KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

ISOLATION AND DEVELOPMENT OF AN HPLC METHOD FOR THE QUANTIFICATION OF A BIOMARKER IN THE ROOTS OF PAULLINIA PINNATA

SUBMITTED BY

CUDJOE EMMANUEL KENNETH

JANUARY, 2013

ISOLATION AND DEVELOPMENT OF AN HPLC METHOD FOR THE QUANTIFICATION OF A BIOMARKER IN THE ROOTS OF *PAULLINIA PINNATA* (SAPINDACEAE)

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By

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KUMASI

JANUARY, 2013

DECLARATION

This thesis is my report of experimental work carried out by myself at the Department of Pharmaceutical Chemistry, KNUST and has not been submitted for any other degree in any other institution.

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ABSTRACT

Paullinia pinnata is an African medicinal plant employed for the management of various ailments in various parts of the continent. It is known locally as Toa-ntini and is normally employed in wound healing remedies and this has been investigated scientifically. The plant is a woody vine with rigid stems. Its leaves are pinnate consisting of five leaflets, the last one being the largest. There are various reports of its use in erectile dysfunction, coughs and pulmonary diseases, and as an antioxidant.

The root bark was provided and authenticated by the department of Pharmacognosy, KNUST. The sample was coarsely powdered and extracted with chloroform using a soxhlet apparatus. The extract was concentrated using a rotavapor and evaporated to dryness. The dry powder obtained was taken through column chromatography (CC) and five compounds isolated from it. The melting points of compounds 1, 3 and 4 were determined and found to be **117-119 °C, 118-120 °C** and **160-161 °C** respectively. An HPLC method was developed for compound 1 and its quantity in the whole chloroform extract determined using the developed method. The mobile phase used is **Methanol:Water (9:1)** with an injection volume of **100µl** and a range of **1.000**. The method employs a **Phenomenex Kromasil C-8, 5µ, 250mm × 4.6mm, 100Å** reverse phase column at a flow rate of **1.3ml/min**, and a wavelength of detection of **210nm**. The method gave a retention time of **2.9687 ± 0.2657** (n=15).

The percentage content of compound 1 in the root of *Paullinia pinnata* was determined to be **0.052009±0.004321% w/w (n=12).** The method was validated for its linearity, repeatability, intermediate precision, and robustness. It was found to be linear for concentrations between **1.296µg/ml** and **11µg/ml.** The relative standard deviation (RSD) of the results obtained for the repeatability study was as **1.886792%** using the peak areas and **1.765537%** using the percentage purity. The RSD of the method with respect to intermediate precision was found to be **1.886792%, 1.183446% and 1.84418%** for days 1, 2 and 3 respectively using the peak areas in the calculation. The limits of detection and quantitation were also determined for the method as **0.464435µg/ml** and **1.40738µg/ml** respectively.

An initial attempt has been made to elucidate the structure of the biomarker with preliminary HNMR data suggesting a 6β-(3-methoxy-4-hydroxybenzoyl) ester of lupeone.

DEDICATION

This work is dedicated to my dad and mum, Mr. and Mrs. Emmanuel Ken Cudjoe, whose financial and spiritual support respectively have brought me this far in my education.

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ACKNOWLEDGEMENTS

I wish to acknowledge first and foremost, the Almighty God who gave me strength and perseverance to go through with this work. When it seemed undoable, He held my hands and took me gently through the storms.

I also wish to convey my sincere gratitude to my supervisor, Prof. N. N. A. Okine, whose admonishing and encouragement guided me throughout the course of the research. I thank him for his understanding, allowing me the opportunity to undertake this research. His ideas and suggestions were a lot of help to me.

I thank also greatly Dr. Kofi Annan, Head of Herbal Medicine department for his help and immense support throughout the course of this research. May the good Lord replenish anything he spent in helping me.

I would also like to thank my friends and lecturers, James Oppong-Kyekyeku and John Addotey. Though above my station, they did not feel a need to lord themselves over me whenever I went to them for advice and help. Their friendship means a lot to me and for that I am truly grateful.

I would again, like to express my gratitude to all my colleague post graduate students whose constructive criticism went a long way into shaping me into the researcher I have become today. Their support during the entire duration of our program must not go unacknowledged and so with this opportunity I want to thank them very much. Principal amongst them are Derrick Afful, Akorfa Ampofo and Michael Worlako Klu.

Last but not least, I'd like to thank the laboratory technicians, Mr. Rashid and Uncle Ben for their service in assisting me with reagents and materials whenever I called upon them.

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Chapter 1
INTRODUCTION

1.1 GENERAL INTRODUCTION

Plant medicine for a very long time has been an intrinsic part of the African's healthcare system. A great many people in Ghana, and Africa for that matter, hold dear the traditions of healing using plant sources passed on from generation to generation. Traditional healing using plant materials has been used to supplement, in some cases, orthodox medicine or substitute completely orthodox medicine for many also who cannot afford it. This is corroborated in the WHO Traditional medicine Factsheet No 134, (2008) which has stated that about 70% to 80% of people in developing countries have resorted to the use of complementary or alternative medicine at one point in time or another. These traditions however rely heavily on empirical evidence and anecdotal perspectives in the use of the various healing materials and techniques.

Over the years however, it has become necessary to obtain scientific evidence for the use of these plant medicines and as such measures have been taken by various stakeholders to provide such evidence. The scientific evidence obtained has not only been useful in providing scientific basis for the use of various plants for various ailments, it has also been useful in improving the production of medicines from these various plants and showing the limits of efficacy and, in some cases, toxicity of various active components of these plants. The stakeholders who have been involved in the search for scientific evidence have come from various disciplines which include pharmaceutical chemistry, pharmacognosy, pharmacology, botany, biology amongst others. Effective collaboration between these disciplines has contributed so much and still has a potential to do more.

Many plant species have now been shown and many more are continually being shown scientifically to possess antimicrobial, anti-inflammatory, analgesic, antiplasmodial and anticancer properties (Cimanga et al. 1997; Wright, 2007; Bayor et al. 2009; Bayor, 2008).. Many components of these plants, called secondary metabolites, have been isolated and also proven to possess some of these activities. Plant secondary metabolites refer to a large class of molecules in plants which contribute to their adaptation to the environment but themselves do not form a part of the plants' primary biochemical processes of cell growth and reproduction. Many terms including plant secondary compounds, phytochemicals, and plant xenobiotics are used generally to refer to this group of compounds. A few examples of plants scientifically proven to possess biological activity through isolation and testing of their secondary metabolites include:

- ✓ Cryptolepine, which was isolated from *Cryptolepis sanguinolenta*, and has been shown to possess antimalarial properties as evidenced by Cimanga et al. (1997) and Wright (2007).
- ✓ Quinine, which was proven a very long time ago to have antimalarial activity, was isolated from the bark of the cinchona tree.
- ✓ The new furano-clerodane diterpenoid crotomembranafuran, [12-oxo-15, 16-epoxy-3, 13(16), 14-clerodatrien-17, 18- dioicacid dimethyl ester], isolated from *Croton membranaceus* has been shown to have anticancer activity (Bayor et al. 2008).

Most research into plant medicines (phytopharmaceuticals) in Ghana has been in the direction of identification and characterization of active principles in the various medicinal plants used by herbalists in the country. Currently most medicines from plant sources are taken in huge doses (measured in cups and number of tablespoons) and this may result in untoward effects for patients. It is necessary therefore to include the quantification of the various active principles

found in the medicinal plants in the research effort as this will seek to enhance safety of the phytopharmaceuticals in use in the country. Knowing the quantity of an active antimicrobial principle in a unit amount of a whole extract of its plant taken for an infection will help to know what dose to take and for what period of time against the background of information regarding the minimum inhibitory concentration, among others, of that active principle.

Quantification of active secondary metabolites in plants may be achieved through a host of techniques principal amongst them being High Performance Liquid Chromatography (HPLC). HPLC has become one of the most employed analytical techniques in the pharmaceutical industry over the years. This has been due to the versatility of the technique, short time required for analysis and the accuracy of results the technique provides.

In this project, a new HPLC method is to be developed for the quantification of the active secondary metabolite after its isolation. The new method will then be validated for its accuracy, precision and robustness.

1.2 AIM

The aim of this project was to identify a biomarker that would be used as a quality control tool for the root bark of *Paullinia pinnata*. A High Performance Liquid Chromatography (HPLC) method was developed for the biomarker obtained and this method was then used to carry out the quality control.

1.2.1 SPECIFIC OBJECTIVES

The specific objectives of the project include:

- ✓ To extract a root sample of *Paullinia pinnata* using a soxhlet apparatus.

- ✓ To isolate the biomarker from the extract using column chromatography and thin layer chromatography.
- ✓ To develop a set of conditions for HPLC work that achieves very good peak separation in quantifying the amount of the biomarker in the raw extract using the isolated biomarker as a secondary standard.
- ✓ To validate the method developed.
- ✓ To determine from the results of the project the weight in grams per kilogram of *Paullinia pinnata* roots.

1.3 PROBLEM STATEMENT AND JUSTIFICATION

With the current rise in the use of herbal medicines in Ghana, it is essential to standardize these herbal products. In light of this fact, any efforts aimed at providing new information to augment and/or increase the repertoire of existing knowledge with regards to the herbal products is commendable.

Again, most herbal products including those that contain extracts of *Paullinia pinnata* are taken in very large doses. Annan & Houghton, (2010) showed that above a certain concentration, the novel compounds isolated from *Paullinia pinnata* showed reduced fibroblast activity and attributed this to possible killing of cells in and around the wound. Information on the quantity of the active secondary metabolites in the plants will therefore be useful in determining accurate dosing regimens for patients using the products in order to avoid toxicity. This information will be useful in combination with information on the minimum effective concentration of the extract and toxicity studies.

Furthermore, in as much as this project seeks to determine the quantity of the given biomarker in the roots of *Paullinia pinnata* which has been scientifically proven to possess antimicrobial effects among others, it will also help to know whether *Paullinia pinnata* is worth pursuing industrially i.e. if the quantity of the biomarker is enough in the roots to warrant commercial production of medicinal products such as ointments from the plant.

Furthermore, in as much as this project seeks to determine the quantity of the given biomarker in the roots of *Paullinia pinnata* which has been scientifically proven to possess antimicrobial effects among others, it will also help to know whether *Paullinia pinnata* is worth pursuing industrially i.e. if the quantity of the biomarker is enough in the roots to warrant commercial production of medicinal products such as ointments from the plant.

Chapter 2
LITERATURE REVIEW

2.1 PAULLINIA SPP

Paullinia spp occur worldwide in the tropical regions of Africa, the Caribbean, Central and South America. The genus Paullinia was first discovered in the Caribbean in the 18th century by Christian Franz Paullini, after whom the genus was named. It is one of many genera in the Sapindaceae family (the soapberry family).

The genus Paullinia has some 150 – 200 species with various pharmacological activities. Paullinia spp have been known to contain purine alkaloids and used to prepare stimulant drinks (Weckerle et al. 2003). Some species of the genus in which purine alkaloids have been isolated and characterized are *Paullinia cupana* and *Paullinia pachycarpa.* There have been reports of the presence of flavonoids, phenols, triterpenes and saponins in a study by Pérez et al. (2010) on extracts from the stem cortex of *Paullinia clavigera*. Some species like *Paullinia guaraná*, (syn. *Paullinia cupana*) have also been shown to possess psychoanaleptic properties (Carlini, 2003).

A lot of work has been done on *Paullinia spp* but most of the work focuses on one species, *Paullinia cupana*. Much work has not been done on most of the other species particularly *Paullinia pinnata*.

Chapter 2
LITERATURE REVIEW

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Table 1 - The scientific classification of Paullinia

Classification	Name
KINGDOM	*Plantae*
PHYLUM	*Magnoliophyta*
CLASS	*Magnoliopsida*
ORDER	*Sapindales*
FAMILY	*Sapindaceae*
SUBFAMILY	*Sapindoideae*
GENUS	*Paullinia*
EPITHET	*Pinnata L.*

2.2 *PAULLINIA PINNATA*

Paullinia pinnata L. is one of the medicinal plants of Ghana folkloric medicine. It is known locally as Toa-ntini. Its English and Portuguese names are Bread and Cheese and Mata Fome respectively. It is a woody vine also found in tropical Africa. It is a climber with rigid stems. It has pinnate leaves with five leaflets the terminal one of which is the biggest. Its leaves are oblong to obovate and are irregularly crenate. The petiole and rachis are winged. Its flowers are white spin-like axillary ~~on long~~ peduncles. The seeds are enclosed in a white substance whereas the fruits are bright red obovate capsules (Dokosi, 1998)

Figure 1 - Top and lower side of leaf of *Paullinia pinnata*

2.2.1 BIOACTIVITY AND FOLKORIC USES

It has a wide variety of uses in folkloric medicine. It is used extensively as an antimalarial in African herbal medicine (Asase et al. 2005; Jimoh et al. 2007). In Ghana, the plant is widely employed in the management of wounds and other diseases of the skin (Annan et al. 2010). There have also been reports of the use of this African plant for the management of erectile dysfunction as well as the possession of antioxidant activity (Zamble et al. 2006). The plant has also been shown to have molluscidal activity (Meléndez & Capriles, 2002) and has been suggested as potential for the relatively cheap control of human schistomiasis. Abbiw (1990) reported that the roots of *P. pinnata* are also chewed for coughs and pulmonary diseases, gonorrhea, abscesses, on open sores and also as an aphrodisiac.

2.2.2 PHYTOCHEMISTRY

Many secondary metabolites have been isolated and identified from *Paullinia pinnata*. Bowden (1962) in an appendix to Broadbent (1962) described the isolation of triterpenes saponins and cardiotonic catechol tannins from *Paullinia pinnata*. Two novel flavone glycosides were also isolated and identified by Abourashed et al. (1999). Paullinoside A and Paullinomide A as well as β-amyrin have been identified in the leaf extract of *Paullinia pinnata* (Miemanang et al. 2006). Again, two new lupane triterpenes have been isolated and characterized by Annan & Houghton (2010). The structures of the two compounds they isolated are shown below.

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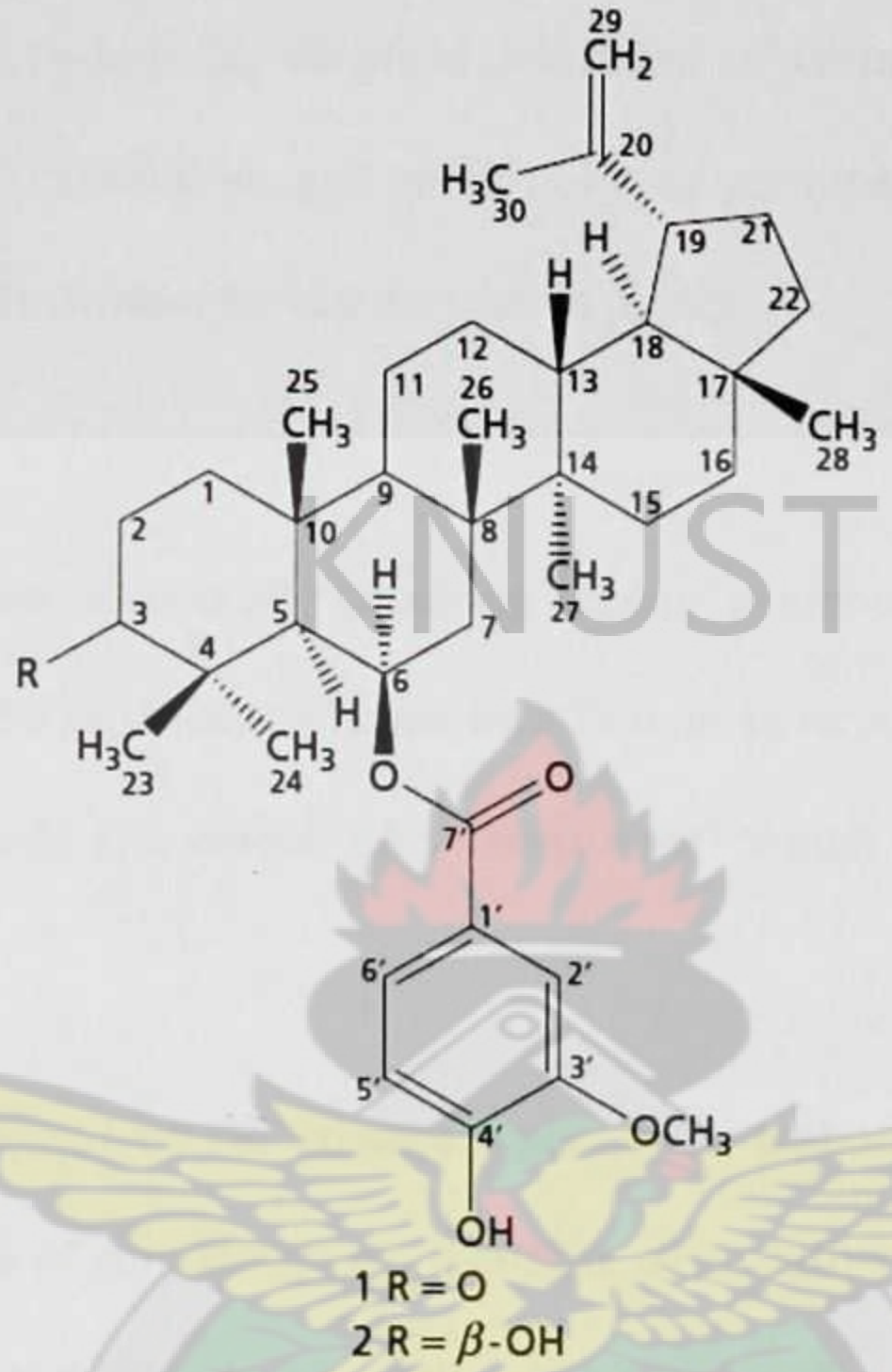

Figure 2 – Two novel lupane terpenoids by Annan & Houghton

2.3 SECONDARY METABOLITES

Plant 'secondary metabolites' is a term used to refer to a diverse class of chemical compounds produced in plants. They may be by-products of certain biochemical processes in the plants and are not involved in the processes themselves. Over 30,000 structures in this class of compounds have been elucidated from the extracts of various plants.

Plant secondary metabolites or phytochemicals as they are sometimes referred to, are classified into many groups examples of which are alkaloids, tannins, non-protein amino acids, protease inhibitors, cyanogenic glycosides, saponins, and lectins. The classification may be due to similarities in chemical structure or the process of production of the metabolite. They may be

against herbivores and pathogens, chemical inhibition of competing plant species known as allelopathy, regulation of symbiosis, and control of seed germination. There are some secondary metabolites however, which have no known uses in plants.

2.3.1 TERPENES

Terpenes are a large class of naturally occurring organic compounds. They are mainly produced by plants but may also be produced by some insects such as termites. Terpenes are the principal components of resins and are named after 'turpentine' which is itself produced from resins (Bano, 2007).

When terpenes are oxidized or the arrangements of the carbon atoms change, terpenoids are formed. The two classes of compounds are therefore chemically related. They occur in relatively large amounts as essential oils, resins, and waxes which offer a wide range of commercially useful products including solvents, flavorings and fragrances, adhesives, coatings, and synthetic intermediates. They may thus be employed in aromatherapy.

Hops are used by some brewers to flavor their beers and the aroma and the flavor of the hops is due to the presence of terpenes (Tinseth, 1993).

Terpenes may serve as biological precursors for the production of steroids and sterols in animals. Though extraordinarily diverse, all terpenes are derived biosynthetically from units of isoprene, C_5H_8 linked either in a 'head-to-tail' or in a cyclic structure. The molecular formula of terpenes is therefore $(C_5H_8)_n$ where n represents the number of isoprene units linked together. Terpenes are formed through the condensation of the precursors (activated forms of isoprene): isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to give geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and/or geranylgeranyl pyrophosphate (GGPP).

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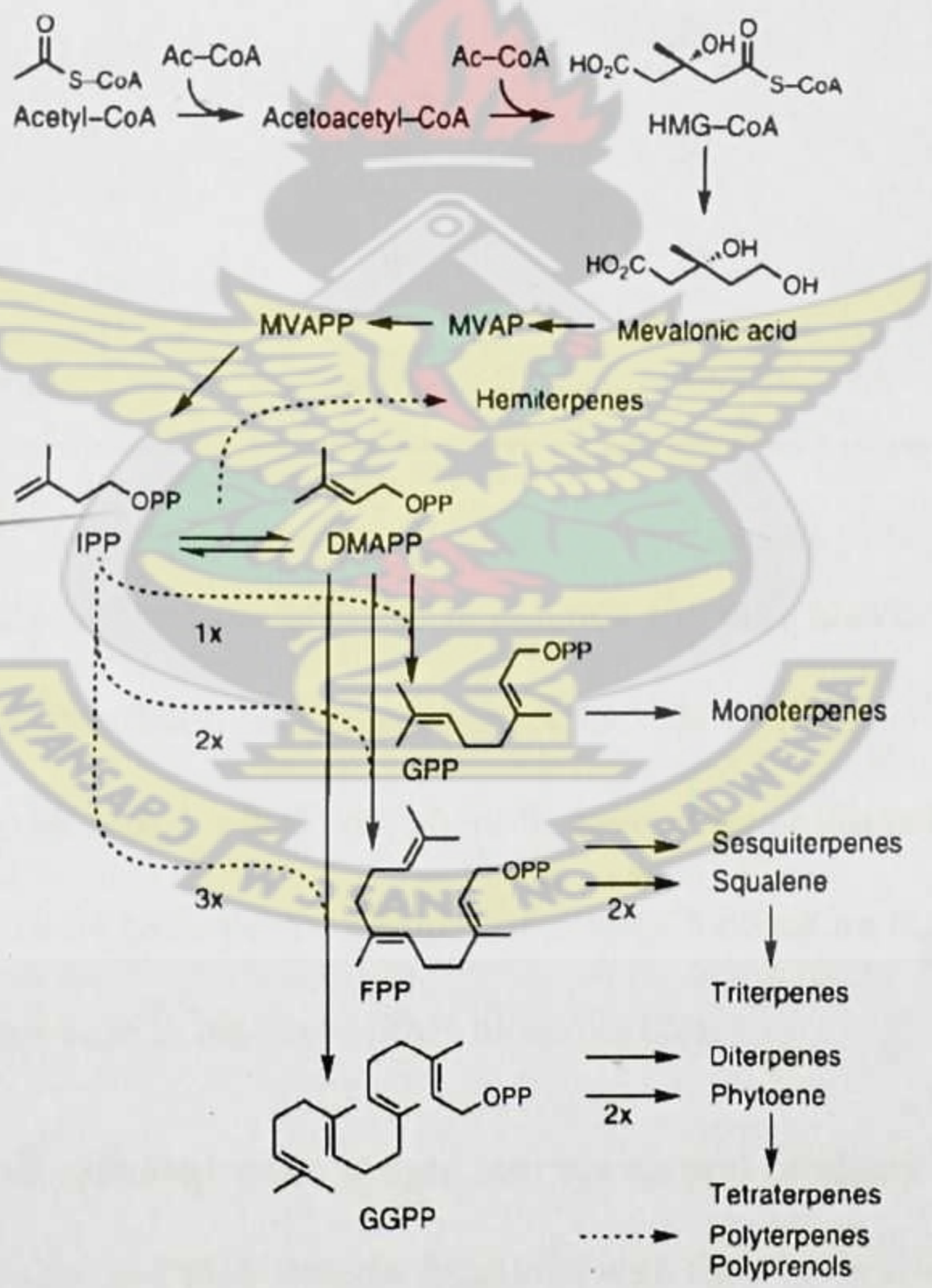
Formation of IPP or DMAPP is via two metabolic pathways; the Mevalonic acid pathway which occurs in the cytosol and the MEP/DOXP or Mevalonate-independent pathway which occurs in plastids. It is generally accepted that the cytosolic pool of IPP serves as a precursor of sesquiterpenes, triterpenes, sterols and polyterpenes whereas the plastid pool of IPP provides the precursors of mono-, di- and tetraterpenes (Bohlmann et al. 1998). The two pathways are principally mutually exclusive but some exceptions have been described showing that interactions between the two biosynthetic pathways may exist (Dudareva N et al. 2005).



