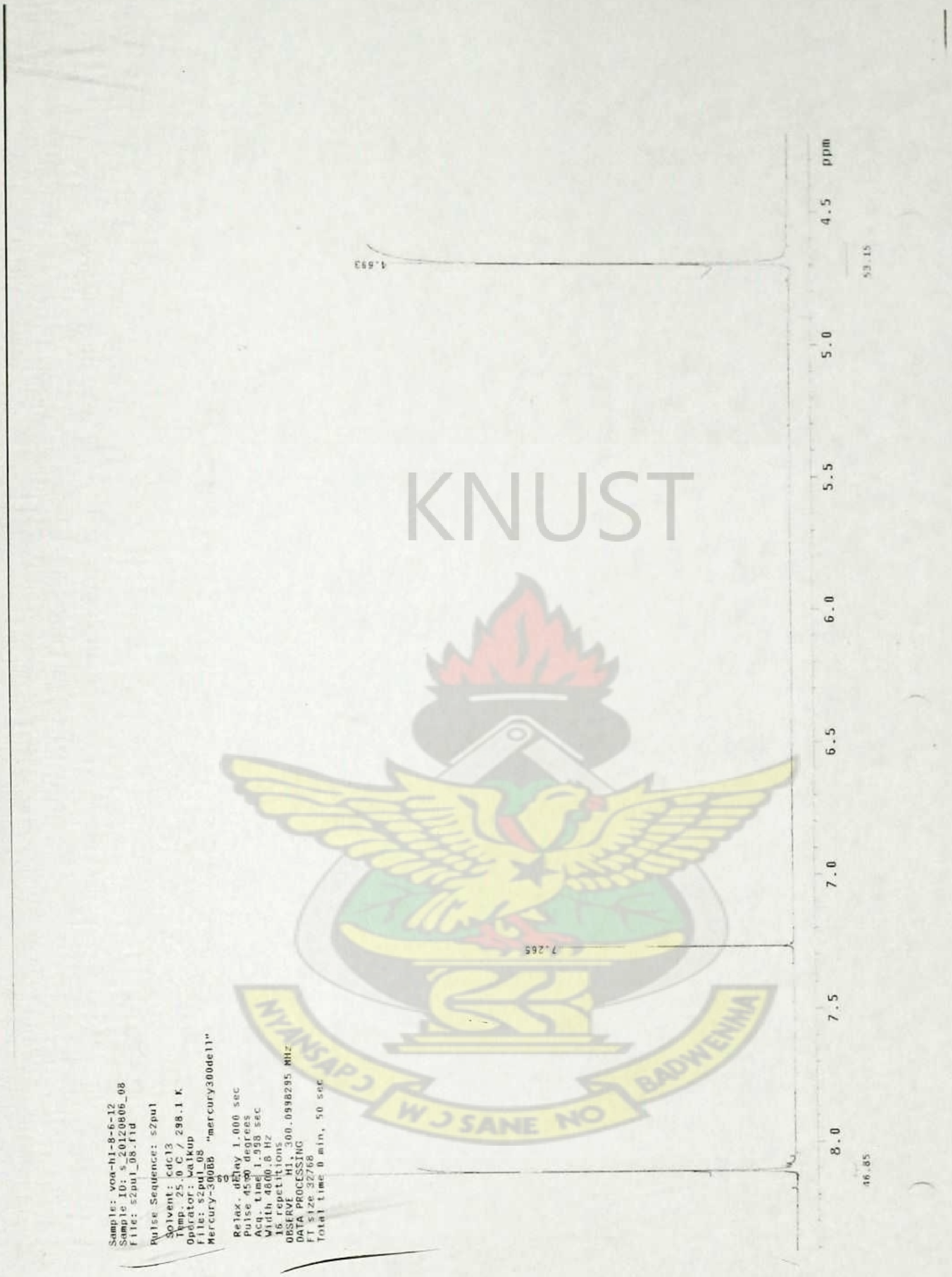
HNMR of Biomarker



HNMR repeat of Bio marker



HNMR 2 of Biomarker



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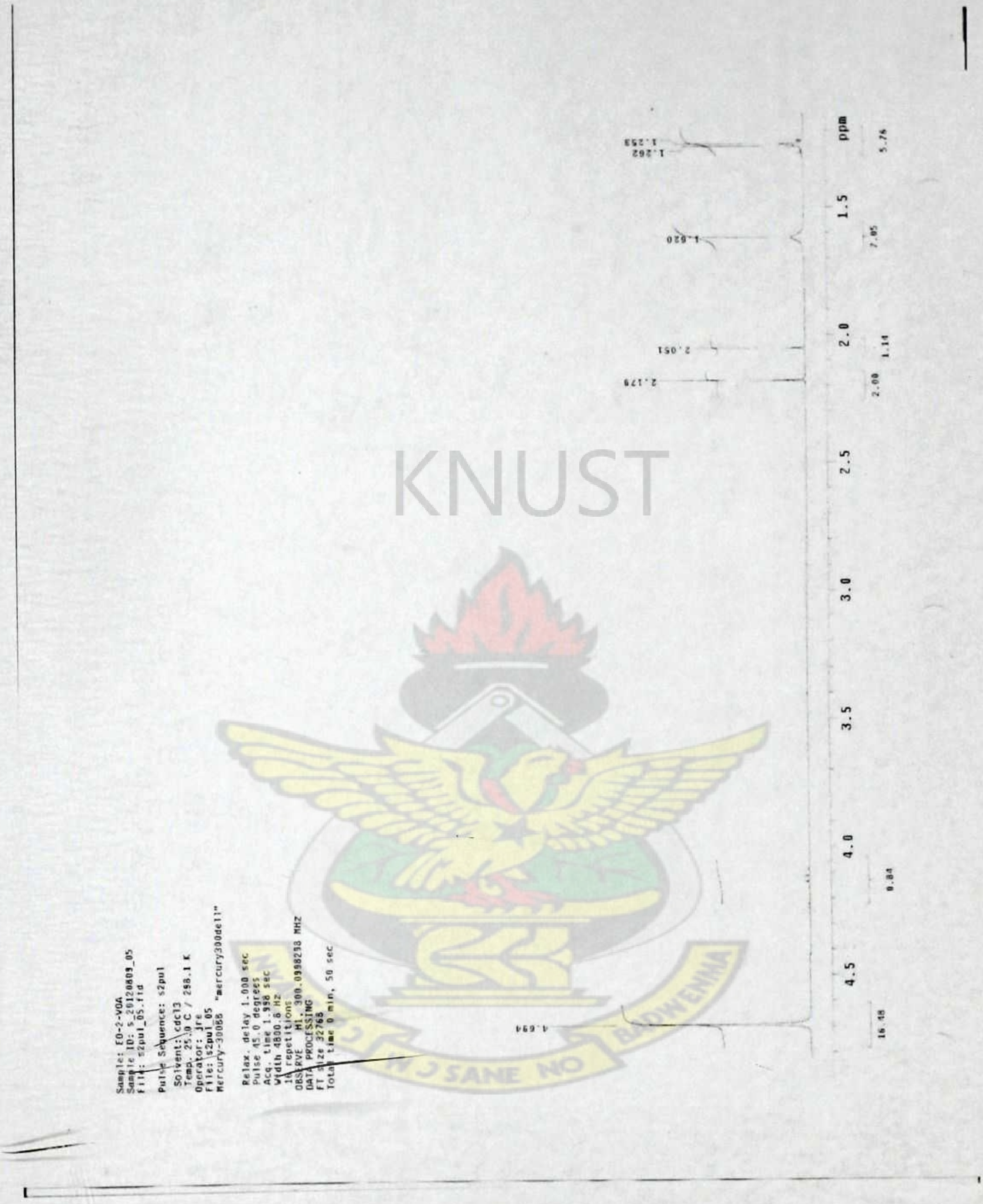