1×, 2× and 3× show the number of IPP units added

Figure 3 – An outline of the Mevalonic Acid Pathway and Terpenoid Skeleton Biosynthesis

(Mcgarvey & Croteau, 1995)

Terpenes have been classified on the basis of the number of isoprene (or isopentane) units incorporated in the basic molecular skeleton:

Table 2 – Classification of terpenes

Terpenes	Isoprene units	Carbon atoms	Examples
Monoterpenes	2	10	Limonene
Sesquiterpenes	3	15	Artemisinin
Diterpenes	4	20	Forskolin
Sesterpenes	5	25	Geranylfarnesol
Triterpenes	6	30	α-amyrin
Carotenoids	8	40	β- carotene
Polymeric terpenoid	> 100	> 500	Rubber

Isoprenoid units are not exclusive to terpenes or terpenoids and may also occur in the structure of some other natural molecules. Such natural molecules include isoprenoid alcohols (also known as terpenols or polyprenols), indole alkaloids, phenols, several quinones (vitamin K), and certain alcohols like vitamin E. There have been a number of studies focused on the diverse nature of the isoprene unit and its conversion in nature to other biomolecules.

Bouvier et al. (2005) have provided some insight into the natural production of plant terpenoids, their regulation within nature and their diverse functions with their review of plant terpenoids.

2.4 SEPARATION TECHNIQUES

2.4.1 LIQUID – LIQUID EXTRACTION

This is a separation technique that is usually employed when the sample to be analyzed is within a liquid solution, usually an aqueous solution, which contains other substances that are going to interfere with the analytical method chosen (Kenkel, 2003). It is also known as solvent extraction.

This analytical technique involves the use of two liquids, the liquid in which the analyte of interest is contained and the extracting solvent into which the analyte of interest is to be retrieved. Two criteria for the selection of the extracting solvent are that it must be immiscible with the solvent in which the analyte is dissolved and the analyte to be extracted must be more soluble in this solvent than the original solvent.

The main apparatus used in liquid-liquid extraction is the separating funnel. It is specially designed for this purpose. It is normally fashioned in a teardrop shape, stoppered at the top end and a stopcock at the lower end. In separation, the solvents are introduced into the funnel and the funnel is stoppered. It is then tilted diagonally and shaken, after which the lower end is pointed upwards and the stopcock opened. This is to allow escape of solvent vapor from the apparatus as one of the solvents used is mostly a volatile organic solvent. This manoeuver is done severally to ensure that the two liquids mix properly and the analyte of interest is able to partition well between the two solvents. The liquids are then separated after clamping the funnel and releasing the stopcock. The extraction is repeated by introducing other aliquots of the extracting solvent until the amount of the analyte of interest is exhausted in the original solvent.

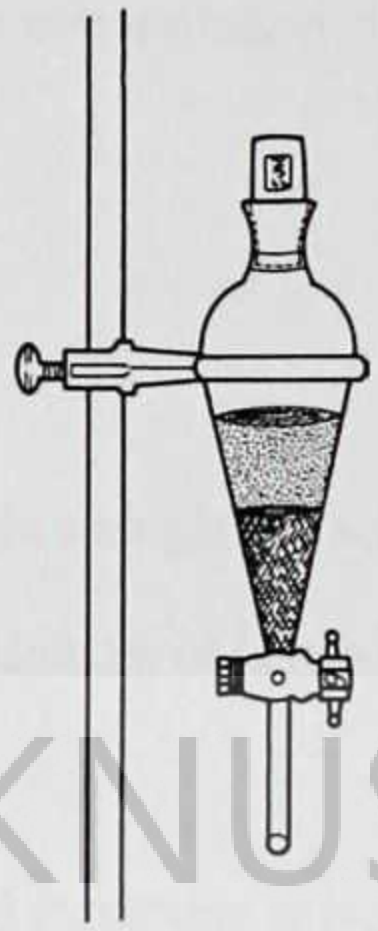


Figure 4 – A separatory funnel containing two immiscible liquids

The process of separation in the separating funnel is an equilibrium process. The analyte of interest moves from the original solvent into the extracting solvent at a rate which declines over time when the liquids are first mixed. After a certain time, the analyte starts migrating back into the original solvent. The rate of 'forwards' and 'backwards' migration becomes equal at a point at which equilibrium is said to have occurred. Owing to the preferential dissolution of the analyte in the extracting solvent, at equilibrium there is more of the analyte in the extracting solvent than the original solvent.

A distribution coefficient known as partition coefficient describes the relative solubility of an analyte in two solvents. The partition coefficient, K is often taken as the ratio of the solubility (i.e. concentration) of the analyte in the extracting solvent to that in the original solvent (Kenkel, 2003).

$$K = \frac{[A]ext}{[A]orig}$$

Where [A]ext and [A]orig refers to the concentration of analyte in the extracting and original solvents respectively.

K may be useful in determining

- ✓ The amount of analyte extracted in a single extraction process
- ✓ The number of extractions needed to obtain a given concentration of analyte in the extracting solvent
- ✓ The ideal volumes of original and extracting solvents to be used for the process

2.4.2 CHROMATOGRAPHY

Chromatography is recorded to have been first employed in 1903 by Mikhail Tswett, a Russian botanist, in the description of a process he carried out: separation of various plant pigments from a petroleum-ether extract by percolating the extract through a glass column packed with powdered calcium carbonate (Ettre, 2003). Tswett's work did not however, gain much interest until in the 1930s when chromatography was employed for biochemical separations. A case was made for liquid-liquid chromatography when in 1941 Martin and Synge established its importance which resulted in the development of a theory regarding chromatographic separation (Harvey, 2000).

Chromatography has been known therefore as essentially a separation technique and is employed principally in analytical work by chemists and other related scientists. It finds wide use in plant work when the separation of secondary plant metabolites from the raw plant extracts is necessary. Chromatography involves the use of a mobile phase, usually a liquid, gas or supercritical fluid, and a stationary phase, usually solid and immobile as well as immiscible with the mobile phase.

There are various forms of chromatography but one basic principle runs through them all; the variation in the rate of migration of the components of the sample through the stationary phase as the mobile phase passes through it. The differing rates of migration are dependent upon the level of interaction between the component and the two phases. This level of interaction depends essentially on the physical and chemical properties of the components of interest. If the interaction with the stationary phase is more i.e. the component has a higher affinity for the stationary phase than the mobile phase, the component is expected to take a longer time to elute/pass through the stationary phase as compared to one which has a higher affinity for the mobile phase than the stationary phase.

There are various forms of chromatography so named by virtue of the stationary phase which can only be liquid (usually on a solid support) or solid and the mobile phase which may be liquid or gas.

Where the stationary phase is contained in a column, it is termed ***column chromatography***. Column chromatography includes *liquid chromatography* (e.g. High Performance Liquid Chromatography and Classical Column Chromatography) where the mobile phase is liquid and *gas chromatography* (GC) where the mobile phase is a gas or supercritical fluid. To distinguish between the stationary phases used in GC, the names *gas-solid chromatography* and *gas-liquid chromatography* are used.

The stationary phase may also occupy a plane surface where it is referred to as ***planar chromatography***. The plane surface may be a paper (paper chromatography) or a glass plate (thin-layer chromatography) (Patnaik, 2004).

2.4.2.1 CHROMATOGRAPHIC MECHANISMS

Every chromatographic process takes place via a number of mechanisms. These mechanisms may be referred to as types of chromatography in some literature. The mechanisms are common to all the forms of chromatography named above. They include adsorption, partition, ion exchange and gel permeation or size exclusion.

2.4.2.1.1 Partition Chromatography

This type of chromatography is based on the idea of partition coefficient. The partition coefficient of a solute or compound is the relative solubility (which is a degree of affinity) of that compound in two immiscible solvents. In partition chromatography therefore, both mobile phase and stationary phase are liquid and immiscible. The stationary phase is a thin film of liquid bonded onto the surface of finely divided solid material like silica gel or aluminium oxide. The sample components therefore partition between the thin liquid film and the liquid mobile phase depending on their solubilities in the two liquids. The different partition coefficients of the sample components therefore results in the separation. Since the relative solubilities of the components in the liquids is essential, it is appropriate to consider the polarities of the components as well as stationary and mobile phase liquids.

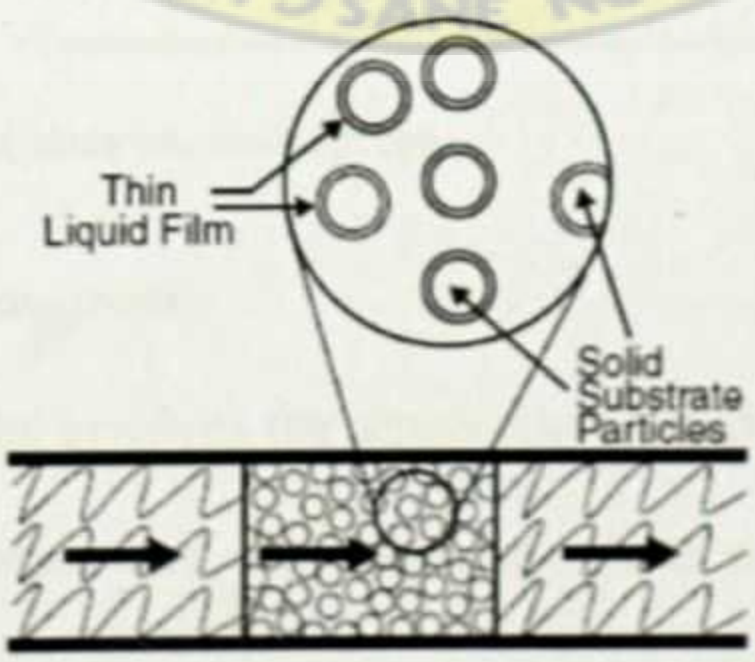


Figure 5 – An illustration of partition chromatography (Kenkel, 2003)

2.4.2.1.2 Adsorption Chromatography

In adsorption chromatography, the stationary phase consists only of finely divided particles to which the components of the mixture adsorb. The degree of adsorption is different owing to the different polarities of the mixture components. Separation then occurs when the liquid mobile phase moves over or through the solid stationary phase. Very weakly adsorbed components are eluted first followed by moderately adsorbed components and finally strongly adsorbed components are eluted. The strength of the mobile phase i.e. the polarity also influences the separation. Adsorption involves an interaction of polar components of the mixture or those with polar groups with the solid stationary phase which is itself very polar. Interactions such as hydrogen bonding, dipolar bonding and van der Waal's forces are involved.

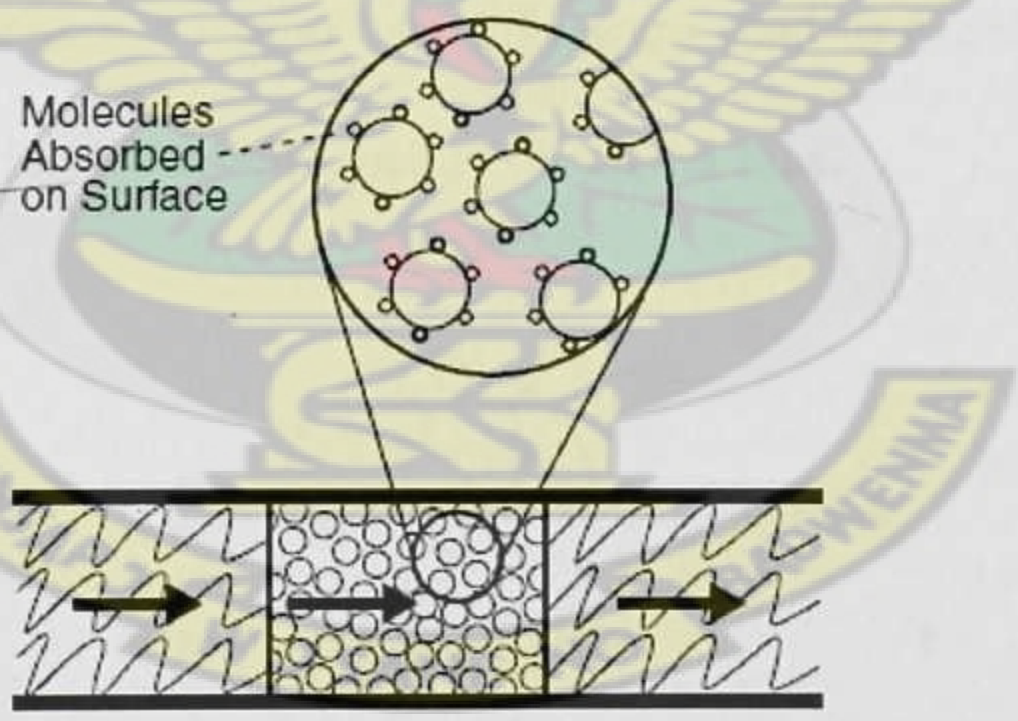


Figure 6 – An illustration of adsorption chromatography

2.4.2.1.3 Ion Exchange Chromatography

Ion Exchange Chromatography involves the separation of mixtures with ionic components which could be inorganic and organic. Here, the stationary phase usually is composed of very small polymer resin beads onto which functional groups bearing charges are bonded. The resins used as stationary phase material may include chemically modified silica or polymers of e.g. styrene

divinyl benzene. The ionic groups form bonding sites which selectively exchange ions with various mobile phases as the mobile phase moves along the length of the stationary phase. Separation then occurs as some ions bond to the charged sites on the resin while others do not. Different mobile phases may be introduced over time which can also further selectively free and exchange bound ions, resulting in their separation. The stationary phase materials used in ion exchange chromatography may be divided into two types depending on the charge (or polarity) of the bonded ionic groups. The two types are **Anion exchange resins** which have positively charged bonded groups to effect exchange with negative ions and **Cation exchange resins** which have negatively charged bonding sites to exchange positive ions.

One of the industrially important applications of ion exchange chromatography is the deionization of water where anion exchangers are used to exchange dissolved anions in the pre-treated water for hydroxide ions and cation exchangers are used to exchange dissolved cations for hydrogen ions thereby giving water without any dissolved ions.

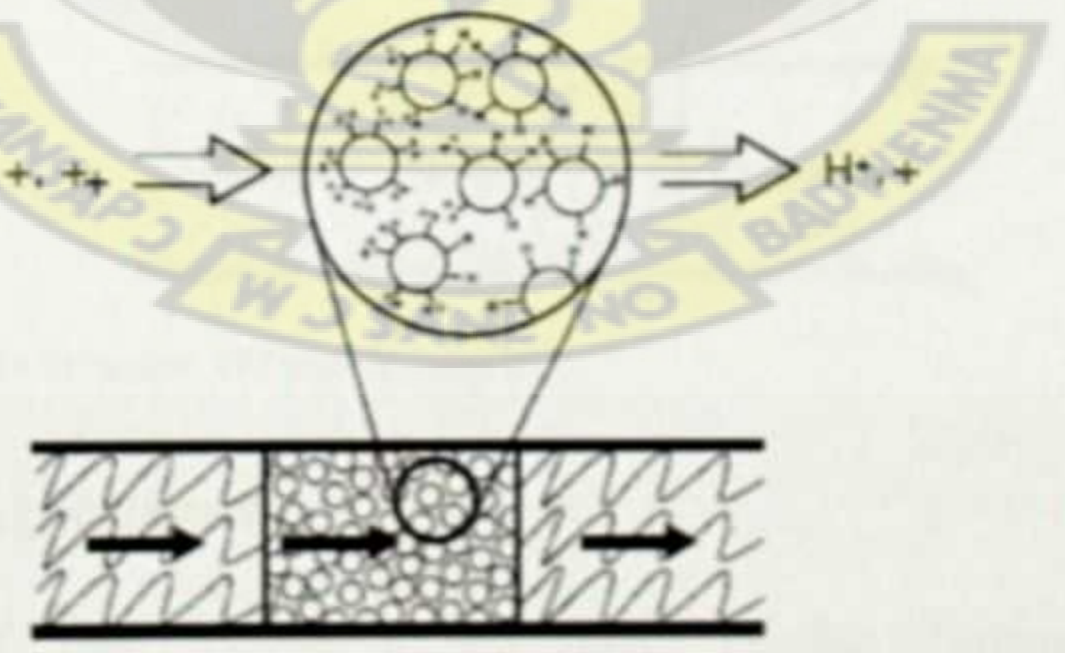


Figure 7 – An illustration of ion exchange chromatography

2.4.2.1.4 Size Exclusion Chromatography

This form of chromatography also known as gel filtration or gel permeation chromatography employs the ability of components of mixtures to penetrate the pores of the stationary phase or otherwise in the separation of mixture components. It is usually used in the separation of high molecular weight compounds (i.e. molecular weight above 10000). The stationary phase used is porous polymer resins. Smaller sized mixture components get caught within the pores of the stationary phase and as a result elute more slowly compared to larger particles or components which do not get trapped in the pores. Separation is thus achieved.

Unlike the other separation mechanisms above, there is no retention or transfer of the components from mobile phase to stationary phase or vice versa (Fifield & Kealey, 2000).

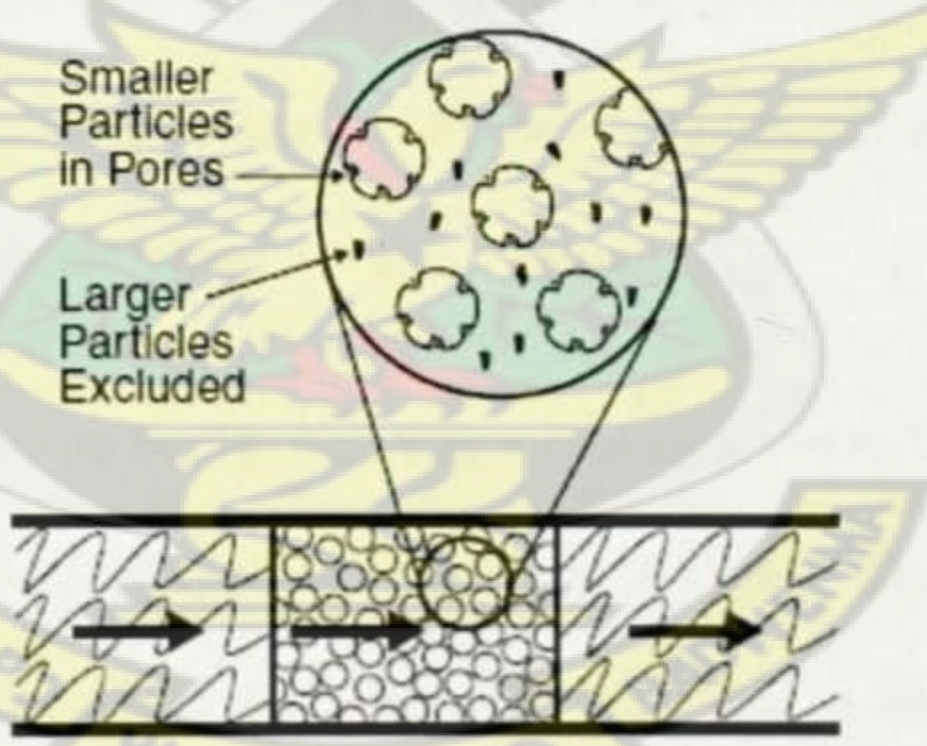


Figure 8 – An illustration of size exclusion chromatography

2.4.2.2 THIN LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) is one of the most popular chromatographic techniques employed in laboratories worldwide owing to its simplicity, flexibility and relative inexpensiveness (Reich & Blatter, 2005). TLC basically involves the separation of substances or mixtures into their component parts as a consequence of the different retention characteristics of the components; the differences arising due to solubility differences, adsorption, size of the

component and/or charge of the component (Fifield & Kealey, 2000). TLC may be used for both qualitative and quantitative analysis but the quantitative information obtained from the technique lacks the accuracy and precision offered by other chromatographic techniques such as High Performance Liquid Chromatography (Vogel, 1989a). TLC finds use in the determination of purity of substances, monitoring of reaction progression as well as the characterization of complex materials (Fifield & Kealey, 2000).

2.4.2.2.1 Stationary Phases in TLC

In TLC, the stationary phase is an adsorbent material such as aluminium oxide or silica gel that is thinly coated onto glass, plastic or aluminium supports usually rectangular in shape (Reich & Blatter, 2005). Silica gel is however, the most used sorbent whereas glass plates are the most popular supports (Adamovics & Eschbach, 1997) though in modern times the use of pre-coated aluminium plates has gained increased popularity. Some terminologies are used in describing TLC plates and some common ones are given in Table 3 below.

Samples to be separated are applied as liquids either as a spot or a band along one of the ends of the rectangular support. The place of application of the spot is usually about 1cm from the edge of the support. A line is drawn, also about 1cm, from the other end to indicate the point where the mobile phase will travel up to. Sample application may be done either manually or by automation. Automation is preferred, especially when amount to be applied is greater than 15µl, as it applies accurate amounts of sample and avoids both overloading, which may result in poor resolution, or inadequate sample application, which may result in diffusion (Adamovics & Eschbach, 1997).

2.4.2.2.2 Development of the TLC plate

The resolution of samples into the components using TLC is referred to as development. Development involves placing the coated support into a chamber known as the chromatank which contains the mobile phase. For good chromatographic separation and reproducibility of the results, the chromatank should be well saturated with the vapour of the mobile phase (Kenkel, 2003). This may be achieved by allowing the well covered chromatank to stand for about 30 – 60 minutes after pouring the mobile phase into it. The mobile phase then rises by capillary action through the adsorbent, the components of the sample separating as it travels to the upper end of the plate.

2.4.2.2.3 Detection methods

After development, the coated support is removed and air dried and one of the detection methods employed to detect the separated components. The most popular detection methods are viewing the plate under ultraviolet/visible light and derivatisation which combines spraying with a reagent and viewing under white or UV light. Derivatisation, via reaction with the components, converts them into a highly colored or fluorescing compound easily detectable (Reich & Blatter, 2005). The choice of reagent to employ in the derivatisation depends largely on the chemistry of the compound.