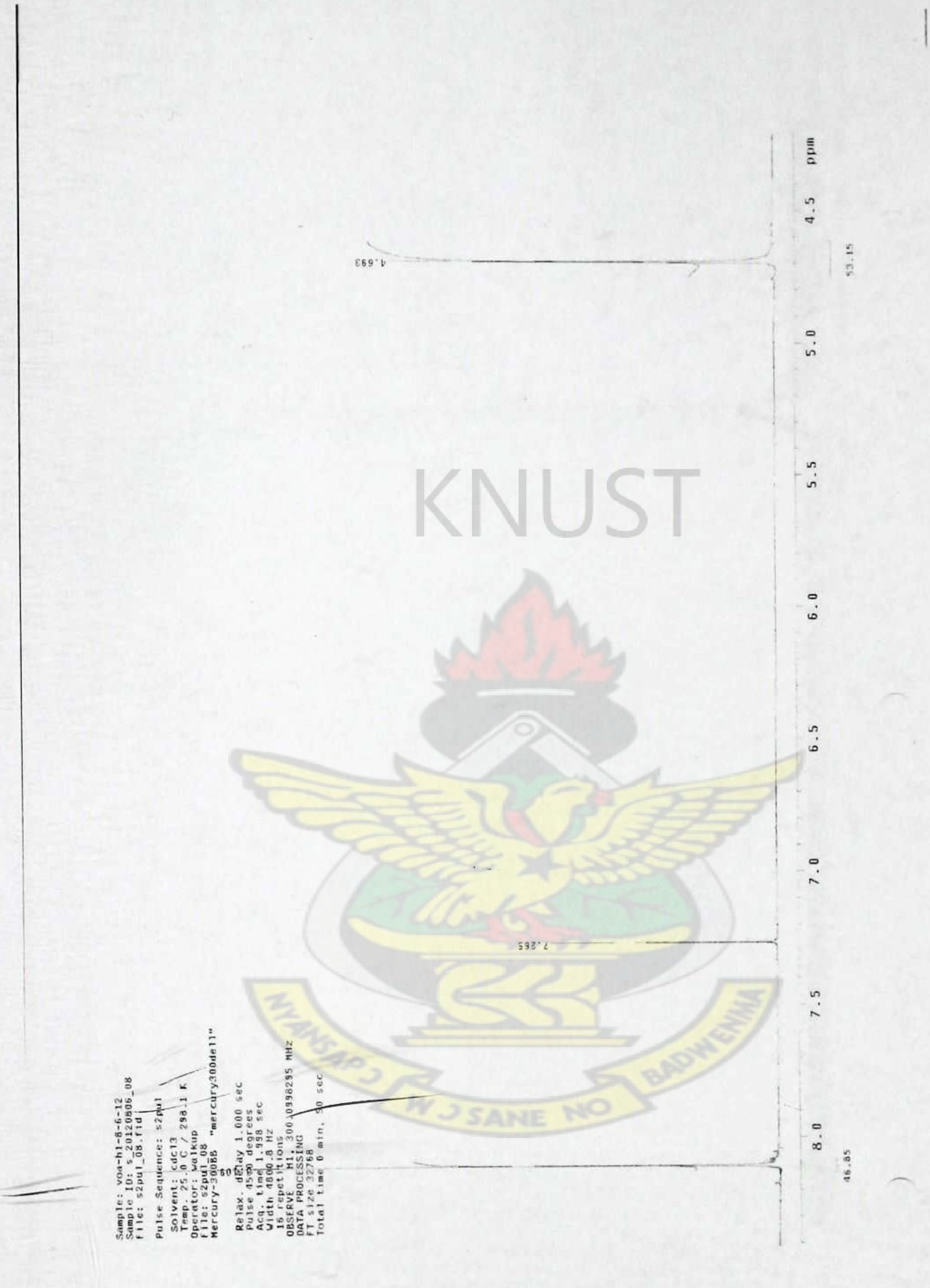


HNMR 3 of Biomarker