2.4.2.2.4 Analysis of the chromatogram

The position of a component spot in the chromatogram after development of a TLC plate is described by a value referred to as the retardation factor, R_f. The R_f value is defined as the ratio of the distance travelled by the component spot to the distance travelled by the solvent front

$$R_f = \frac{Z_x}{Z_m}$$

where,

R_f = retardation factor, Z_x = distance of the component spot from the starting point [mm] and

Z_m = distance between the solvent front and the starting point [mm]

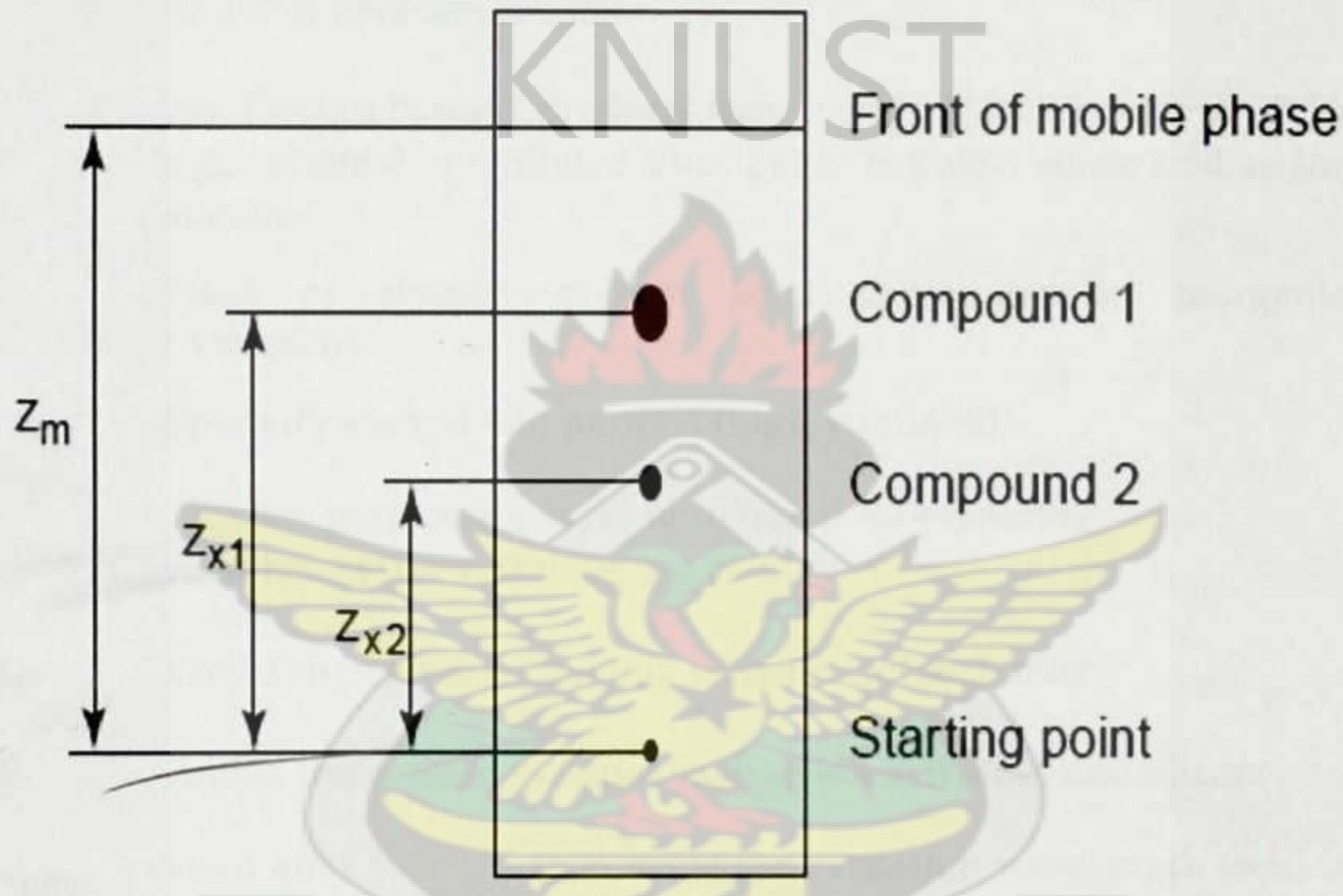


Figure 9 – A schematic planar chromatogram

Table 3 – Nomenclature of TLC plates (Adamovics & Eschbach, 1997)

NAME	MEANING
"Sil"	A product composed of silica gel, e.g., Anasil from Analabs
G	Gypsum ($CaSO_4 \cdot Vi\ H_2O$) binder ("soft" layers)
S	Starch binder
Q	Organic binder, such as polymethacrylate or polycarboxylate; layers are "hard" or abrasion resistant
H or N	No "foreign binder"; products may contain a different form of the adsorbent, e.g., colloidal or hydrated silica gel or colloidal silicic acid, to improve layer stability
HL	Hard or abrasion-resistant layer containing an inorganic hardener (Analtech)
HR	Specially washed and purified (highly refined)
P	Thicker, preparative layer or material for preparing such layers (for cellulose, see below)
P + $CaSO_4$	Preparative layer containing calcium sulfate binder
F or UV	Added fluorescent material such as Mn-activated zinc silicate
254 and 366	Used after F or UV to indicate the excitation wavelength (nm) of the added phosphor
60	Silica gel 60 (Merck) has pore size of 60 A (1OA =1 nm). Other pore size designations are 40, 80,100
D	Plates divided into a series of parallel channels
L	Layer with a pre-adsorbent sample dispensing area (Whatman)
K	Symbol used in all Whatman products
RP	Reversed-phase layer; $RP_{,8}$ or RP-C_{18} would indicate that octadecylsilane groups are chemically bound to silica gel
4, 7, 9	These numbers after the adsorbent name usually indicate pH of a slurry

2.4.2.3 CLASSICAL COLUMN CHROMATOGRAPHY

Classical column chromatography is one of the forms of column chromatography available for the separation of components of mixtures. It finds wide use in the separation of components of plant extracts. It may be seen as a form of pre-treatment for some mixtures.

Here, the setup consists of an open glass tube placed vertically in a clamp or any holder in which silica gel or other adsorbent (which is the stationary phase) and the sample to be separated are placed. The sample is placed on top of the stationary phase as shown in Fig. 8.

The mobile phase is poured onto the sample and allowed to elute through the stationary phase separating the mixture. The eluting components are collected in either beakers, test tubes or any suitable containers. The setup uses gravity for the elution.

The elution process results in the separation of components of the mixture based mainly on their polarities. Gradient elution is normally employed in column chromatography. The elution usually begins with a non-polar mobile phase, the polarity being increased over time. Non-polar components elute first followed by medium polar and highly polar components. The elution power of a solvent depends largely on its polarity as well as the polarity of the stationary phase and the nature of the components of the mixture (Fifield & Kealey, 2000).

However, there's a modification which employs pressure to push the solvent through the silica gel column and this is known as flash chromatography. It offers better separation as well as reduced separation time.

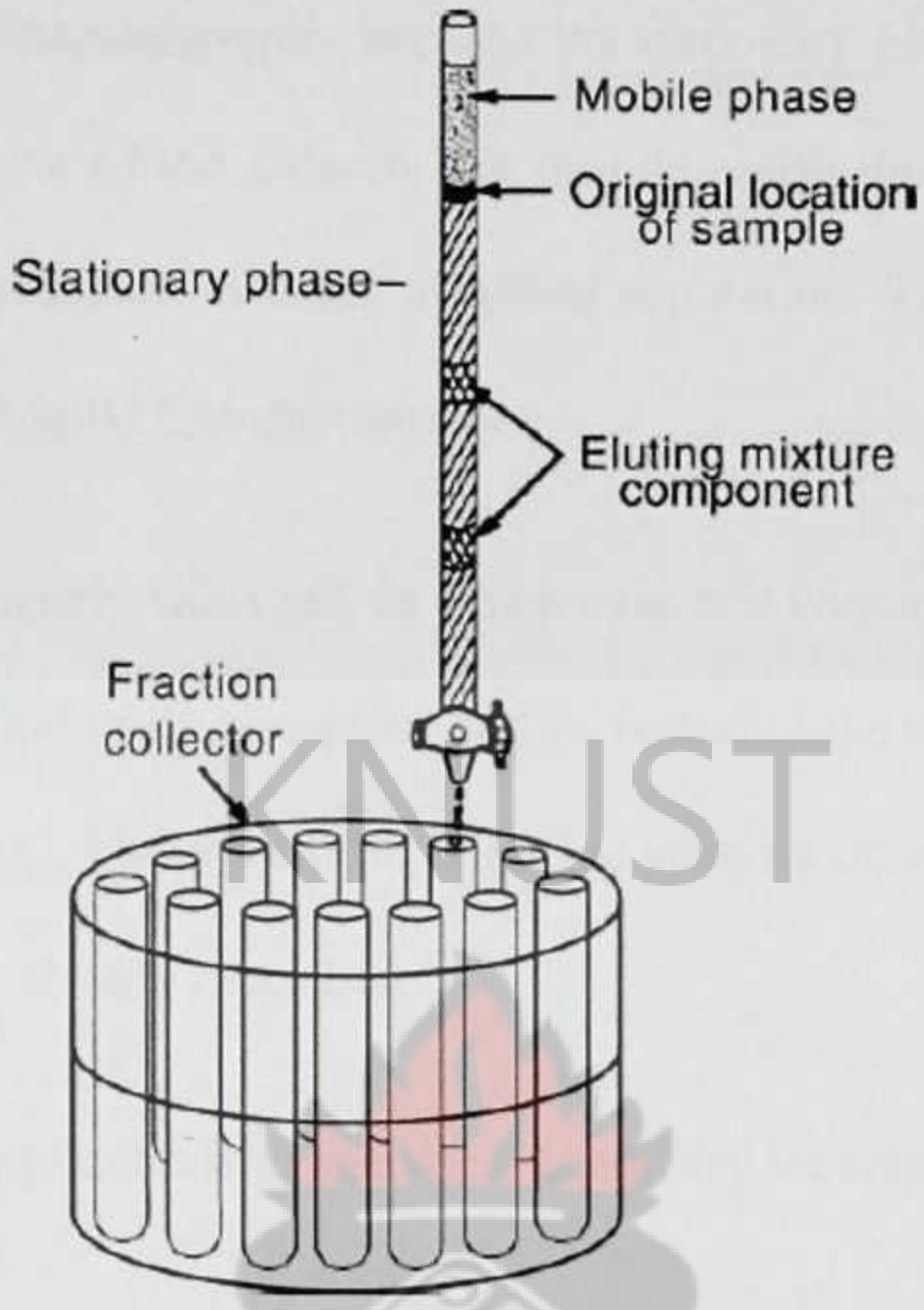


Figure 10 – An example of a classical column chromatography setup

2.4.2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is one of the most widely used chromatographic methods of analysis in modern day analytical chemistry. It is the preferred method for the analysis (or separation) of the components of complex mixtures containing non-volatile compounds (Stefova et al. 2001).

As one of the improvements in column chromatography, HPLC as is now known became available in 1969 but its acceptability came later and in the 1990s became the most widely employed analytical technique evidenced by the sale of HPLC instruments and its importance to analytical science (Galichet et al. 2005). Its popularity was due to its application to a wide array of compounds, its high resolving power, its accuracy, precision and robustness in the analysis of pharmaceuticals, its speed of analysis as well as its ability to detect nanomolar concentrations of compounds (Galichet et al. 2005; Watson, 1999).

t is also a form of column chromatography because the stationary phase is packed into a very narrow column. Due to the size of the column, the mobile phase must be pushed through the stationary phase under high pressure in order to afford separation. This technique is therefore known also as High Pressure Liquid Chromatography.

The stationary phase used is mostly silica gel, in which case it is known as Normal Phase HPLC, or chemically modified silica gel (with non-polar groups bound to the surface) in which case it is known as Reverse Phase HPLC. The separation in HPLC analysis occurs via one or more of the chromatographic mechanisms already described.

HPLC has a wide variety of applications in analytical chemistry including but not limited to

- ✓ The purification of synthetic and natural products including plant extracts.
- ✓ Pharmacodynamic and pharmacokinetic studies.
- ✓ Monitoring of stability of pure drug substances as well as drugs in formulations by quantitative analysis of any degradation products.
- ✓ Analysis of drugs and their metabolites in biological fluids.
- ✓ Determination of partition coefficients and pKa values of drugs.

2.4.2.4.1 Normal Phase HPLC (NP-HPLC)

This is the first form of HPLC that was employed in analytical chemistry. In NP- HPLC, the stationary phase is a polar material, mostly silica gel, and the mobile phase less polar or non-polar. The extent to which a component of a mixture or sample is retained depends largely therefore on its polarity (Watson, 1999).

he stationary phase may also be made of bonded phase silica gel where polar groups such as mino, cyano and diol groups are bound to the surface of the silica gel. This overcame an earlier roblem of the stationary phase washing off the column (Patnaik, 2004).

.4.2.4.2 Reverse Phase HPLC (RP-HPLC)

n RP-HPLC, the stationary phase is made of a non-polar material, mostly silica gel with highly ıon-polar groups such as long alkyl chains bound to the surface. It is the more preferred mode of HPLC analysis.

The most employed stationary phase material for RP-HPLC is Octadecylsilane (ODS), which is an 18-carbon alkyl chain, bonded silica gel. Other materials that are used in place of ODS include an 8-carbon alkyl chain, a cyclohexyl group and a phenyl group. The 18-carbon alkyl chain is introduced by reacting the OH groups on the surface of the silica gel with a silane, which contains the 18-carbon alkyl chain, side groups such as methyl groups and a group that can react with the surface of ~~the silica~~ (Cl groups are normally used) (McPolin, 2009). Not all the silanol groups of the silica gel may react and so this poses a problem of secondary retention during analysis particularly when basic compounds are present. The secondary retention stems from the interaction of some of the sample components with the unreacted or free silanol groups. This problem may be overcome by endcapping, where the residual silanol groups are reacted with a group such as trimethylchlorosilane. (McPolin, 2009)

The extent of retention of a compound on the stationary phase depends on its lipophilicity which determines whether it partitions more into the ODS-bonded silica gel or the relatively more polar mobile phase (Watson, 1999).

2.4.2.4.3 Instrumentation

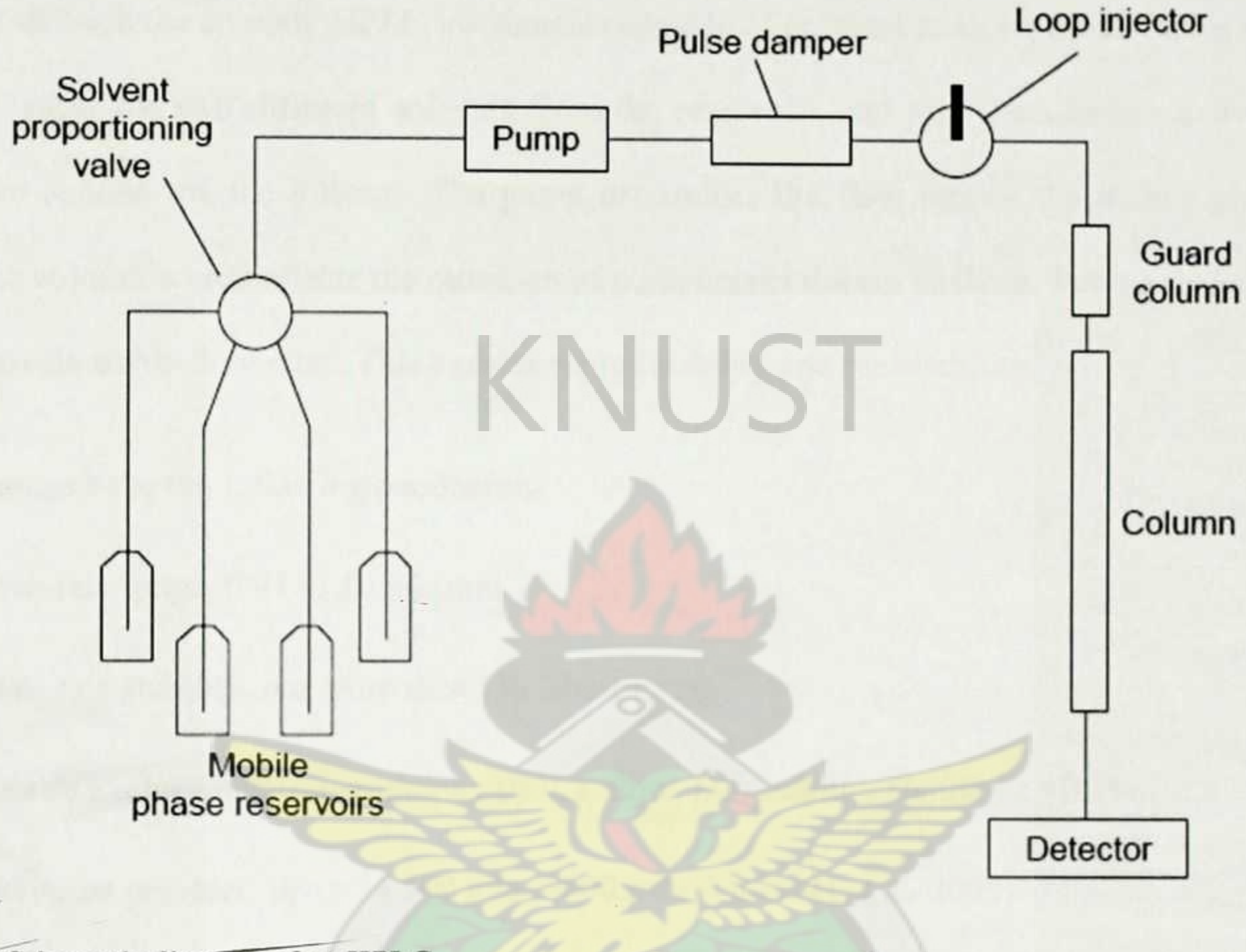


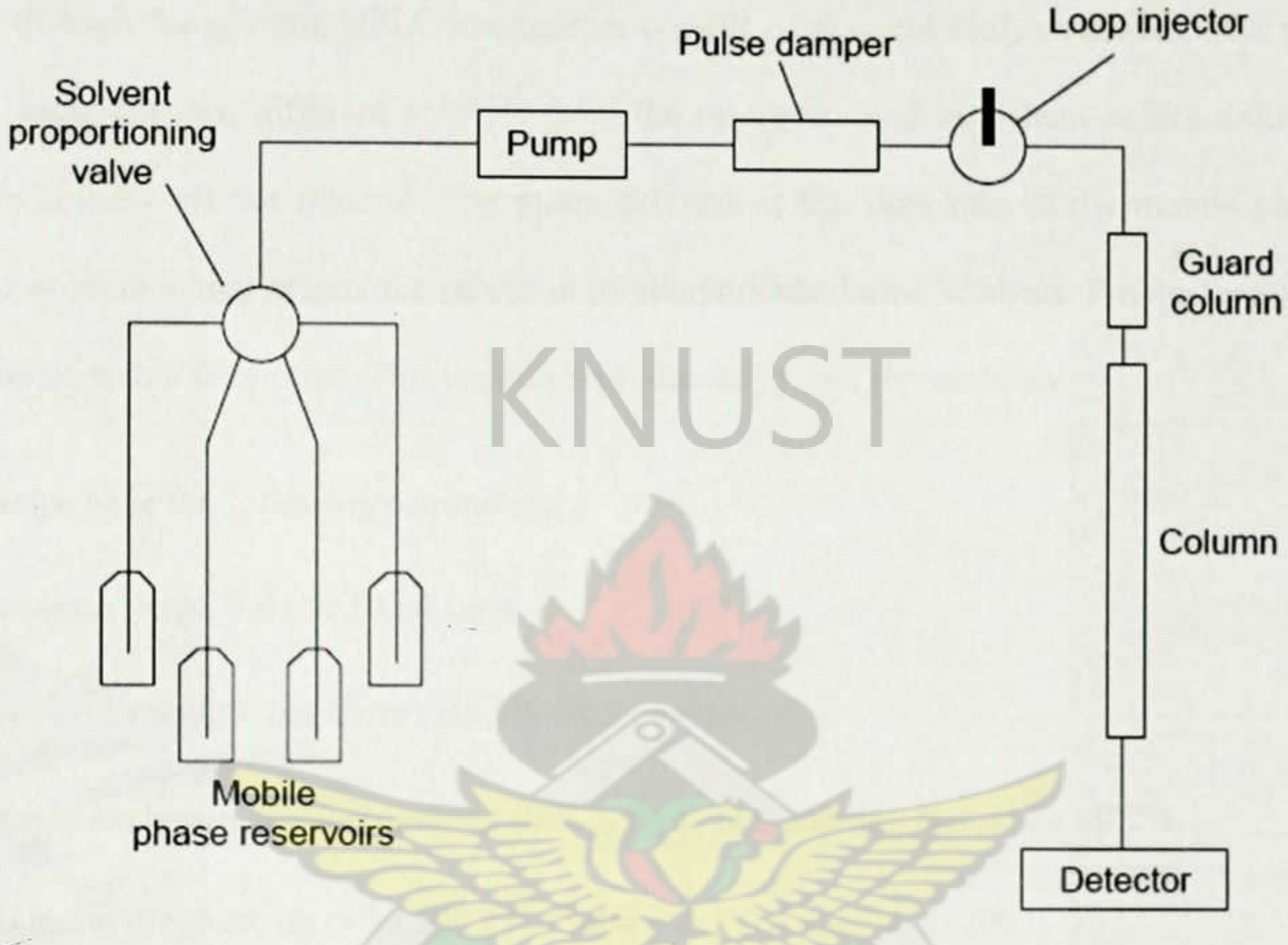
Figure 11 – Schematic diagram of an HPLC

The main components of a high performance liquid chromatograph are the mobile phase reservoir(s), the pump, the injector, the column and the detector.

MOBILE PHASE RESERVOIR

The reservoir is the 'storage' vessel for the mobile phase(s) during the analytical process. The pump draws the solvent(s) from the reservoir through the column to afford separation of the sample introduced into the instrument. With isocratic elution, only one reservoir is needed. However, when gradient analysis (i.e. more than one mobile phase system is used) is to be carried out, more than one reservoir is required to hold the solvents used to make up the solvent systems.

4.2.4.3 Instrumentation



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PUMP

The pump is that part of the instrument that sucks the mobile phase from the reservoir and 'pushes' it through the column. HPLC instruments capable of gradient analysis usually have two pumps to 'suck' the two different solvents from the reservoirs and mix them before delivery through the column via the injector. The pump determines the flow rate of the mobile phase through the column which affects the retention of components during analysis. Pumps therefore need to provide stable flow rates. This ensures reproducibility and repeatability.

Modern pumps have the following parameters:

- ✓ Flow–rate range, 0.01 to 10 mL/min.
- ✓ Flow–rate stability, not more than 1% (short term).
- ✓ For size exclusion chromatography (SEC), flow–rate stability should be <0.2%.
- ✓ Maximum pressure, up to 34 500 kPa (5000 psi). (Galichet et al. 2005)

There are two elution methods in HPLC analysis and the method to be employed determines the choice of pump used. The two methods are isocratic and gradient elution. In isocratic elution, one mobile phase system is used throughout the entire separation process (Kenkel, 2003). If the mobile phase combination is to be changed, the pump would have to be stopped and the mobile phase reservoir refilled with the new mobile phase or replaced with one that contains the new mobile phase. In gradient elution, the mobile phase combination is changed in the course of the analysis and this is often done gradually with the aid of two or more pumps (depending on the number of solvents in the mobile phase combination).

INJECTOR

The injector is where the sample to be analyzed is introduced into the system. A solution of the sample is made in the appropriate solvent (could be the mobile phase or any other solvent though the mobile phase is preferred if it is capable of dissolving the sample) if the sample is solid and it is injected into the instrument where the mobile phase drives it through the column to afford separation of the components.

The injector is required to deliver the liquid sample in the range of 0.1ml – 100ml of volume with high pressure and should be reproducible (Galichet et al. 2005).

The standard loop injector is a valve fitted with a fixed volume loop which delivers fixed volumes of sample into the system for analysis.