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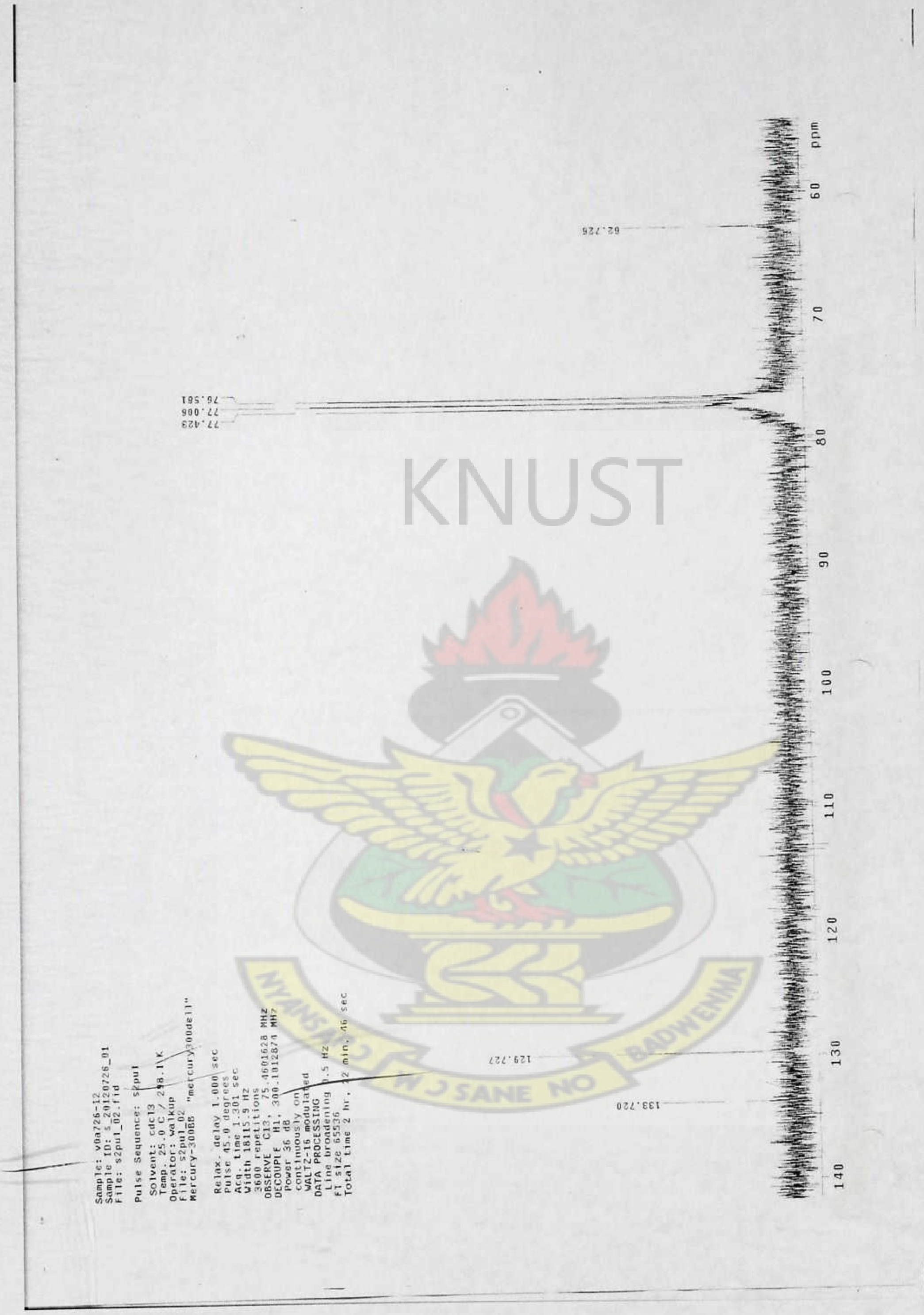
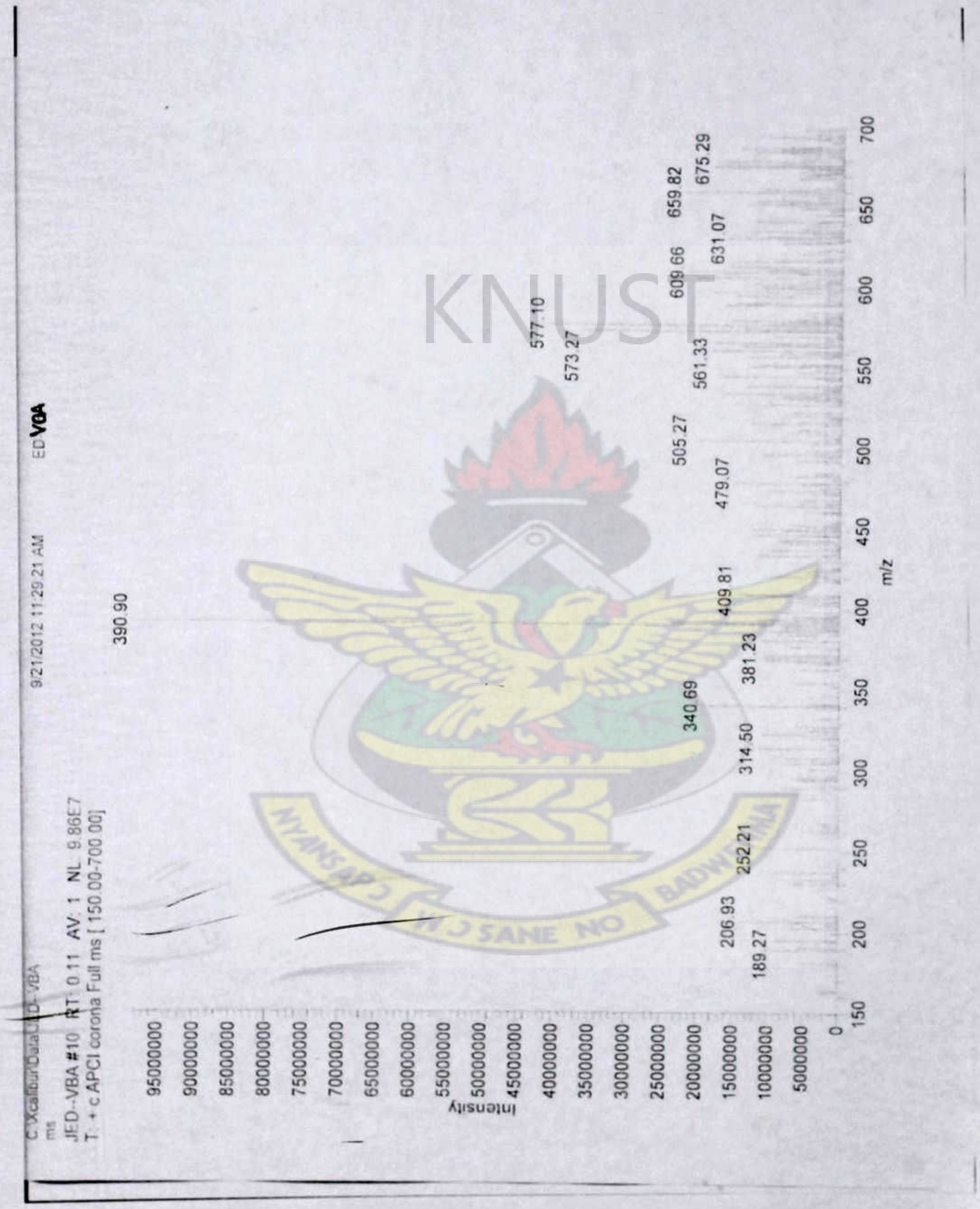


C13 NMR 1 of Biomarker

C13 NMR of Biomarker



Mass Spectroscopy data of Biomarker