COLUMN

The column is the support in which the stationary phase is 'stationed'. The columns come pre-packed with the stationary phase and depend on the type of analysis desired i.e. whether normal or reverse phase analysis. The column normally used is made of stainless steel and the stationary phase, ODS-bonded silica gel with an average particle size distribution of 3, 5 or 10µm (Watson, 1999). There are various stationary phases suited for various separations and therefore the column used for a particular separation process depends largely on the sample to be analyzed, the chromatographic mechanisms to be employed i.e. whether adsorption, partition, ion exchange or size exclusion.

Chromatographic columns typically come in twos; one being the analytical column and the other the guard column. The analytical column is that column which does the actual separation whereas the guard column, as its name suggests, is used to protect the analytical column from

contamination. Contamination may result from irreversibly adsorbed compounds and/or particles injected with the liquid sample which may clog the analytical column (Harvey, 2000). The guard column therefore prolongs the 'life' of the analytical column by preferentially being contaminated.

Some columns are fitted with thermostats to control temperature fluctuation as large temperature variations may tend to affect measurement of peaks in analysis in terms of size or area. It is often desirable that the temperature fluctuation be within ±0.2°C (Galichet et al. 2005).

DETECTOR

The detector is the part of the instrument that notices the presence of compounds as they elute from the column by measuring a given property of the compound. The detector then relays this information to a recorder which gives a visual representation of the detection which is referred to as the chromatogram. The chromatogram is therefore essentially a plot of the detector's signal as a function of the time of elution or the volume of mobile phase required for elution (Harvey, 2000).

There are many types of detectors used with HPLC systems. The most common ones are based on spectroscopic measurements. The UV/Vis and fluorescence detectors are therefore very popular. The UV/Vis detector measures absorbance and thus the chromatogram obtained is a plot of absorbance versus the retention time. The absorbance is proportional to area of the peak obtained and so the plot may be of peak area against retention time instead. UV/Vis detectors measure the absorbance at given wavelengths and so multicomponent samples with different wavelengths of absorption can be quite difficult to detect. Diode array detectors (DAD) capable of measuring absorbance over the entire ultraviolet wavelength range using photodiodes are

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preferred for multicomponent analysis. They present a three-dimensional chromatogram of absorbance or peak area as a function of wavelength and retention time. Fluorescence detectors detect fluorescent emission resulting from excitation of the compound at a given wavelength.

Other detectors such as electrochemical detectors measure different properties of the eluting components based on amperometry, voltammetry, coulometry and conductivity. Their detection limits are quite good; the detection limit for amperometry being between 10pg and 1ng of injected analyte (Harvey, 2000).

There are still other detectors that measure other properties of the components. An example is refractive index detector that measures the refractive index of the eluting components. It is suitable for practically every sample but their detection limit of 100ng - 1µg of injected analyte is not comparable to other detectors. It is however, not suited for gradient elution unless the refractive indices of the component solvents in the gradient are identical (Harvey, 2000).

2.4.2.5 SOME TERMS USED IN CHROMATOGRAPHY

There are a host of terms used to describe the chromatographic columns as well as the chromatogram obtained during analysis. If a compound, during a chromatographic analysis, does not partition significantly or at all into the stationary phase, then it will travel through the column at the same rate as the mobile phase. The volume or time of mobile phase then, that is needed to pass an unretained compound through the column, is referred to as the **void volume, V_o** or **void time, t_o** of the column. The **retention time**, t_r of an analyte is the time required for the analyte to pass through the column, i.e. the time between injection of the analyte and the appearance of the peak maximum. The **retention volume, V_r** is then the volume of mobile phase that moves a retained analyte from the point of injection to the point of detection.

2.4.2.5.1 Retention Factor

The retention factor is also known as the capacity factor in some literature. It gives a measure of the distribution or partitioning of a compound between the stationary and mobile phases. The retention time of an analyte is therefore dependent upon the retention factor.

The retention factor designated K' is given by the equation

$$K' = \frac{V_r - V_o}{V_o} = \frac{t_r - t_o}{t_o} = \frac{t'_r}{t_o}$$

The parameters are as described above. t'_r is the adjusted retention time of the component.

2.4.2.5.2 Column Efficiency

Column efficiency is expressed in terms of the number of theoretical plates of an HPLC column. The column may be divided into sections of equal dimensions at which partitioning of the components occurs between the stationary and mobile phases. The number of theoretical plates, N is related to the height of each plate H, and the length of the column L, by the equation

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

Column efficiency also describes quantitatively how broad the band of a component is. During separation, the band of an analyte, which is small at the start of the analysis, broadens as the component travels along the column. The width of the band increases. The broadening occurs as the individual molecules of the analyte spread out in the column in the course of the analysis. A column is said to be less efficient if a peak broadens rapidly as the molecules of the compound travel along it (Watson, 1999).

For a peak with a Gaussian shape, the height of the theoretical plate H, is related to the width of the peak, the retention time and the length of the column by the equation

$$H = \frac{Lw^2}{16t_r^2}$$

Substituting H into the previous equation gives

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

Alternatively, the width of the peak at half its height $w_{1/2}$ may be used to make the equation

$$N = 5.545\left(\frac{t_r}{w_{1/2}}\right)^2$$

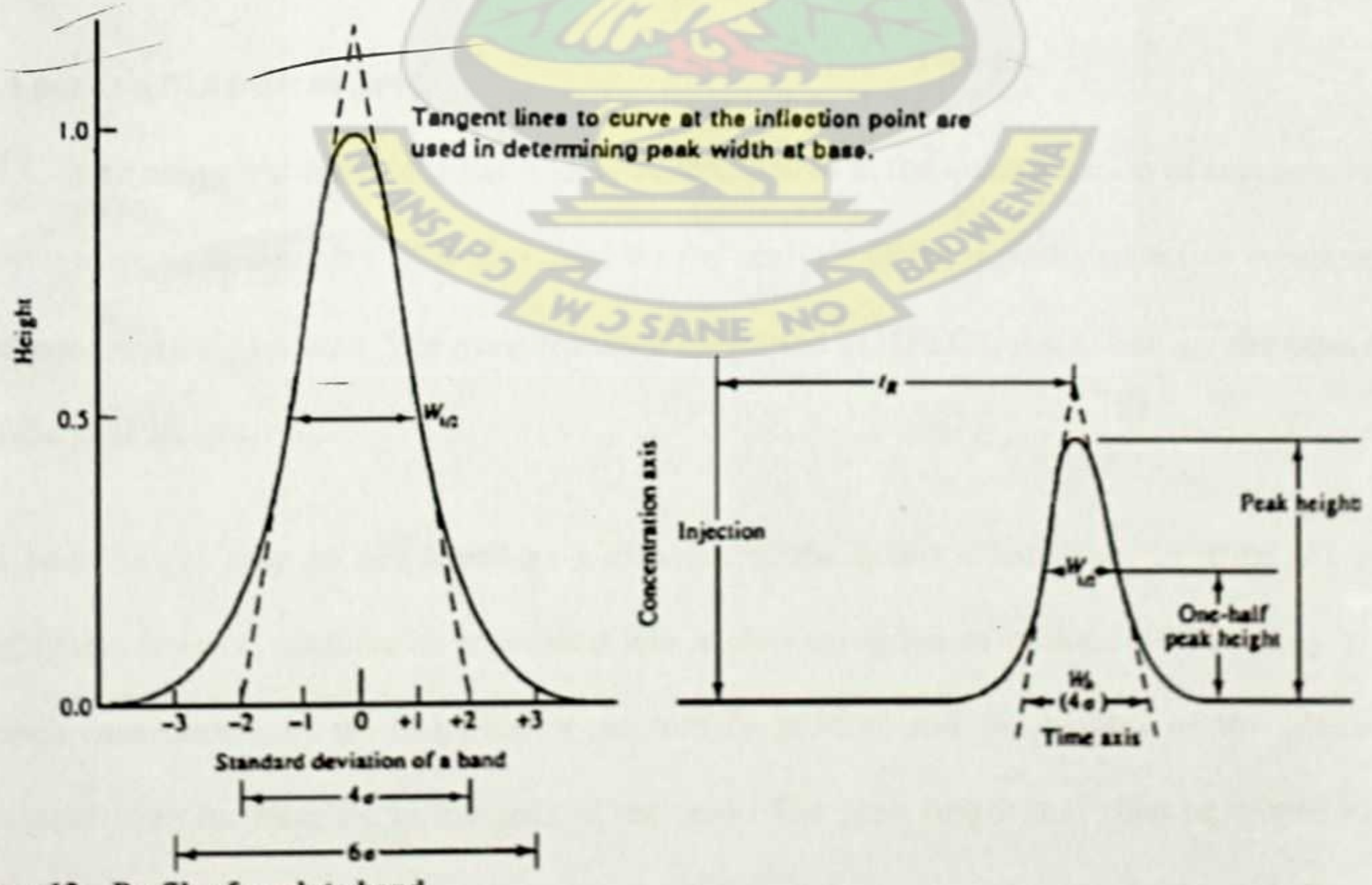

Figure 12 – Profile of a solute band

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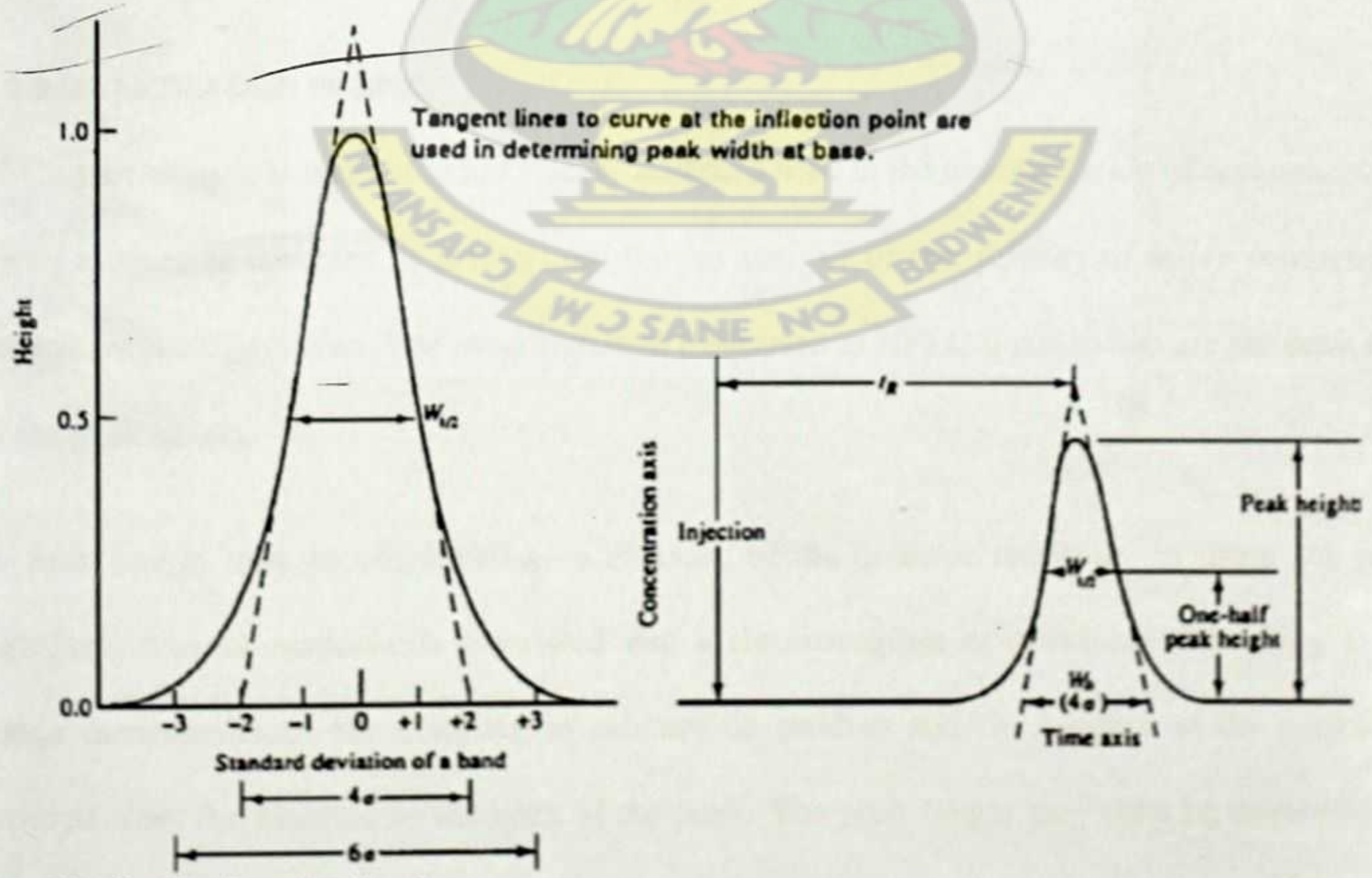

Figure 12 – Profile of a solute band

2.4.2.5.3 Column Selectivity

The selectivity of a column describes its relative selectivity with respect to a pair of solutes. It is represented as relative retention α and given by the equation

$$\alpha = \frac{t'_{r,2}}{t'_{r,1}}$$

where solute 1elutes faster than solute 2.

2.4.2.5.4 Chromatographic Resolution

Resolution describes the ability of a column to resolve peaks due to two components with similar elution time. The chromatographic resolution Rs, is defined as the difference between the retention times of the two peaks and the average of their peak widths. This is written as

$$Rs = \frac{t'_{r,2} - t'_{r,1}}{0.5\,(w_2 + w_1)}$$

2.4.2.6 QUANTITATION IN HPLC

HPLC as an analytical technique has widely accepted uses in the quantification of components of a given mixture or complex. It is also used for the analysis of the quantity of active components in pharmaceutical products. The most common tools used in HPLC quantitation are the peak area and the peak height.

The peak height may be employed as a measure of the detector response. In using the peak height, the detector response is translated into a chromatogram of peaks corresponding to the various components of the complex or mixture or product and the heights of the peaks are measured from the baseline to the apex of the peak. The peak height may then be related to the concentrations of the components accordingly. The peak height may be obtained manually or by

ıe use of computer integrated systems connected to the detector. It is one of the easiest ways of uantitation in HPLC analysis. It is preferred in the analysis of trace amounts of substances.

'he peak area may also be used in quantitation and is in fact, the most used of the methods of uantification in HPLC. The area of the peak is calculated like as the area of a triangle seeing as ı symmetrical peak takes the shape of a triangle. There are now computer softwares that do the :alculation of the peak areas quite efficiently and easily. This approach is based on the fact that for a given compound, the ratio of the peak areas of the test and reference samples is proportional to the ratio of the concentrations of the test and reference samples. A calibration curve is plotted in most cases to determine the range of concentration which the above statement holds very true. The calibration curve approach may be done in three ways discussed below:

2.4.2.6.1 Calibration by external standards

Here, various concentrations of the external standard of the analyte in question are prepared, commonly by serial dilution. The solutions are then injected and their peak areas noted and used to plot the calibration curve. After this, the sample of interest is also injected and the peak area noted. The concentration of the analyte in the sample is traced from the calibration curve with the aid of the peak area measured.

2.4.2.6.2 Calibration by internal standards

In this method, a fixed amount of an internal standard, a different compound from the analyte of interest which is well separated from the analyte in the chromatogram, is added to varying concentrations of standard solutions of the analyte. The ratio of the peak areas of the standard and internal standard are calculated for each standard solution concentration used and a calibration curve plotted of the ratio obtained against the concentrations used. An unknown

concentration of the sample to be analyzed is also mixed with the same fixed amount of the internal standard and its peak area noted. The concentration of the analyte in the sample is then determined by extrapolation of the ratio of the peak areas of sample and internal standard.

2.5 SPECTROSCOPIC ANALYSIS OF SECONDARY METABOLITES

Before the 20th century, most quantitative analytical techniques involved titrimetry and gravimetry. This was good for analysis of samples occurring in major or minor quantities but not for trace amounts of materials. Early in the 19th century, chemical analysts began developing new methods for the analysis of trace amounts, one of which is spectroscopy but even this was limited to ultraviolet, visible and infrared radiations only. Spectroscopy has now been developed even further to include other radiations in the electromagnetic spectrum such as x-rays, microwaves and radio waves. (Harvey, 2000)

Spectroscopy basically involves the study or measurement and detection of the interactions between electromagnetic radiations and various chemical substances. The interactions results in either absorption or emission and this forms the basis of most, if not all, spectroscopic methods.

2.5.1 ULTRAVIOLET/VISIBLE SPECTROSCOPY

Absorption of radiation in the ultraviolet and visible regions of the electromagnetic spectrum is widely employed in the pharmaceutical and biomedical industries for the analysis of compounds quantitatively. It also has applications in the analysis of drugs, their impurities as well as their metabolites, albeit with varied limitations (Galichet et al. 2005).

UV/visible spectroscopy deal with absorption of radiation in the ultraviolet/visible range of the electromagnetic spectrum. Absorption occurs when electrons, arranged in energy levels in the orbitals around the atom, gain energy in order to move to higher energy levels. This causes

excitation and is referred to as absorption. Some electrons also lose energy in order to drop back into orbitals of lower energy and this is known as emission.

When light passes through a solution of an analyte, the intensity of the radiation that comes out of the sample is reduced. This occurs due to absorption of the radiation by the solute particles. The absorption that occurs is referred to as absorbance and measured and plotted against another parameter, often wavelength, to give a spectrum.

Two common terms employed in absorption and emission spectroscopy are transmittance and absorbance. Transmittance (T) is the ratio between the intensity of light passing through a solution without the analyte species incident on a detector (I_o) and the intensity of light passing through a solution of the analyte species incident on the detector (I). I_o represents the maximum intensity of the incident light since there's no absorbing species in the blank solution used for measuring I_o (Kenkel, 2003).

$$T = \frac{I_o}{I}$$

Transmittance does not vary linearly with the concentration of analyte species. However, the logarithm does and this is referred to as absorbance (Kenkel, 2003). Transmittance is therefore measured and in the UV spectrophotometer converted to absorbance.

$$A = -\log T$$

A law governs the application of UV spectroscopy for the quantitative analysis of compounds using the absorbance or transmittance. This law is usually referred to as the Beer-Lambert Law. They are two different laws combined into one.

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Beer's law states that the transmittance of a stable solution is an exponential function of the concentration C of the absorbing solute while Lambert's law states that for parallel, monochromatic radiation that passes through an absorber of constant concentration, the radiant power decreases logarithmically as the path length b increases arithmetically (Patnaik, 2004).

An equation is derived which relates the absorbance A, concentration C, and the path length b. A constant is introduced into the equation known as the absorptivity or molar absorption coefficient depending on the unit of concentration used.

$$A = a\,b\,C$$

2.5.2 INFRARED (IR) SPECTROSCOPY

Like UV spectroscopy, infrared spectroscopy involves the measurement of absorption of energy. However, the energy is within the infrared region of the electromagnetic spectrum. Interaction of molecules with infrared radiation results in changes in the energy of vibration of these molecules (Fifield & Kealey, 2000).

2.5.2.1 BOND VIBRATIONS

Atoms within a molecule are bonded together and these bonds are constantly in vibrational and rotational motion. The shortest distance between two vibrating, bonding atoms in the ground state of the atoms is referred to as the bond length. The vibrations occur at certain frequencies that represent particular bonds. Different frequencies occur within the infrared region of the electromagnetic spectrum. Absorption in the infrared region then occurs when the frequency of vibration of a bond corresponds to the frequency of an externally applied infrared radiation.

Molecules undergo two main forms of vibrational motions: bending and stretching. The two motions require different amounts of energy for absorption; stretching requires more energy.

IR active bonds are polar bonds and possess dipole moments which change periodically with their vibrations. The absorption or interaction of the infrared radiation with the dipoles of the polar bond causes an increase of decrease in the stretching (amplitude) of the bond. Different bonds and vibrational modes have different absorption characteristics and so present with different intensities in the spectrum (Galichet et al. 2005). The carbonyl (C=O bond) stretches at around 1650 cm^{-1} and generates a strong dipole moment which is prominent in a spectrum. The C-H bond on the other hand generates only a small or relatively weak dipole moment but the abundance of this bond in many molecules results in an additive value which then features prominently in the spectrum (Galichet et al. 2005).

Symmetrical bonds such as H–H (hydrogen gas), –C–C– (ethane), O–O (oxygen) and N–N (nitrogen) possess no dipole moments and therefore their stretching is not observed (Galichet et al. 2005).

2.5.2.2 WAVELENGTH AND WAVENUMBER

The infrared region of the electromagnetic spectrum extends from 800 nm to 1,000,000nm. IR radiation was formerly expressed in wavelength terms but is now almost always referred to in terms of wavenumber ($\tilde{\nu}$) which is the inverse of the wavelength. Wavenumbers are expressed in units of cm^{-1}. The region may be subdivided into three regions which are 12 500 to 4000 cm^{-1} (0.8 to 2.5 μm; near IR), 4000 to 400 cm^{-1} (2.5 to 25 μm; mid IR), and 400 to 10 cm^{-1} (25 to 1000 μm; far IR) (Galichet et al. 2005). The energy of an infrared radiation varies proportionally as its wavenumber.

2.5.2.3 IR REGIONS: FINGERPRINT AND FUNCTIONAL GROUP

The mid IR portion is the portion of interest and is therefore simply referred to as the IR region. Within the mid IR portion, the range 400 to 1400 cm^{-1} is referred to as the fingerprint region. In this region, many absorption bands are obtained and so spectra of unknown samples can be matched with spectra of known samples for identification. The region from 1400 to 4000 cm^{-1} is filled with relatively fewer absorption bands and is known as the functional group region. In this region, only the vibrations associated with certain functional groups register absorption bands which also make such spectra ideal for the identification of functional groups peculiar to compounds.

2.5.2.4 SAMPLE PREPARATION AND ANALYSIS

IR spectroscopy can be employed in the analysis of solids, liquids and gases. The cells that hold the samples are made from salts such as NaCl and KBr which do not absorb IR radiation (Harvey, 2000). Considering the high water solubility of these salts, water must be prevented at any cost from gaining access to the cells.

In analyzing liquid samples, one may use the pure liquid for the analysis or as a solution. The former is preferred for identification purposes or for the determination of purity. When the latter is employed, the solvent in which the analyte is dissolved may pose slight difficulty as it may be IR active and so will give bands together with the analyte. For this reason, solvents such as CCl_4, $CHCl_3$, and CH_2Cl_2 which have very simple IR spectra are used (Kenkel, 2003). For non-volatile liquids, a drop of the liquid may be placed between two NaCl discs to form a thin film for analysis. For volatile liquids however, the sample is placed into the cell which must be sealed (Harvey, 2000).

For solid samples, a solution may be prepared and treated as above. The sample may alternatively be dissolved in a suitable solvent and a few drops of the solution placed on one salt disc or plate. The solvent is then allowed or forced to evaporate forming a thin film of the solid sample. This disc is then placed in the path of the infrared radiation for analysis. Again, the solid sample may be premixed with finely powdered KBr and pressed into a wafer which is then placed in the path of the radiation for analysis. Solid materials may also be mixed with mineral oil to form a smooth paste which is then squeezed lightly between two salt discs. The solid sample must be finely powdered in this case. (Kenkel, 2003)

2.5.2.5 IR SPECTRA

The spectrum obtained from infrared spectroscopy differs slightly from that obtained in UV spectroscopy due to the fact that the IR spectrum is a transmission spectrum and not an absorption spectrum. Percentage transmittance is plotted against wavenumbers and not wavelength on the horizontal axis.

2.5.3 MASS SPECTROMETRY

Mass spectrometry is one of the most versatile analytical techniques with varied applications in physics, biology, chemistry as well as medicine. Its main applications include the analysis of chemicals and the identification of trace amounts of impurities whether in drug samples or biological fluids.

Mass spectrometry offers a number of advantages over other analytical techniques. Amongst its advantages are its high specificity in the determination of the identity, structure of compounds and the presence of impurities in compounds.

A mass spectrometer functions by producing charged or ionized fragments of a molecule which pass through a magnetic field within the instrument and are directed towards the ion detector. The charged fragments may be produced either in or prior to reaching a high vacuum (Galichet et al. 2005). The ions are produced when electrons, produced from hot tungsten or rhenium wire or filament, collide with the molecules of the sample introduced. The ions are then repelled through accelerating plates (see Fig. 13) which cause them to gain kinetic energy. This energy gained propels them through a curved path along the mass analyzer. As they move through the magnetic field and collide with the ion detector, they are detected as a function of their mass to charge ratios (m/z). The m/z values of the ions generated is related to the radius of the magnetic sector, the strength of the magnetic field, and the acceleration voltage within the instrument (Mikkelsen & Corton, 2004).

The mass spectrometer explained above is a single-focusing mass spectrometer which employs electron-impact for ionization. These spectrometers are not suitable or preferred when the sample to be analyzed is non-volatile or unstable (Galichet et al. 2005). There are other mass spectrometers that employ other ionization techniques. For instance, soft ionization techniques are employed for the analysis of biomolecules which are macromolecular in size.

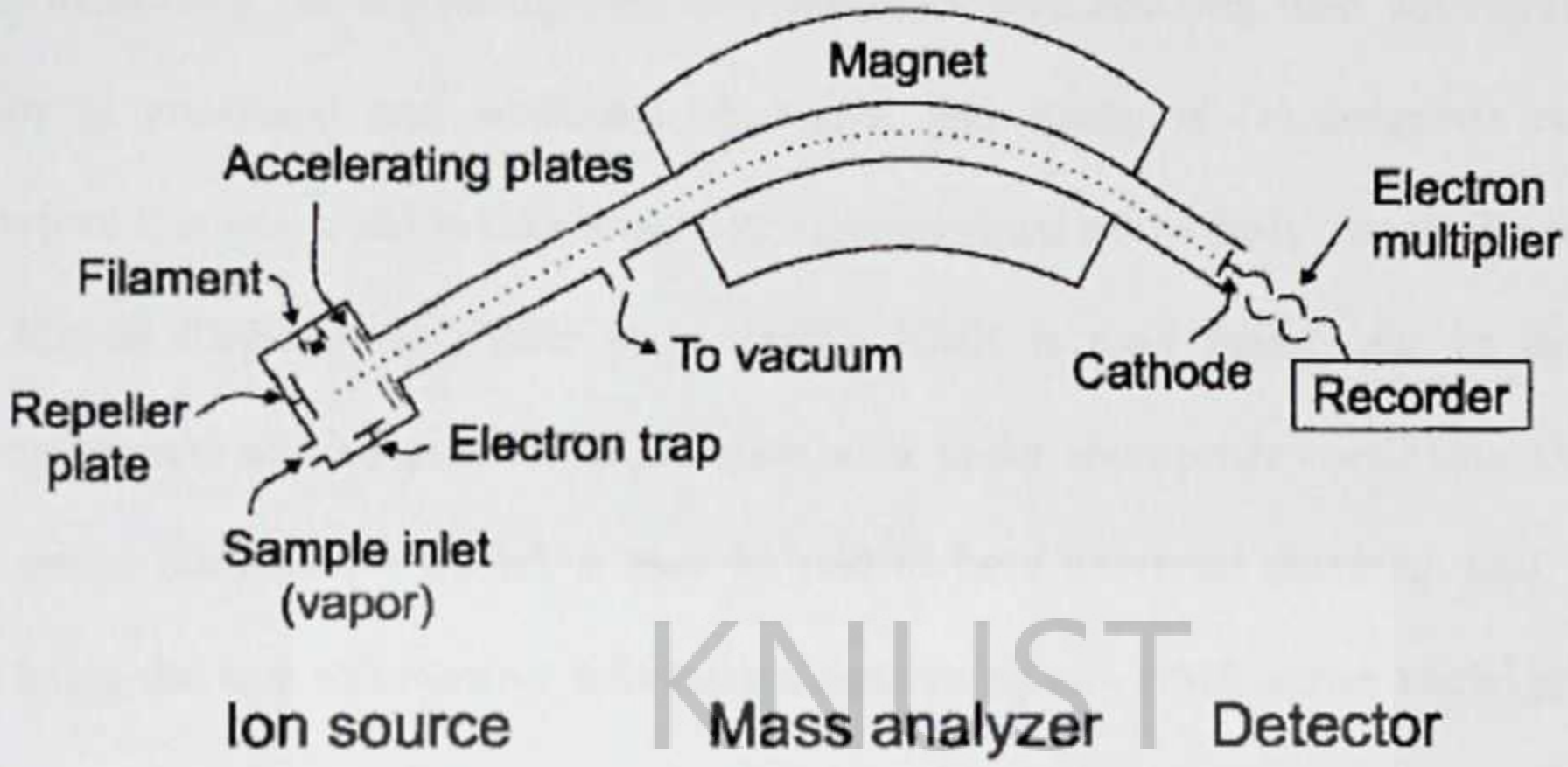

Figure 13 – Schematic diagram of a mass spectrometer

2.5.3.1 MASS SPECTRA

A mass spectrum is a plot of m/z ratio against the signal intensity recorded and consists of sharp peaks due to fragments of a molecule under study. A specific molecule will always give the same mass spectrum during analysis because the pattern of fragmentation is the same in every analysis for the same molecule or compound.

The most abundant ion or fragment is given a relative abundance of 100% against which all other peaks are determined.

The molecular ion is the ion with the highest m/z ratio of all the ions in the spectrum. It is represented by M^+, the positive sign referring to the loss of an electron.

2.5.4 NUCLEAR MAGNETIC RESONANCE

Nuclear magnetic resonance (NMR) is a spectroscopic technique that is employed principally for structural elucidation of various chemical species or compounds (Patnaik, 2004). It has gained wide acceptance since it was first observed in 1945 and is now one of the principal methods of

dentifying molecules, understanding their conformations and detailing their structures. It has ıpplications in analytical and medicinal chemistry, and medicine (as magnetic resonance maging) where it is employed to take images of various organs of the body, especially the brain, to make clinical diagnosis (Galichet et al. 2005). NMR is used mainly for its qualitative applications but may also be used for quantitative work under appropriate conditions. Due to its ability to detect NMR-active nuclei, it may be said to be a universal detection tool, its only limitation being the lack of objective information concerning non NMR-active nuclei groups on a molecule.

Amongst other things, NMR seeks to answer questions pertaining to the location, identity and number of specific nuclei in a given molecule. It also describes neighboring nuclei and the relationship between the identified nuclei (Patnaik, 2004).

2.5.4.1 THEORY

Nuclei are positively charged species that possess a quantized parameter known as spin. As the nuclei spin, a small/local magnetic moment (field) is generated.

The magnetic field of the spinning nuclei tends to align itself with an external, more powerful applied magnetic field. This behaviour is like the compass needle which aligns itself in the same direction or along the same plane as the earth's more powerful magnetic field. The nuclei tend to assume an opposite alignment against the external magnetic field when they are excited through the absorption of a small amount of energy that corresponds to the radio frequency range of the electromagnetic spectrum. The two alignment configurations represent different energy states. These energy states are constantly in resonance as nuclei jump from one state to another.

The resonance frequency denoted as v, that causes the jump from one state to another is represented as

$$\Delta E = hv = \frac{\mu H_o}{I}$$

- ✓ h = Planck's constant,
- ✓ μ = magnetic moment of the nucleus,
- ✓ I = spin quantum number (h/π)
- ✓ H_o = strength of the external magnetic field

In most NMR instruments, a fixed radio frequency transmitter is employed and the strength of the external magnetic field varied in the course of the analysis. At a fixed radio frequency, absorption of energy by the nuclei occurs at different field strengths which is measured and plotted to give the spectrum – absorption vs. field strength (Patnaik, 2004).

There are many NMR active nuclei available for NMR studies including fluorine 19, nitrogen 15, phosphorous 31 amongst others but the two most commonly used ones are proton NMR and ^{13}C NMR.

In proton NMR, the focus is on hydrogen atoms bonded covalently to other atoms, mostly carbon. Proton NMR finds widespread use due to its sensitivity and detailed information concerning the structure of compounds that it affords (Watson, 1999).Protons and hydrogen nuclei may be used interchangeable in some texts to refer to the hydrogen atoms under study.

In ^{13}C NMR, the focus is on carbon atoms with atomic number 13. These carbon atoms do not occur in abundance in nature compared to ^{12}C; they are just about 1.1% of the latter. Chemical

shifts of ^{13}C NMR spectra spread through to 180ppm which causes relatively few, if any, overlaps in the spectra.

2.5.4.1 SAMPLE PREPARATION AND ANALYSIS

The quantity of material needed for NMR analysis is usually small ranging between 10mg and 50mg (Kenkel, 2003). Only about 0.5ml of the sample is used for analysis.

The sample to be analyzed is dissolved in a solvent prior to analysis. Solvents used should not contain hydrogen atoms as these are capable of absorbing at the resonance frequency. Solvents such deuterated chloroform $CDCl_3$, deuterated water D_20, and CCl_4 are therefore the solvents of choice. The D is the symbol for deuterium, the isotope of hydrogen that has one proton and one neutron. The absence of ^1H nuclei in these solvents means they do not absorb resonance frequencies and do not interfere with the analysis or results.

2.5.4.2 CHEMICAL SHIFTS

In a molecule, the hydrogen nuclei or the NMR-active nuclei are 'surrounded' by electrons and other nuclei that also possess spin and magnetic moments. The effective strength of the external applied magnetic field therefore experienced by the hydrogen nuclei is less than the applied strength (Kenkel, 2003). The absorption pattern therefore shifts or changes, which is referred to as the chemical shift. This shift in the absorption is measured in parts per million (ppm) and recorded.

The electrons and other nuclei present near the hydrogen nuclei differ for each hydrogen nucleus. This results in different absorption and therefore different peaks for each hydrogen nucleus or NMR-active nucleus under study. Hydrogen nuclei or atoms within the same environment therefore resonate at the same frequency and give rise to one major absorption peak.

Protons that absorb at the same resonance frequency are referred to as equivalent protons while those within different chemical environments which give rise to different absorption peaks are known as non-equivalent protons.

The absorption pattern of nuclei under study is recorded and compared to a standard, tetramethylsilane (TMS) whose protons are in the same environment and therefore absorb at the same frequency.

The horizontal scale of the NMR spectrum is in ppm and goes from 0 on the right to 10 on the left. TMS is given a ppm value of zero. TMS is said to resonate upfield of all measured nuclei with positive ppm values. The nuclei are also said to resonate downfield relative to TMS. Nuclei under study may resonate either upfield or downfield to each other depending on their ppm values.

Chemical shifts of protons are affected by the extent of shielding or deshielding offered by the groups attached to it. The higher the electron density around a proton, the more shielded it is and the lower its ppm value. The lower the electron density, the more deshielded it is and the higher its ppm value which means it resonates at a lower frequency. Electron withdrawing groups surrounding a proton can therefore be said to make that proton resonate at a lower frequency i.e. deshield it.

2.5.4.3 PEAK SPLITTING AND INTEGRATION

A peak that appears on the spectrum at a particular chemical shift does not always appear as one peak though it may be resulting from just one proton. It may appear split into two, three, four or any number of peaks. When it splits into two, the peak is referred to as a duplet, triplet when it is three, quadruplet when it is four, quintet when it is five and multiplet when it is many.

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Chemical shifts of protons are affected by the extent of shielding or deshielding offered by the groups attached to it. The higher the electron density around a proton, the more shielded it is and the lower its ppm value. The lower the electron density, the more deshielded it is and the higher its ppm value which means it resonates at a lower frequency. Electron withdrawing groups surrounding a proton can therefore be said to make that proton resonate at a lower frequency i.e. deshield it.

2.5.4.3 PEAK SPLITTING AND INTEGRATION

A peak that appears on the spectrum at a particular chemical shift does not always appear as one peak though it may be resulting from just one proton. It may appear split into two, three, four or any number of peaks. When it splits into two, the peak is referred to as a duplet, triplet when it is three, quadruplet when it is four, quintet when it is five and multiplet when it is many.

Peak splitting occurs as a result of the effects of other hydrogen nuclei around the nucleus or nuclei of interest (Kenkel, 2003). The effect of adjacent protons on the absorption of the proton of interest is referred to as coupling. The number of hydrogen nuclei on the carbon atom adjacent the carbon of the proton of interest determines the number of peaks the major peak will be split into according to the n+1 rule where n refers to the number of hydrogen atoms on the adjacent carbon (Kenkel, 2003).

2.6 ANALYTICAL METHOD VALIDATION

Analytical method validation includes the processes through which an analytical method is passed to confirm its suitability for its intended purposes ICH Harmonised Tripartite Guideline Validation of Analytical Procedures: Text And Methodology Q2(R1), 1994. An analytical method is defined or described by the **ICH guidelines** as the procedure for the performance of the analysis which includes the details of steps included in performing each analytical test.

Many analytical methods are used in chemistry and industry but the **ICH guidelines** discuss the validation for the four most common of these methods. They include:

- ✓ Identification tests;
- ✓ Quantitative tests for impurities' content;
- ✓ Limit tests for the control of impurities;
- ✓ -Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

2.6.1 SPECIFICITY

The specificity of an analytical method refers to its ability to detect and/or quantitate the analyte of interest in the presence of expected 'impurities'.

2.6.2ACCURACY

According to the ICH guidelines, accuracy refers to the proximity of agreement between a value obtained by the analytical method and that which is accepted as a reference value or true conventional value.

2.6.3 PRECISION

This refers to the proximity of agreement between a series of values obtained from different aliquots of the same homogeneous sample under the given conditions. Precision is usually expressed as variance, standard deviation or coefficient of variation and may be viewed at in three levels namely repeatability, reproducibility and intermediate precision.

2.6.2.1 REPEATABILITY

Repeatability may also be referred to as intra-assay precision or intra-day precision. Repeatability expresses the precision under the same operating conditions over a short interval of time within the same day.

2.6.2.2 REPRODUCIBILITY

This gives a measure of the precision of an analytical method between different laboratories.

2.6.2.3 INTERMEDIATE PRECISION

This gives a measure of the precision (or variations between results) of an analytical method carried out within the same laboratory. Values are normally obtained on different days, by different analysts and/or different equipment amongst others.

2.6.4 LIMITS OF DETECTION AND QUANTITATION (LOD)

The LOD refers to the lowest amount of an analyte that may be detected by the method but not exactly quantified by the method whereas the LOQ refers to the least amount of an analyte that may be detected by the method and quantified with good accuracy and precision.

The LOD and LOQ may be determined in three ways: based on visual examination, signal-to-noise ratio or the standard deviation of the response and the slope.

Based on the standard deviation of the response and the slope, the LOD is calculated

$$\text{LOD} = \frac{3.3\sigma}{S}$$

$$\text{LOD} = \frac{10\sigma}{S}$$

where σ = the standard deviation of the response S = the slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte. The estimation of σ may be done based on the calibration curve.

2.6.5 LINEARITY

The ability of an analytical method to obtain results (e.g. peak area) that are directly proportional to the amount of analyte in the sample describes its linearity.

2.6.6 RANGE

The interval between the least and largest concentration for which an analytical process has a suitable degree of accuracy, precision and linearity is known as the range of the method.

2.6.7 ROBUSTNESS

This refers to the ability of an analytical method to withstand small, intended changes in certain parameters. For instance, if an HPLC method gives peak areas which do not differ significantly for a given concentration despite small deliberate changes in the pH of the mobile phase, the method can be said to be robust. Since during everyday analysis, certain parameters may change without intention, the robustness of a method gives an idea of its reliability in everyday use.

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Chapter 3
EXPERIMENTAL

3.1 MATERIALS

- ✓ *Paullinia pinnata* root sample
- ✓ Methanol
- ✓ Distilled water
- ✓ Petroleum ether 40°-60°
- ✓ Ethylacetate
- ✓ Chloroform
- ✓ Cyclohexane
- ✓ Hexane
- ✓ Acetone
- ✓ Silica gel for column and thin layer chromatography

3.2 EQUIPMENT

- ✓ Soxhlet apparatus (extractor)
- ✓ Hot air oven
- ✓ Bucchi rotavapor
- ✓ Desiccator
- ✓ Glass column for column chromatography
- ✓ UV viewing system (CHROMATO-VUE® C-70G)
- ✓ Glass plates for TLC
- ✓ Desaga spreader
- ✓ High Performance Liquid Chromatography apparatus (Shimadzu).

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3.2 METHODS

3.2.1 PREPARATION OF PLANT MATERIAL

A sample of *Paullinia pinnata* roots was obtained and accordingly authenticated at the Department of Herbal Medicine, KNUST. The root sample was then coarsely powdered using the mill at the Pharmaceutical Chemistry department of KNUST and kept in large paper envelopes for later extraction.

3.2.2 EXTRACTION OF POWDERED MATERIAL

The coarsely powdered plant material (5.4kg) was extracted with about 15L of chloroform using a soxhlet extractor. The extract obtained was concentrated using a Bucchi rotavapor under reduced pressure to a syrupy mass. The syrupy mass was then transferred into a crucible and air dried for some time to obtain a brown powder of weight 41.67g which was transferred into a glass bottle and kept in a desiccator for later use.

3.2.3 ISOLATION OF BIOMARKER

3.2.3.1 LIQUID – LIQUID EXTRACTION

About 30g of the brown powder was dissolved in just enough chloroform and transferred into a separating funnel containing water and petroleum ether in a 1:1 ratio (200ml each). The green petroleum ether layer was collected and further aliquots of petroleum ether added and collected until the green colour of the layer was discharged upon addition of more petroleum ether. Chloroform was then added to the remaining mixture in the separating funnel and allowed to stand overnight. The chloroform layer was then drained and more chloroform added until the chloroform soluble fraction was exhausted.

The petroleum ether and chloroform fractions thus obtained were then concentrated and air-dried. The petroleum ether fraction resulted in a green syrupy mass of weight 15g and the chloroform fraction resulted in a brown powder of weight 8g.

3.2.3.2 COLUMN CHROMATOGRAPHY

7g of the concentrated chloroform fraction was dissolved in a little amount of chloroform/ and a suitable amount of silica gel added. It was then heated gently on a thermostatic water bath while stirring constantly until a dry mass was obtained. The silica gel-adsorbed extract was then air dried for some time to ensure that all the solvent had evaporated from the mixture and the mixture was free flowing powder. A glass column, well washed and dried with acetone, was packed to a height of 25cm with silica gel, the sides of the column being tapped after filling to ensure even and compact packing of the silica gel and minimization of air spaces in the packed silica gel. The silica gel adsorbed extract was then transferred into the glass column and a wad of cotton placed on top of it to prevent splashing of silica gel with the introduction of the various solvents.

Gradient elution was carried out using petroleum ether containing increasing amounts of ethyl acetate. One hundred and thirty (130) fractions were collected and bulked according to similarities in their profiles after thin layer chromatography (TLC) was carried out on the various fractions using pre-coated analytical plates. The bulked fractions were labeled $\mathbf{C_1}$ **to** $\mathbf{C_{18}}$.

3.2.3.3 ISOLATION BY PRECIPITATION

After drying the bulked fractions, the components were re-dissolved using petroleum ether, ethyl acetate, chloroform and methanol. Addition of methanol or chloroform/methanol combinations to the concentrated bulked fractions resulted in the precipitation of compounds from the mixtures. The precipitates were then filtered out and dried. The TLC profiles of the precipitates

were determined in various solvent systems and bulked to afford compounds. Fractions **C_3 - C_{11}** excluding **C_5 and C_{10}** afforded the compounds here reported.

Compounds **1** and **2** were isolated from fraction **C_3** thus: chloroform was used to dissolve the contents of fraction **C_3.** Insoluble crystals were observed at the base of the container. These crystals were filtered out. Methanol was then added to the filtrate after which a white powdered substance precipitated out and was filtered. The filtrate was washed repeatedly with methanol to purify it. The two compounds were labeled **2** and **1** respectively.

Compound **3** was isolated from fraction **C_4** thus: chloroform was used to dissolve the contents of the container. Due to the insolubility of some components, methanol was added to aid the dissolution. A clear solution was obtained which turned cloudy upon standing. Precipitation was observed after a while and filtered out and the powdered substance labeled compound **3**.

Compound **4** was isolated from fraction **C_7** thus: an available mixture of petroleum ether and ethyl acetate (20/80) was used to dissolve the contents of **C_7** and some amount of chloroform added when not all the components of the components dissolved. This did not dissolve everything and so methanol was added to aid dissolution. This caused precipitation and the amorphous material was filtered out and labeled **4**.

Compound **5** from fraction **C_{11}** thus: an amount of a chloroform and methanol mixture was poured into an ethyl acetate solution of the contents of **C_{11}**. Precipitation occurred and the precipitate filtered out of the solution and labeled **5**.

3.2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.2.4.1 PREPARATION OF 'STANDARD' SOLUTION AND DILUTIONS

A stock solution of the isolate, here referred to as standard solution, was prepared by dissolving about 5mg of compound 1 in about 1ml of ethyl acetate, transferred into a 50ml volumetric flask and topping up to the 50ml mark using Methanol:Water (90:10) to give a concentration of 0.01% (w/v). 10ml of this solution was pipetted and transferred into a 100ml volumetric flask and made to volume with Methanol:Water (60:40). This solution was labeled solution A. 15ml of solution A was pipetted and transferred into a 25ml volumetric flask and made up to volume to give solution B. 15ml of solution B was pipetted and transferred into a 25ml volumetric flask and made up to volume to give solution C. 15ml of solution C was pipetted and transferred into a 25ml volumetric flask and made up to volume to give solution D.15ml of solution D was pipetted and transferred into a 25ml volumetric flask and made up to volume to give solution E.

The final concentrations of the solutions are as follows:

Table 4 – Concentrations for calibration curve

SOLUTION	CONC. / % (w/v)
A	0.0010000
B	0.0006000
C	0.0003600
D	0.0002160
E	0.0001296

3.2.4.2 PREPARATION OF EXTRACT FOR INJECTION

361.67g of milled *P. pinnata* roots was exhaustively extracted with 2.5L of chloroform. The extract was then concentrated to a volume of 500ml. 10ml of the resulting solution was pipetted into a 100ml volumetric flask and made up to volume using methanol. The resulting solution was labeled E_A. 10ml of E_A was then pipetted and transferred into a 100ml volumetric flask and also made to volume using methanol and labeled E_B.

3.2.4.3 DETECTION OF WAVELENGTH OF MAXIMUM ABSORPTION

Solution A (0.001%) was scanned between a wavelength range of 200nm and 400nm with the T90+ UV-VIS Spectrometer (PG Instruments) using 1ml ethyl acetate in 25ml methanol as the blank. Wavelengths between 230nm and 210nm gave appreciable absorption. A wavelength of 210nm was settled on as the preferred wavelength of detection for the HPLC analysis since it gave maximum absorption.

3.2.4.4 MOBILE PHASE

Various mobile phase solutions were tried including 60% methanol in water and 90% methanol in water. A mobile phase of 90% methanol in water gave the best results and was chosen for the analysis. About 1L of this solution was prepared and used for the chromatographic analysis.

3.2.5 METHOD VALIDATION

3.2.5.1 LINEARITY

100µl of each of solutions A-E were injected into the HPLC system and the peak areas obtained. The peak areas were then plotted against their concentrations and used for assessing the linearity and range of the method.

3.2.5.2 LIMITS OF DETECTION AND QUANTITATION (LOD and LOQ)

Data from the calibration curves was used to calculate the LOD and LOQ using the appropriate equations.

3.2.5.3 PRECISION (REPEATABILITY)

Solution E was injected six times and the peak areas noted and recorded. The standard deviation and the relative standard deviation were then calculated.

3.2.5.4 PRECISION (INTERMEDIATE)

The intermediate precision was also determined by injection of solution E six times for three different days. The standard deviation and relative standard deviation were then calculated and recorded.

3.2.5.5 ROBUSTNESS

The wavelength of detection was varied and used to run the sample and the results recorded. The standard deviation and relative standard deviation were then calculated and recorded.

Chapter 4
RESULTS AND CALCULATIONS

4.1 EXTRACTION AND FRACTIONATION

Weight of ground root material	Weight of extract obtained	Weight of Pet ether fraction	Weight of chloroform fraction
5.4kg	41.67g	15g	7.8g

4.2 MELTING POINT DETERMINATION

Table 5 – Melting points of isolated compounds

COMPOUND	MELTING POINT/°C
1	117-119
3	118-120
4	160-161

*Compounds 2 and 5 charred above 200 °C

4.3 SOLUBILITY PROFILE

Table 6 – Solubility profile of isolated compounds

SOLVENT	SOLUBILITY				
	1	2	3	4	5
Petroleum ether	±	±	±	±	-
Chloroform	+	-	±	+	-
Ethyl Acetate	+	+	+	±	-
Methanol	-	+	±	-	-

+ Soluble in solvent, - Insoluble in solvent ± Sparingly soluble

4.4 HPLC ANALYSIS

4.4.1 CONDITIONS FOR CHROMATOGRAPHIC ANALYSIS

- ✓ Column: Kromasil C-8, 5µ, 250mm × 4.6mm, 100Å by Phenomenex
- ✓ Flow rate: 1.3ml/min
- ✓ Wavelength of detection: 210nm
- ✓ Mobile phase: Methanol (90%) and Water (10%)
- ✓ Injection volume: 100µl
- ✓ Range: 1.000

4.4.2 RETENTION TIME

Retention time (mean ± SD) = **2.9687 ± 0.2657 ; n=15**

4.5 METHOD VALIDATION

4.5.1 CALIBRATION CURVE

Table 7 – Values for Calibration Curve Plot

SOLUTION	CONCENTRATION	PEAK AREA
A	0.0010000	8.12
B	0.0006000	4.77
C	0.0003600	2.95
D	0.0002160	1.55
E	0.0001296	1.08

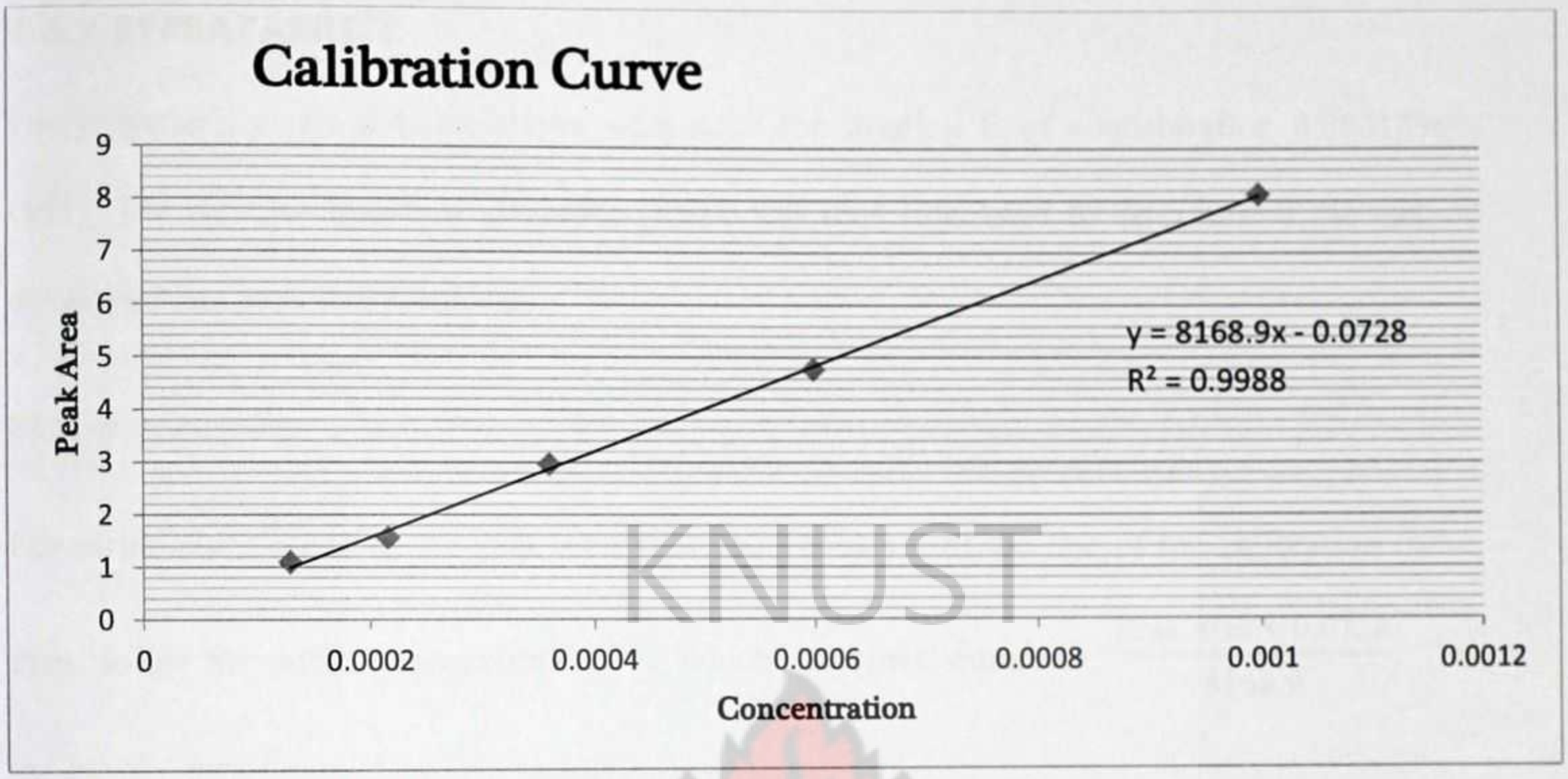


Figure 14 – Calibration Curve

Slope of the curve = **8168.9**, Coefficient of regression = **0.9988**, Range = **1.296µg/ml - 11µg/ml**

4.5.2 LOD and LOQ

The formulae for LOD and LOQ are

$$LOD = \frac{3.3\sigma}{S}$$

$$LOD = \frac{10\sigma}{S}$$

σ = 0.11496692 and **S** = 8168.87432 as calculated using Microsoft Excel

Therefore, the limit of detection (**LOD**) = 0.0000464435 = **0.464435µg/ml** and

the limit of quantification (**LOQ**) = 0.000140738 = **1.40738µg/ml**

4.5.3 REPEATABILITY

For repeatability, six determinations were done for solution E of concentration 0.0001296% (w/v). The Relative Standard Deviation (RSD) was then calculated to determine if the method developed has intra-day precision.

Sample calculation:

For actual concentration, put peak area value into equation of the line of the calibration curve, then solve for actual concentration, X which becomes equal to $\frac{\text{Peak area} + 0.0728}{8168.9}$. The percentage purity is calculated as $\frac{\text{actual concentration}}{0.0001296} \times 100$.

Therefore for determination 1, actual concentration $= \frac{1.08 + 0.0728}{8168.9} = 0.000141\%$ and the percentage purity $= \frac{0.000141}{0.0001296} \times 100 = 108.8893$

Table 8 – Repeatability of the method for the isolated biomarker

Determination	Peak Area	Actual Concentration	% Purity
1	1.08	0.000141%	108.8893
2	1.05	0.000137%	106.0557
3	1.03	0.000135%	104.1665
4	1.05	0.000137%	106.0557
5	1.07	0.000140%	107.9448
6	1.08	0.000141%	108.8893

	Mean	Standard Deviation	RSD (%)
Using Peak Area	1.0600	0.0200	1.8868
Using % Purity	107.0002	1.8891	1.7655

4.5.4 INTERMEDIATE PRECISION

The intermediate precision of the method developed was also determined. Solution E was run on three different days.

Table 9 – Intermediate precision using peak areas

Day	Mean Area (n=6)	SD	RSD (%)
1	1.0600	0.0200	1.8868
2	1.0233	0.0121	1.1835
3	0.9700	0.0179	1.8442

Table 10 – Intermediate precision using % purity

Day	Mean % Purity (n=6)	SD	RSD (%)
1	107.0002	1.8891	1.7655
2	103.5368	1.1439	1.1048
3	98.4991	1.6897	1.7154

4.5.5 ROBUSTNESS

Table 11 – Robustness using wavelength for biomarker concentration 0.000236% (w/v)

Wavelength (nm)	Effect on peak	SD	RSD (%)
210	Symmetrical peak with no significant tailing	0.028577	1.894633
220	Symmetrical peak with no significant tailing	0.012111	1.942877

Wavelength (nm)	210	220	P value
Mean	0.000194	0.0000195	0.59107

4.6 PERCENTAGE CONTENT OF BIOMARKER

Sample calculation

Equation of the line is y = 8168.9x - 0.0728

Therefore for peak area 25.62, the concentration of dilute solution of extract = $\frac{25.62+ 0.0728}{8168.9}$ = 0.003145% (w/v).

The dilution factor is 10 so the concentration of the stock solution = 10 × 0.003145% (w/v) = 0.03145% (w/v).

The concentration of the stock solution is in grams per 100ml of solution but the volume of the stock used is 500ml. The amount of the biomarker in the stock solution is therefore 5 times the concentration and is given by 5 × concentration of stock solution.

Table 13 - % content of biomarker for 1 in 100 dilution of stock solution

Peak Area	Concentration of solution (% w/v)	Concentration of stock	Actual Amount of biomarker in stock solution	% w/w of biomarker in roots
2.88	0.000361	0.03614685	0.18073425	0.049972
2.91	0.000365	0.036514096	0.18257048	0.05048
3.22	0.000403	0.040308977	0.20154488	0.055726
2.67	0.000336	0.033576124	0.16788062	0.046418
3.19	0.000399	0.03994173	0.19970865	0.055218
3.34	0.000418	0.041777963	0.20888981	0.057757

The mean content of biomarker in the roots of *Paullinia pinnata* is therefore **0.052009±0.004321% w/w (n=12)**

Chapter 5
DISCUSSION

5.1 ISOLATION AND CHARACTERIZATION OF BIOMARKER

The biomarker, after successful isolation from the plant extract was taken through a number of procedures for identification and characterization.

5.1.1 MELTING POINT

The melting points of compounds 1, 3 and 4 were determined and found to be 117-119 °C, 118-120 °C and 160-161 °C respectively. Compounds 2 and 5 charred above 200 °C.

5.1.2 SOLUBILITY

The compounds were dissolved in a number of common solvents to ascertain their solubility profiles. It was found that compound 1 is soluble in chloroform and ethyl acetate but not methanol. Compound 2 was soluble in ethyl acetate and methanol but not chloroform. Compounds 3 and 4 are soluble in ethyl acetate and chloroform respectively whereas compound 5 is insoluble in all the solvents used.

5.2 HPLC METHOD DEVELOPMENT AND VALIDATION

Following the isolation of the biomarker, an HPLC method was developed and used for the quantification of the amount of the biomarker in the roots of *Paullinia pinnata*. A number of conditions were tried during the method development and in the end the method that yielded the best results with respect to the consistency of the peak shape and symmetry chosen.

The method/conditions chosen eluted the biomarker at a time of **2.9687 ± 0.2657mins** (n=15). This is quite short and can therefore afford a lot of 'runs' in the space of a short time. This is good for analytical work as long retention times are undesirable (Fifield & Kealey, 2000).

5.2.1 LINEARITY

The biomarker was used as a secondary standard and the method developed used to determine the amount of the biomarker in the root extract. A calibration curve was therefore drawn to determine the concentration range within which the method gives a good linear relationship between the concentration of the biomarker and the peak area obtained. The results indicate that the method has a coefficient of regression (R^2) of **0.9988**. The **ICH guidelines (1994)** recommend a coefficient of regression value above 0.998 to indicate good linearity. The linearity of the methods holds true for concentrations between **1.296µg/ml** and **11µg/ml.**

5.2.2 LOD and LOQ

The LOD and LOQ represent a measure of the sensitivity of a given method. The lesser the LOD, the more sensitive a method is. The LOD of the method developed was calculated as **0.464435µg/ml** which means that the method is very sensitive to the biomarker being analyzed. The LOQ, much like the LOD, gives accurate information regarding the quantity of the analyte that can be correctly determined quantitatively. The lower the LOQ the more sensitive therefore the method is. The LOQ was calculated as **1.40738µg/ml** which is low and signifies that using this method one can accurately determine microgram quantities of the biomarker.

5.2.3 PRECISION

Precision may be in the form of the intra-day precision (repeatability) of the method or the inter-day precision (intermediate). The intra-day day precision judges the repeatability (consistency of results) of the analytical method being used using results obtained on the same day of analysis whereas the inter-day precision determines the consistency of results obtained on different days of analysis. The **ICH guidelines (1994)** stipulate an RSD of $\leq$2% for both intra- and inter-day precision to make an analytical method repeatable or reproducible.

The RSD of the results obtained for the repeatability study was calculated as **1.886792%** and **1.765537%** using the peak areas and the % purity respectively.

The intermediate precision of the method was also determined using six replicate determinations of one concentration level on three days. The RSD of the method in this respect was found to be **1.886792%, 1.183446%** and **1.84418%** for days 1, 2 and 3 respectively using the peak areas in the calculation. All values were below the recommended ≤2 mark and suggest good between-day precision of the method developed. The method showed good between-day precision as well when the %purity values were used in the calculation with RSD values of **1.765537%, 1.104847%** and **1.715434%** representing days 1, 2 and 3 respectively.

5.2.4 ROBUSTNESS

Robustness gives a measure of the ability of the method to give accurate results within variations of certain conditions. The method developed was found to be robust with respect to wavelength change. The RSD determined using 210nm and 220nm were found to be **1.894633%** and **1.942877%** respectively indicating that the method can be used satisfactorily at any of these wavelengths of measurement.

5.3 STRUCTURE ELUCIDATION

Structure elucidation is done with information from different techniques including nuclear magnetic resonance (NMR), ultraviolet/visible light (UV/Vis), mass spectrometry (MS) and infra-red spectroscopy (IR).

The NMR spectra were determined at the College of Pharmacy and Pharmaceutical Sciences of Florida A&M University using a Mercury-300BB Varian Spectrometer with the samples

dissolved in deuterated chloroform ($CDCl_3$) using tetramethylsilane (TMS) as the reference standard. A UV spectrum was obtained on T90+ UV/VIS Spectrometer (PG Instruments Ltd.) with UV Win 5.2.0 software.

5.3.1 PROTON NMR

With the knowledge of various compounds isolated from *Paullinia pinnata* by Annan & Houghton (2010), the HNMR spectra obtained were compared to those they obtained in their work. Compounds 1 and 3 appear to have similar spectra. They are both white to off-white amorphous powders.

5.3.1.1 COMPOUND 1

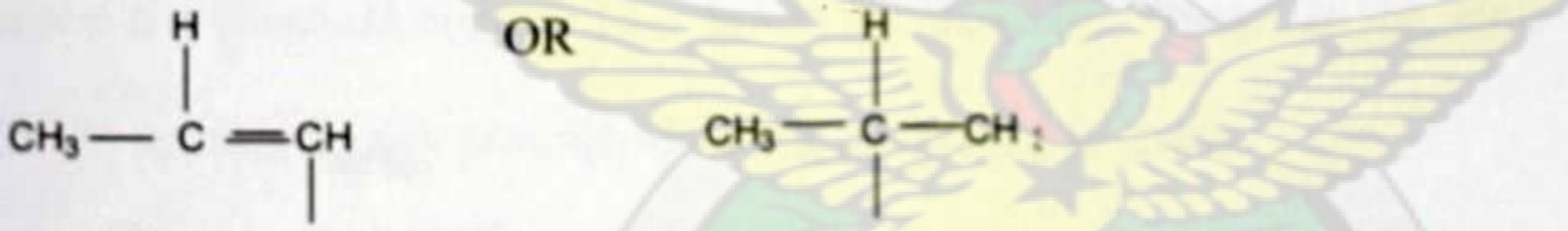
The HNMR spectra of compound 1 reveal a large cluster at chemical shifts between δ 0.8 and δ 2.4 which is in the methylene envelope (chemical shifts between δ 2.6 and δ 0.7). This suggests the likelihood of a steroidal or triterpenoid structure. Again, the signals obtained at δ 4.597, δ 4.586, δ 4.456 and δ 1.563 are similar to the signals obtained by Annan & Houghton, (2010) at δ 4.67, δ 4.54 and δ 1.68 and suggest the presence of a lup-20(29)-ene system. The chemical shift obtained at δ 5.301 deviates only slightly from that obtained at δ 5.22 by Annan & Houghton (2010) which was similar to the chemical shift of carbinol methine proton at H-6 in the 6β hydroxyl lupane derivatives reported by Dantanaranaya et al. (1982). The signal between δ 8 and δ 7 in the spectrum may be resulting from aromatic protons and may signify the presence of a substituted aromatic ring attached to a triterpenoid structure but is also characteristic of chloroform or deuterated chloroform, the solvent used for the analysis.

Using the HNMR spectra, compound 1 is suspected to be a 6β-(3-methoxy-4-hydroxybenzoyl) ester of lupeone. However, this cannot be confirmed in the absence of ^{13}CNMR data and/or mass spectroscopy data.

5.3.1.2 COMPOUND 2

The 1H-NMR spectrum of compound 2 shows four peaks: two very small peaks at δ1.3 and δ 2.15, and two very pronounced peaks at δ 3.3 and δ 4.932.

The extended spectrum shows the peak at δ 3.3 is a quintet and the five 'sub' peaks are narrow and symmetrical. The peaks start at δ 3.293 and end at δ 3.315. The five 'sub' peaks is an indication of coupling with four (4) equivalent hydrogen atoms on adjacent carbon atoms. The signal could therefore be from a proton on a secondary or tertiary carbon atom thus:



The signal at δ 4.932 could also be of a proton on an sp2 hybridized carbon atom (Antipolis, 2012).

5.3.1.3 COMPOUND 3

The 1H-NMR spectra (contracted and expanded) of compound 3 are very identical to those of compound 1. This suggests that the two compounds are most likely the same. The COSY spectrum of compound 3 is also very similar to that of compound 1.

The two compounds may however, be different and represent two compounds as for the 'ketone' and 'phenol' derivatives of lupane triterpene isolated by Annan & Houghton (2010) . This

difference may only be detected by the careful analysis of information from 1H-NMR spectra, ^{13}CNMR, infra-red spectra and mass spectroscopy data.

5.3.2 COSY

1H-1H COSY spectra (correlation spectroscopy) give an indication of the correlation in space between hydrogen atoms on adjacent carbon atoms or in some cases three to four bonds away depending on the arrangement of the groups on the molecule.

In COSY spectra, the 'spots' or 'dots' found on the diagonal line represent the same hydrogen atoms in space as the proton spectrum of a molecules is plotted against itself. There are also 'dots' either side of the diagonal which provide the information regarding which protons are in the same plane in space or not and which protons are coupling with which protons. A 'spot' above the diagonal appears also below the diagonal. 'Spots' in the same line or plane in the COSY spectra signify protons which are interacting in space.

COSY spectra are used by many for the confirmation of a structure that has been proposed using the data from ^{13}CNMR, HSQC, HMBC, Mass Spectroscopy and IR spectroscopy. Other scientists use the COSY spectra to 'build' in a stepwise manner the structure of the compound after analyzing information from the other above-mentioned spectral data.

From the COSY spectra obtained the diagonal can be seen clearly but also clearly visible is a lack of 'dots' or 'spots' on either side of the diagonal. This suggests an absence of correlation between any of the hydrogen atoms present in the compounds and this is highly unlikely.

5.3.3 ULTRAVIOLET-VISIBLE SPECTROSCOPY

UV spectroscopy helps in the structural elucidation of compounds by the identification of the presence of chromophores which give rise to absorption within the ultraviolet region of the electromagnetic spectrum. The presence of auxochrome(s) on the structure also gives rise to increased absorption by a molecule.

Carbonyl groups exhibit absorption bands around 190nm and 280nm. However, the absorption pattern is changed with conjugation of the carbonyl bond with a carbon-carbon double bond. In this instance, absorption resulting from promotion of the carbon-carbon π-system to the antibonding orbitals of the carbonyl group is found in from 220nm – 250nm (Vogel, 1989b).

Compound 1 gave absorbances at wavelengths between 205nm and 236nm. The λmax appears to be 236nm which is indicative of the presence of conjugation in the compound. This may be indicative of the presence of a carbonyl functional group bonded to a carbon-carbon unsaturated bond in the molecule. This is in agreement with the structure of the suspected structure of compound 1 in figure 2 where the isolated benzene ring is attached to a carbonyl carbon atom. Again, benzene exhibits some absorbance at 204nm (Vogel, 1989b) which is in agreement with the suspected structure of compound 1.

5.3.4 CARBON-13 NMR (^{13}CNMR)

^{13}CNMR could not be obtained and seeing as it is very essential in establishing the structure of compounds. Data from ^{13}CNMR provides information regarding the number of carbon atoms in the molecule/structure. These spectra may be plotted (combined) with proton NMR spectra to provide information on which proton atom is bonded to which carbon atom. This gives a more detailed basis for the elucidation of the structure.

Insufficient amounts of the isolate prevented generation of ^{13}CNMR. The identity of the biomarker is therefore yet be confirmed.

5.3.5 MASS SPECTROSCOPY

Mass spectroscopy data give the molecular weight of the compound and hence, help in the identification of compounds.

The mass spectra could not be obtained for any of the compounds as a result of a breakdown of the instrument and insufficient amounts of some compounds.

5.3.6 IR SPECTROSCOPY

IR spectra give accurate information about the functional groups present on a molecule. IR spectra however could not be obtained for the isolates.

In light of the fact that not all spectroscopic data necessary for the identification of the compounds were obtained, further spectra need to be run to help provide data that will enable accurate elucidation and valid identification of the structures of the compounds isolated.

Chapter 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Five isolates were obtained from the chloroform extract of the root bark of the plant. The melting points of compounds 1, 3 and 4 were determined and found to be **117-119 °C, 118-120 °C** and **160-161 °C** respectively.

Spectroscopy results obtained i.e. HNMR and COSY were inadequate for valid and conclusive identification of the isolates. A reverse phase HPLC method was developed for compound 1 with the following parameters: **Column**: Kromasil C-8, 5µ, 250mm × 4.6mm, 100Å by Phenomenex, **Flow rate**: 1.3ml/min, **Wavelength of detection**: 210nm, **Mobile phase**: Methanol (90%) and Water (10%), **Injection volume**: 100µl and **Range**: 1.000.

The method was validated according to ICH guidelines (1994) and was found to be precise, accurate, robust and linear for concentrations between **1.296µg/ml** and **11µg/ml.** The limits of detection and quantitation were also found to be **0.464435µg/ml** and **1.40738µg/ml** respectively.

The percentage content of compound 1 in the root bark of *P. pinnata* was determined to be **0.052009±0.004321% w/w (n=12)** using the RP-HPLC method developed. This information will be useful in the determination of dosage regimen for products containing *P. pinnata* and chemists may employ this information to standardize such products for quality control purposes.

6.2 RECOMMENDATIONS

Based on the work that has been carried out in this project, I make the following recommendations

- ✓ The identity of the compounds isolated in this work should be determined using NMR data (i.e. HSQC, HMBC, ^{13}CNMR) that will help to conclusively identify them.
- ✓ The seasonal variations in the amount of the isolates in the root should be determined to ascertain the best time to harvest the roots of *P. pinnata* for medicinal purposes.
- ✓ Different and possibly easier/faster methods of isolation of the compounds obtained from the root bark should be investigated.
- ✓ Stability studies should be done on extracts of *P. pinnata* and on the isolates to determine the effect of temperature and storage conditions on the isolates.
- ✓ Where stability studies reveal degradation with respect to temperature or time, suitable methods should be developed for detection of possible degradation products and establish limits of toxicity for those that may be toxic.

REFERENCES

Abbiw, D. K. (1990). *Useful plants of Ghana: West African uses of wild and cultivated plants*. Intermediate Technology Publications. Retrieved from http://books.google.com.gh/books?id=k-0HAQAAMAAJ

Abourashed, E. A., Toyang, N. J., Choinski, J., & Khan, I. A. (1999). Two new flavone glyosides from Paullinia pinnata. *Journal of Natural Products*, *62*(8), 1179–1181.

Adamovics, J. A., & Eschbach, J. C. (1997). Planar Chromatography. In J. A. Adamovics (Ed.), *Chromatographic Analysis of Pharmaceuticals* (2nd ed., pp. 58–67). Marcel Dekker Inc.

Annan, K., Govindarajan, R., & Kisseih, E. (2010). Wound Healing and Cytoprotective Actions of Paullinia pinnata L . *Phcog J*, *2*(10), 345–350.

Annan, K., & Houghton, P. J. (2010). Two novel lupane triterpenoids from Paullinia pinnata L. with fibroblast stimulatory activity. *The Journal of pharmacy and pharmacology*, *62*(5), 663–668.

Antipolis, S. (2012). Multispectroscopy. *Universite Nice*. Retrieved from www.uel-pcsm.education.fr/consultation/reference/chimie/multispectro/en/multispectroscopie_book_resources

Asase, A., Oteng-Yeboah, A. a, Odamtten, G. T., & Simmonds, M. S. J. (2005). Ethnobotanical study of some Ghanaian anti-malarial plants. *Journal of ethnopharmacology*, *99*(2), 273–9. doi:10.1016/j.jep.2005.02.020

Bano, S. (2007). Chemistry of natural products. Retrieved June 21, 2012, from http://www.scribd.com/doc/46472860/Chemistry-of-Natural-Products

Bayor, M. T. (2008). *The anticancer and other bioactivity investigations on the extract and some compounds of Croton membranaceus (Euphorbiaceae)*. Kwame Nkrumah University of Science and Technology.

Bayor, M. T., Gbedema, S. Y., & Annan, K. (2009). The antimicrobial activity of Croton membranaceus , a species used in formulations for measles in Ghana. *Journal of Pharmacognosy and Phytotherapy*, *1*(4), 47–51.

Bohlmann, J., Meyer-Gauen, G., & Croteau, R. (1998). Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(8), 4126–33. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=22453&tool=pmcentrez&rendertype=abstract

Bouvier, F., Rahier, A., & Camara, B. (2005). Biogenesis, molecular regulation and function of plant isoprenoids. *Progress in lipid research*, *44*(6), 357–429. doi:10.1016/j.plipres.2005.09.003

Bowden, K. (1962). Isolation from P. pinnata Linn. of material with action on frog isolated heart. *British journal of pharmacology and chemotherapy*, *18*(1), 173–174.

Broadbent, J. L. (1962). Cardiotonic action of two tannins. *British journal of pharmacology and chemotherapy*, *18*(1), 167–174.

Carlini, E. A. (2003). Plants and the central nervous system. *Pharmacology Biochemistry and Behavior*, *75*(3), 501–512. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S0091305703001126

Cimanga, K., De Bruyne, T., Pieters, L., Vlietinck, A. J., & Turger, C. A. (1997). In vitro and in vivo antiplasmodial activity of cryptolepine and related alkaloids from Cryptolepis sanguinolenta. *Journal of Natural Products*, *60*, 688–691.

Dantanaranaya, A. P., Kumar, N. S., Muthukuda, P. M., & Wazeer, M. I. M. (1982). A lupane derivative and the 13 C-NMR chemical shifts of some lupanols from Pleurostylia opposita. *Phytochemistry*, *21*, 2065–2068.

Dokosi, O. B. (1998). *Herbs of Ghana*. Accra: Published for Council for Scientific and Industrial Research, Ghana by Ghana Universities Press.

Ettre, L. S. (2003). M . S . Tswett and the Invention of Chromatography, (September). Retrieved from http://www.lcgceurope.com/lcgceurope/data/articlestandard/lcgceurope/382003/69718/article.pdf

Fifield, F. W., & Kealey, D. (2000). *Principles and Practice of Analytical Chemistry* (5th ed.). Blackwell Science.

Galichet, L. Y., Moffat, A. C., Osselton, M. D., & Widdop, B. (Eds.). (2005). *Clarke's Analysis of Drugs and Poisons* (3rd ed.). Pharmaceutical Press.

Harvey, D. (2000). *Modern Analytical Chemistry* (1st. ed.). McGraw Hill.

ICH Harmonised Tripartite Guideline Validation of Analytical Procedures: Text And Methodology Q2(R1). (1994). Retrieved from http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

Jimoh, F. O., Sofidiya, M. O., & Afolayan, A. J. (2007). Antioxidant properties of the methanol extracts from the leaves of Paullinia pinnata. *Journal Of Medicinal Food*, *10*(4), 707–711. Retrieved from

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18158845

Kenkel, J. (2003). *Analytical Chemistry for Technicians Third Edition* (3rd ed.). CRC Press LLC.

Mcgarvey, D. J., & Croteau, R. (1995). Terpenoid Metabolism. *The Plant Cell*, *7*(July), 1015–1026.

McPolin, Oo. (2009). *An Introduction to HPLC for Pharmaceutical Analysis*. Mourne Training Services.

Meléndez, P. A., & Capriles, V. A. (2002). Molluscicidal activity of plants from Puerto Rico. *Annals of tropical medicine and parasitology*, *96*(2), 209–18. doi:10.1179/000349802125000600

Miemanang, R. S., Krohn, K., Hussain, H., & Dongo, E. (2006). Paullinoside A and Paullinomide A : A New Cerebroside and a New Ceramide from Leaves of Paullinia pinnata, *13*, 1123–1127.

Mikkelsen, S. R., & Corton, E. (2004). *Bioanalytical Chemistry* (1st online.). Wiley-Interscience.

Patnaik, P. (2004). *Dean's Analytical Chemistry Handbook* (2nd ed.). McGraw Hill. Retrieved from www.digitalengineeringlibrary.com

Pérez, D., Iannacone, J., & Pinedo, H. (2010). Toxicological effect from the stem cortex of the amazonic plant soapberry Paullinia clavigera (Sapindaceae) upon three arthropods. *Ciencia e Investigacion Agraria*, *37*(3), 133–143.

Reich, E., & Blatter, A. (2005). Thin-Layer Chromatography: Overview. (P. Worsfold, A. Townshend, & C. Poole, Eds.)*Encyclopedia of Analytical Science*. Elsevier Ltd.

Stefova, M., Stafilov, T., & Kulevanova, S. (2001). HPLC of Flavonoids. (J. Cazes, Ed.)*Encyclopedia of Chromatography*. Dekker.com. doi:10.1081/E-ECHR-120016287

Tinseth, G. (1993). The Essential Oil of Hops: Hop Aroma and Flavor in Hops and Beer. Retrieved June 21, 2012, from http://realbeer.com/hops/aroma.html

Vogel, A. I. (1989a). *Vogel's Textbook of Quantitative Chemical Analysis*. (G. H. Jeffery, J. Bassett, J. Mendham, & R. C. Denney, Eds.) (5th ed., pp. 229 – 234). Longman Scientific and Technical and John Wiley & Sons.

Vogel, A. I. (1989b). *Vogel's Textbook of Practical Organic Chemistry*. (B. S. Furniss, A. J. Hannaford, P. W. G. Smith, & A. R. Tatchell, Eds.) (5th ed., p. 390). Longman Scientific and Technical and John Wiley & Sons.

Watson, D. G. (1999). *Pharmaceutical_Analysis A Textbook For Pharmacy Students and Pharmaceutical Chemists*. Churchill Livingstone, Harcourt Publishers.

Weckerle, C. S., Stutz, M. A., & Baumann, T. W. (2003). Purine alkaloids in Paullinia. *Phytochemistry*, *64*(3), 735–742. Retrieved from http://www.sciencedirect.com/science/journal/00319422/64/3

WHO | Traditional medicine Factsheet No 134. (2008). World Health Organization. Retrieved July 4, 2012, from http://www.who.int/mediacentre/factsheets/fs134/en/

Wright, C. W. (2007). Recent developments in naturally derived antimalarials: cryptolepine analogues. *Journal of Pharmacy and Pharmacology*, *59*, 899–903.

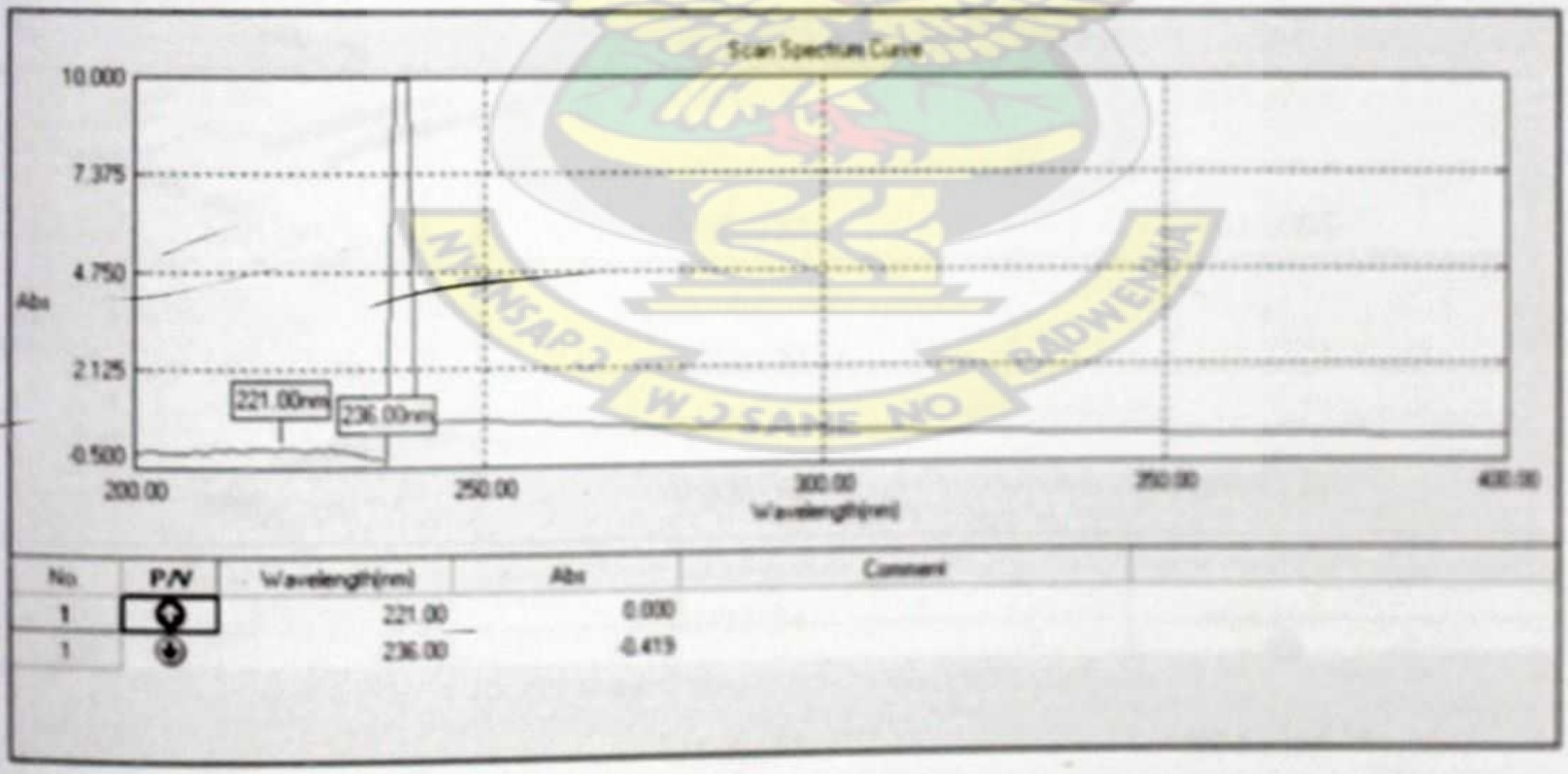
Zamble, A., Carpentier, M., Kandoussi, A., Sahpaz, S., Petrault, O., Ouk, T., Hennuyer, N., et al. (2006). Paullinia pinnata extracts rich in polyphenols promote vascular relaxation via endothelium-dependent mechanisms. *Journal of Cardiovascular Pharmacology*, *47*(4), 599–608. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16680075

APPENDIX

APPENDIX I – UV-VIS SPECTRA

No	P/V	Wavelength(nm)	Abs	Comment
1	◆	203.00	0.023	
1	◆	236.00	-0.296	

Figure 15 – UV-Vis spectrum of Compound 1



No	P/V	Wavelength(nm)	Abs	Comment
1	◆	221.00	0.000	
1	◆	236.00	-0.419	

Figure 16 – UV-Vis spectrum of Compound 1

Appendix II - INTER-DAY PRECISION

DAY ONE

PEAK AREA	ACTUAL CONCENTRATION	% PURITY
1.08	0.000141	108.8893
1.05	0.000137	106.0557
1.03	0.000135	104.1665
1.05	0.000137	106.0557
1.07	0.00014	107.9448
1.08	0.000141	108.8893

DAY TWO

PEAK AREA	ACTUAL CONCENTRATION	%PURITY
1.01	0.000133	102.2774
1.03	0.000135	104.1665
1.01	0.000133	102.2774
1.04	0.000136	105.1111
1.02	0.000134	103.222
1.03	0.000135	104.1665

DAY THREE

PEAK AREA	ACTUAL CONCENTRATION	%PURITY
0.99	0.00013	100.3883
0.97	0.000128	98.49914
0.99	0.00013	100.3883
0.95	0.000125	96.61001
0.97	0.000128	98.49914
0.95	0.000125	96.61001

Appendix III - THIN LAYER CHROMATOGRAMS

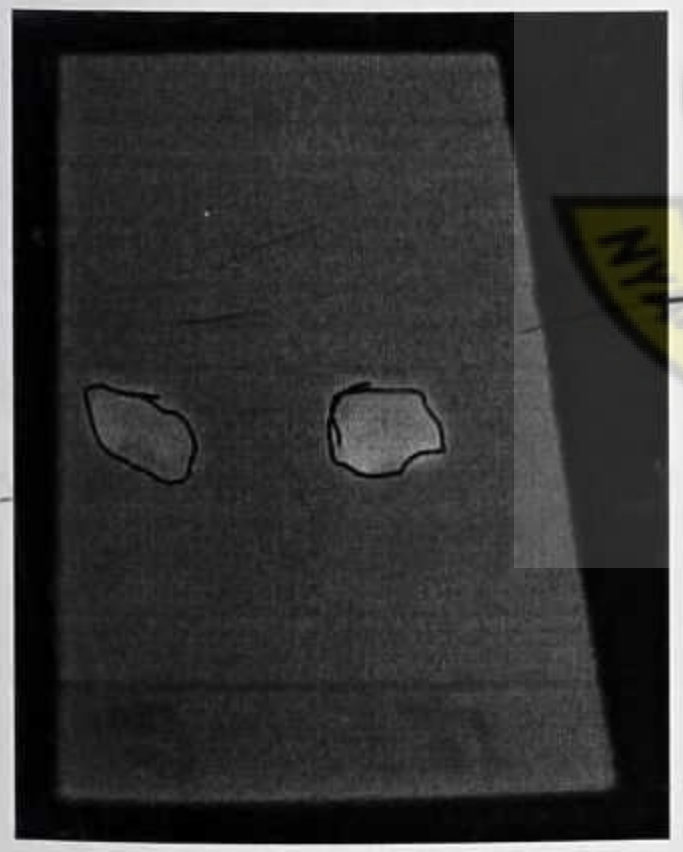


Figure 18 - TLC of two isolates

Figure 17 - TLC of some CC fractions

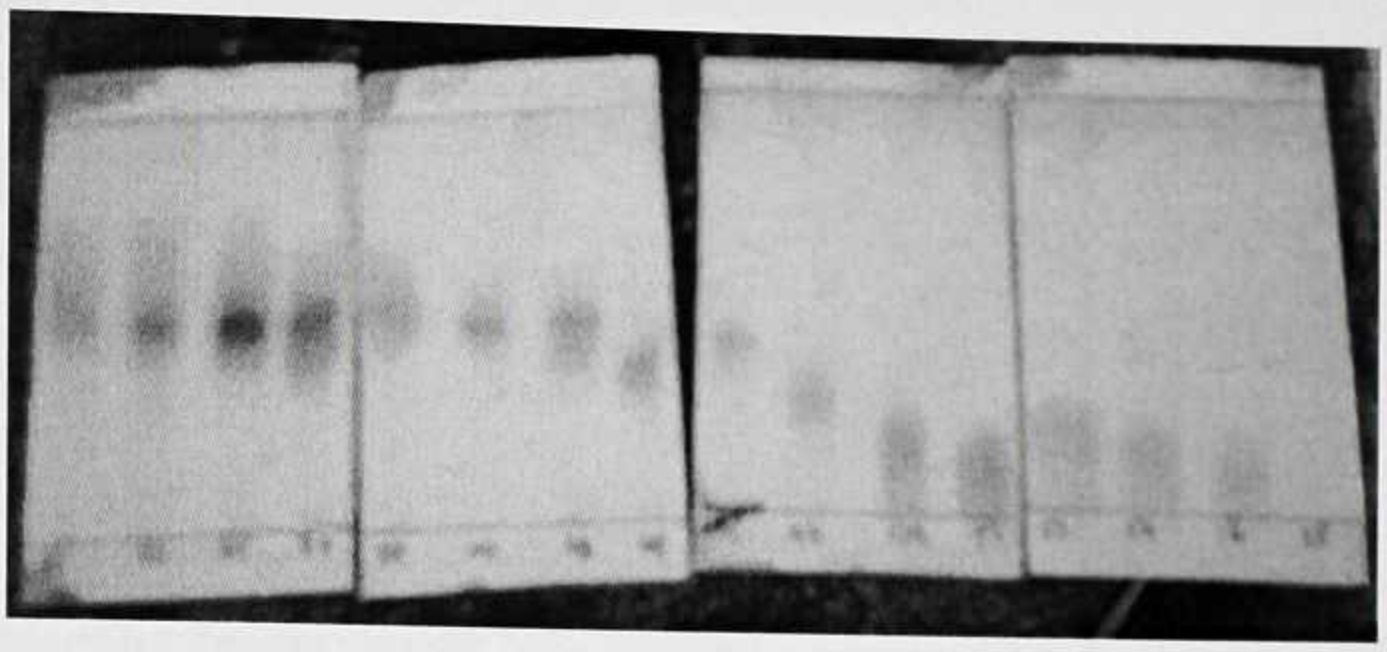


Figure 19 - TLCs of some CC fractions

Appendix IV - HPLC CHROMATOGRAMS

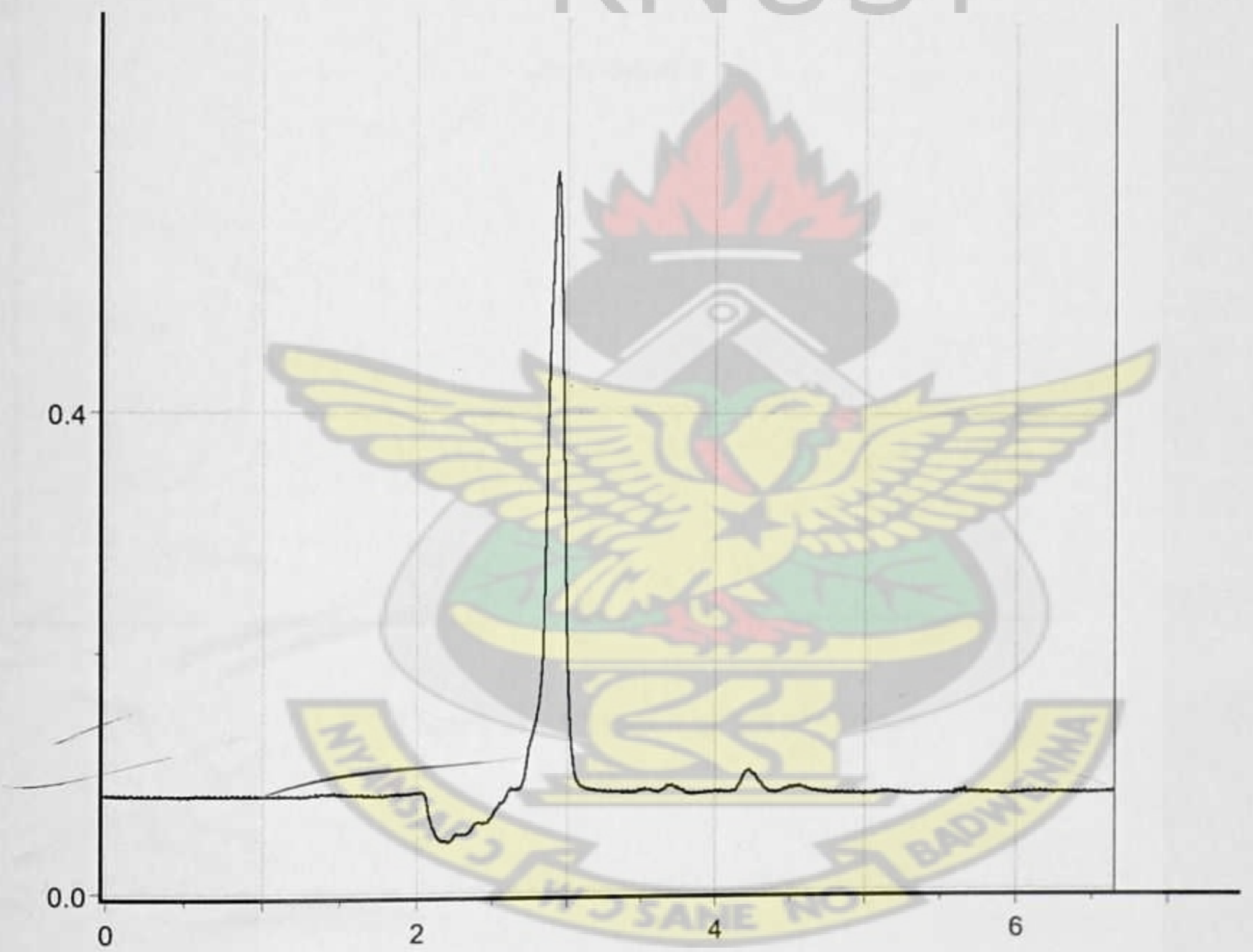


Figure 20 - Chromatogram of isolate

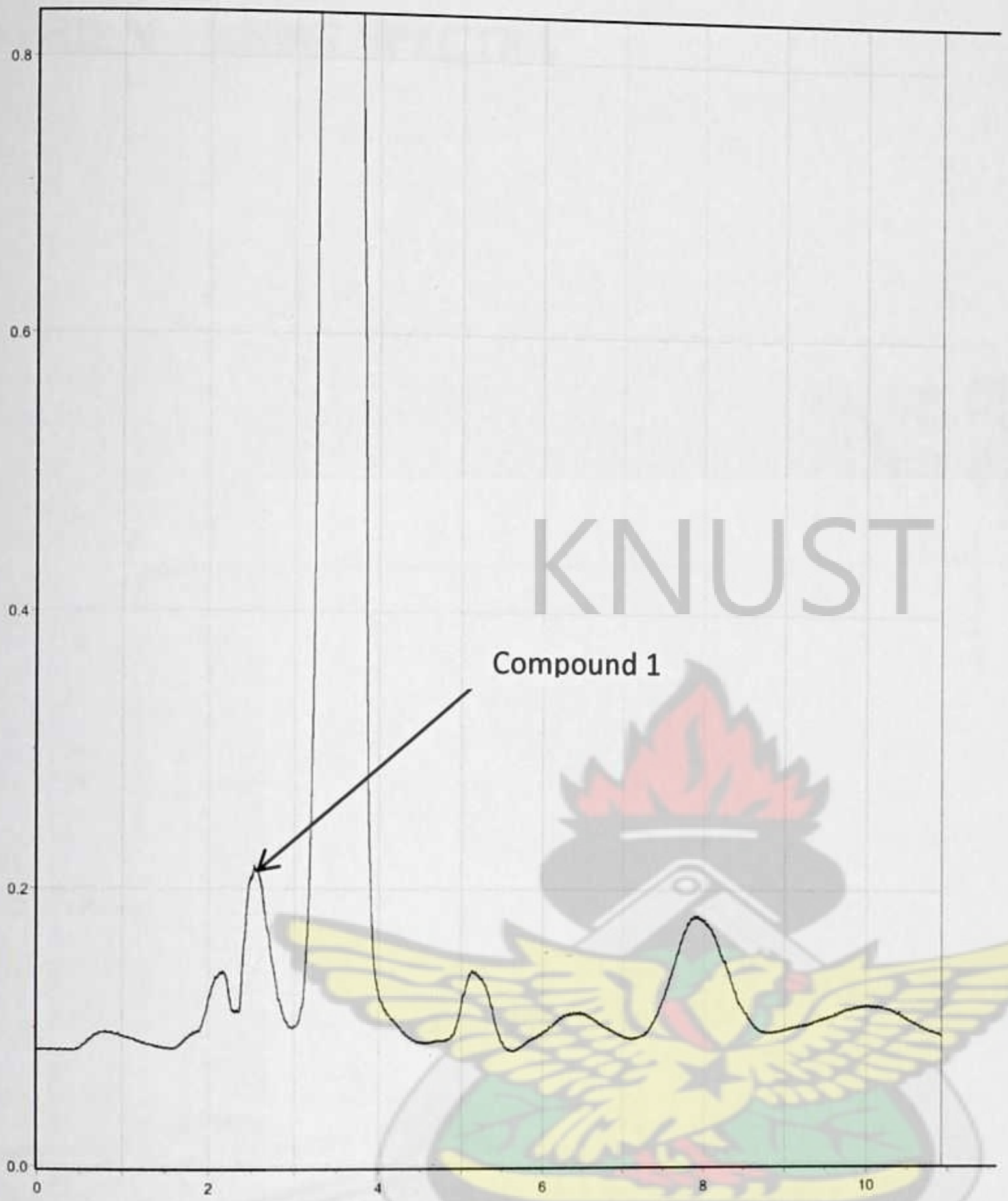


Figure 21 - Chromatogram of extract

Appendix V - HNMR SPECTRA

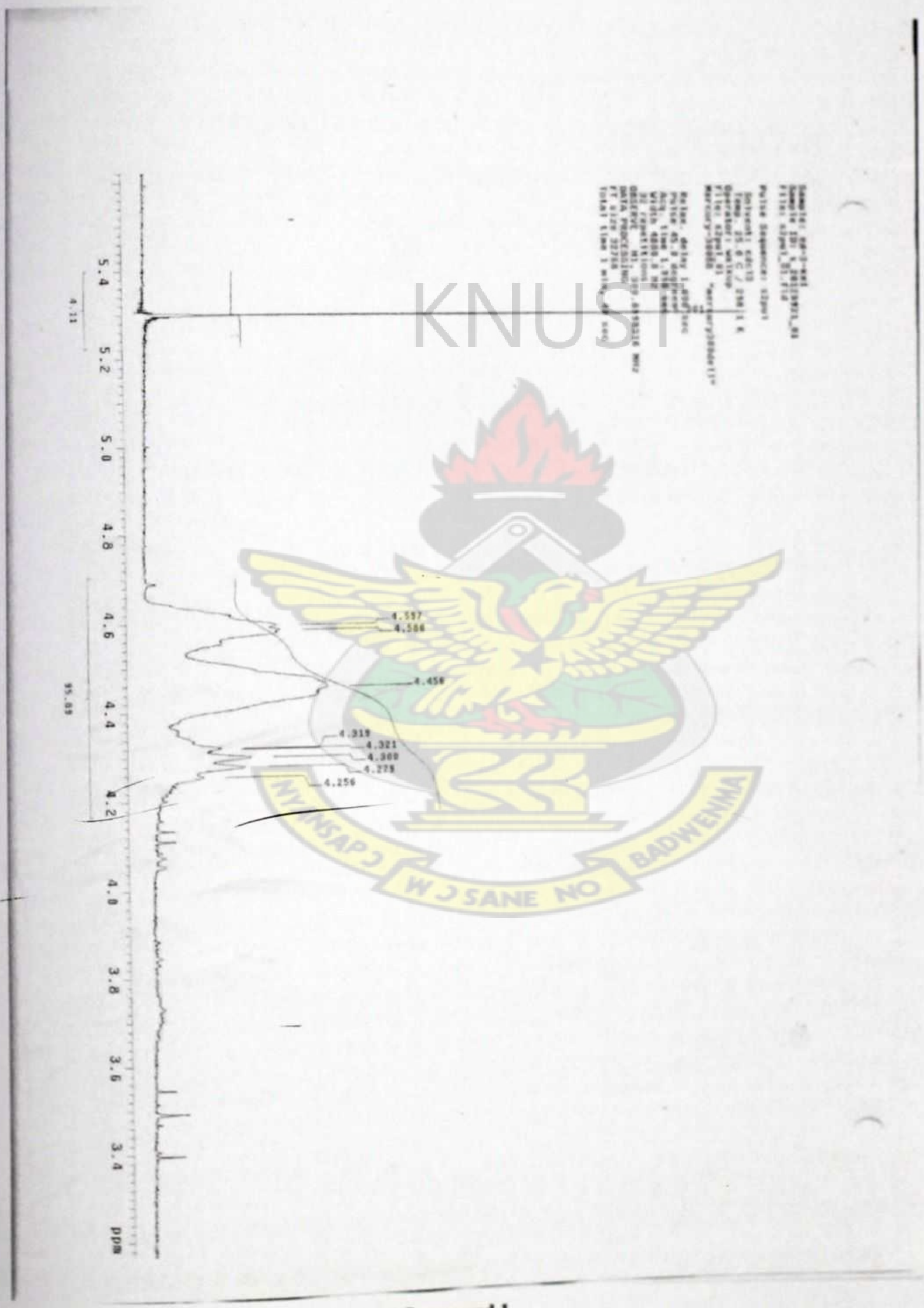


Figure 22 – Extended 1H-NMR spectrum of Compound 1

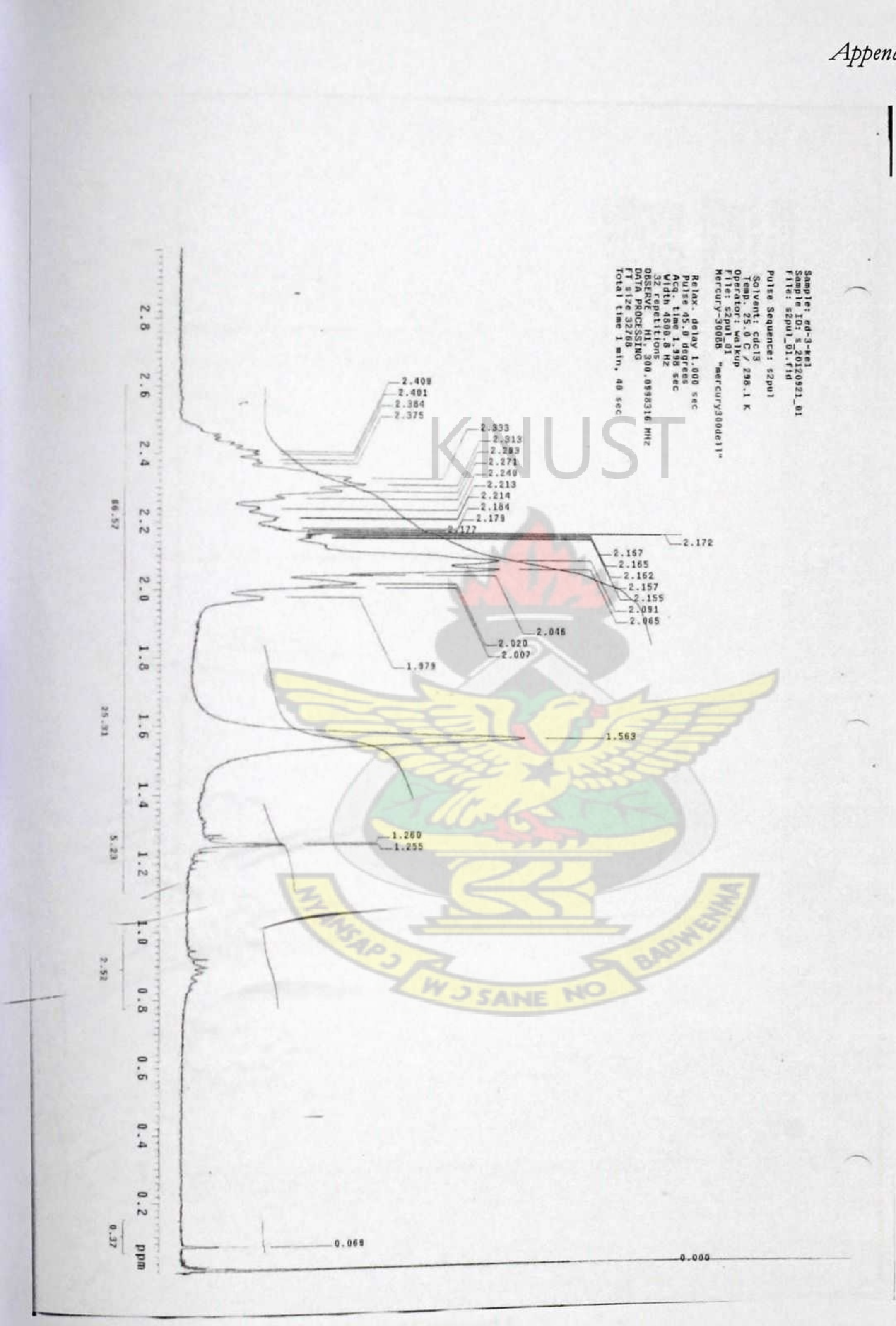


Figure 23 - Extended 1H-NMR spectrum of Compound 1

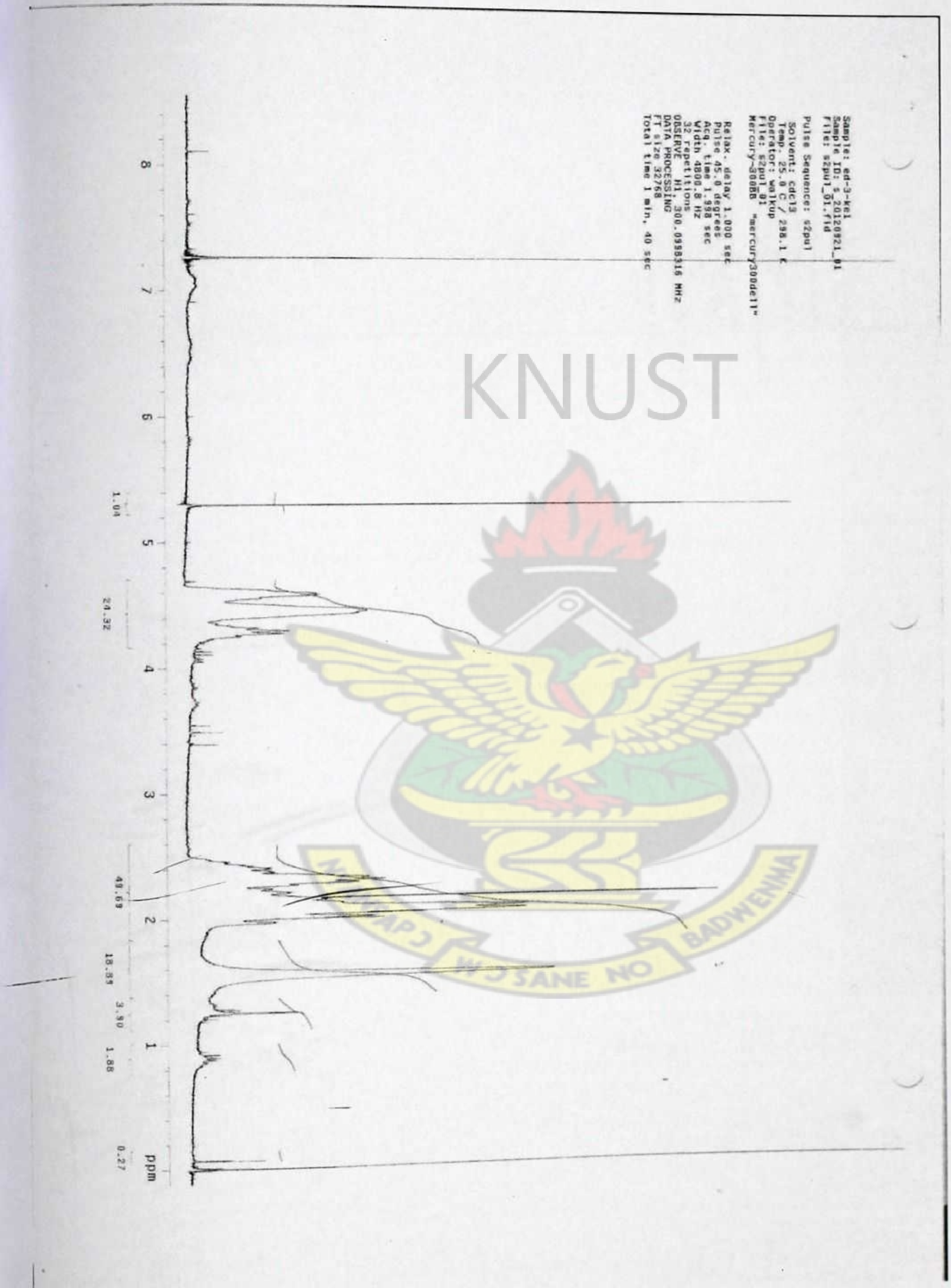


Figure 24 - Contracted 1H-NMR spectrum of Compound 1

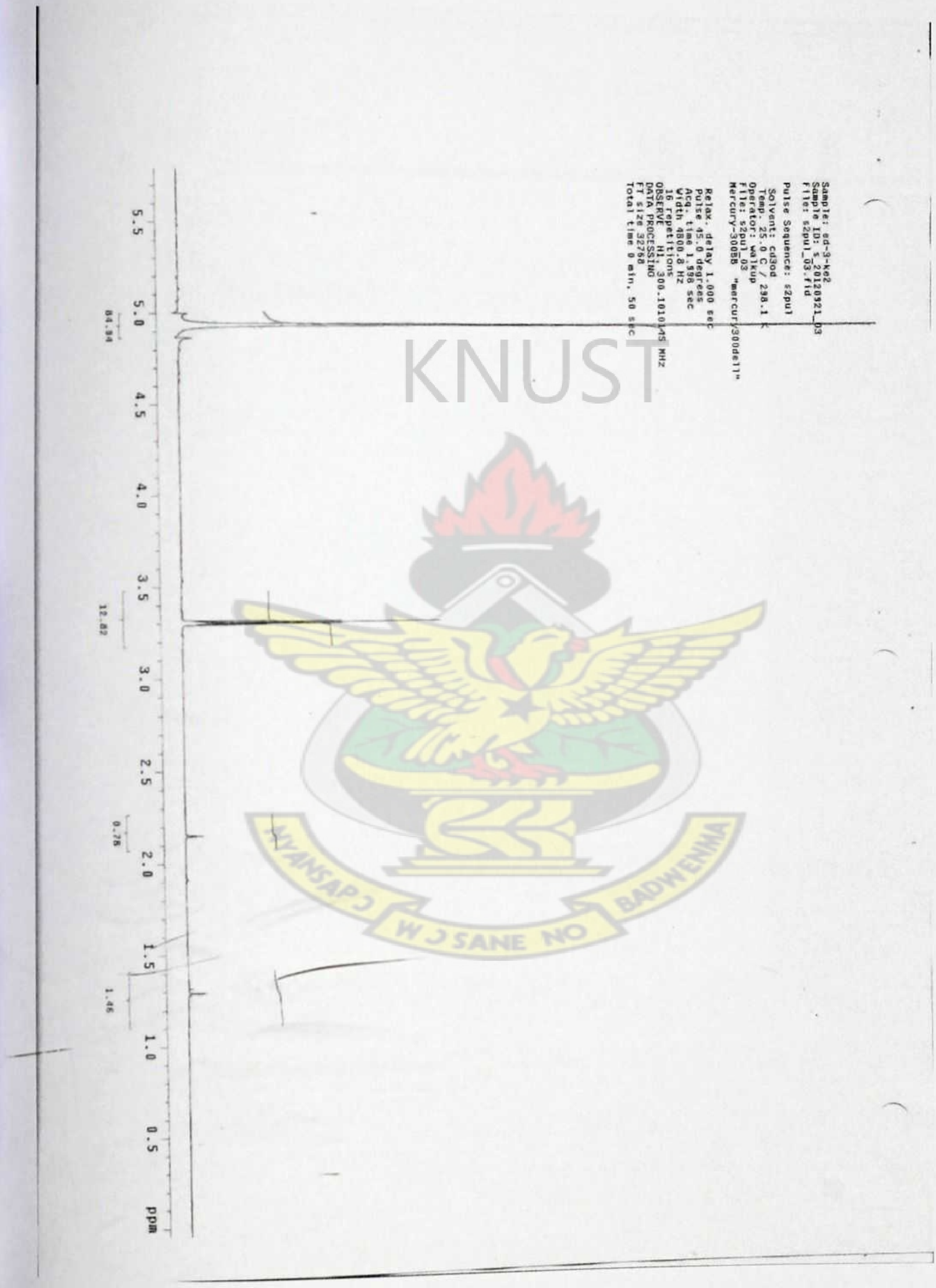


Figure 25 – Contracted 1H-NMR spectrum of Compound 2

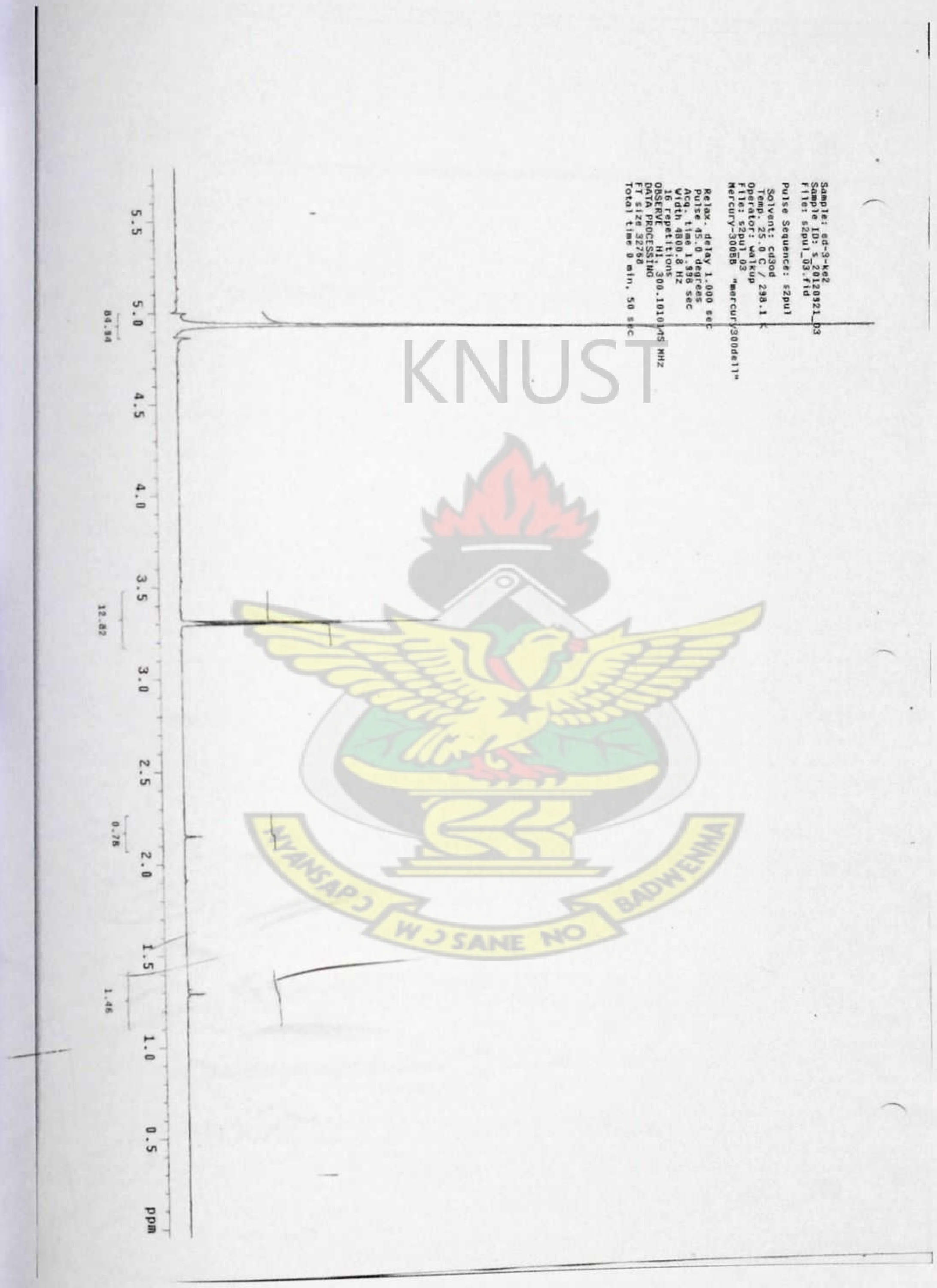


Figure 25 – Contracted 1H-NMR spectrum of Compound 2

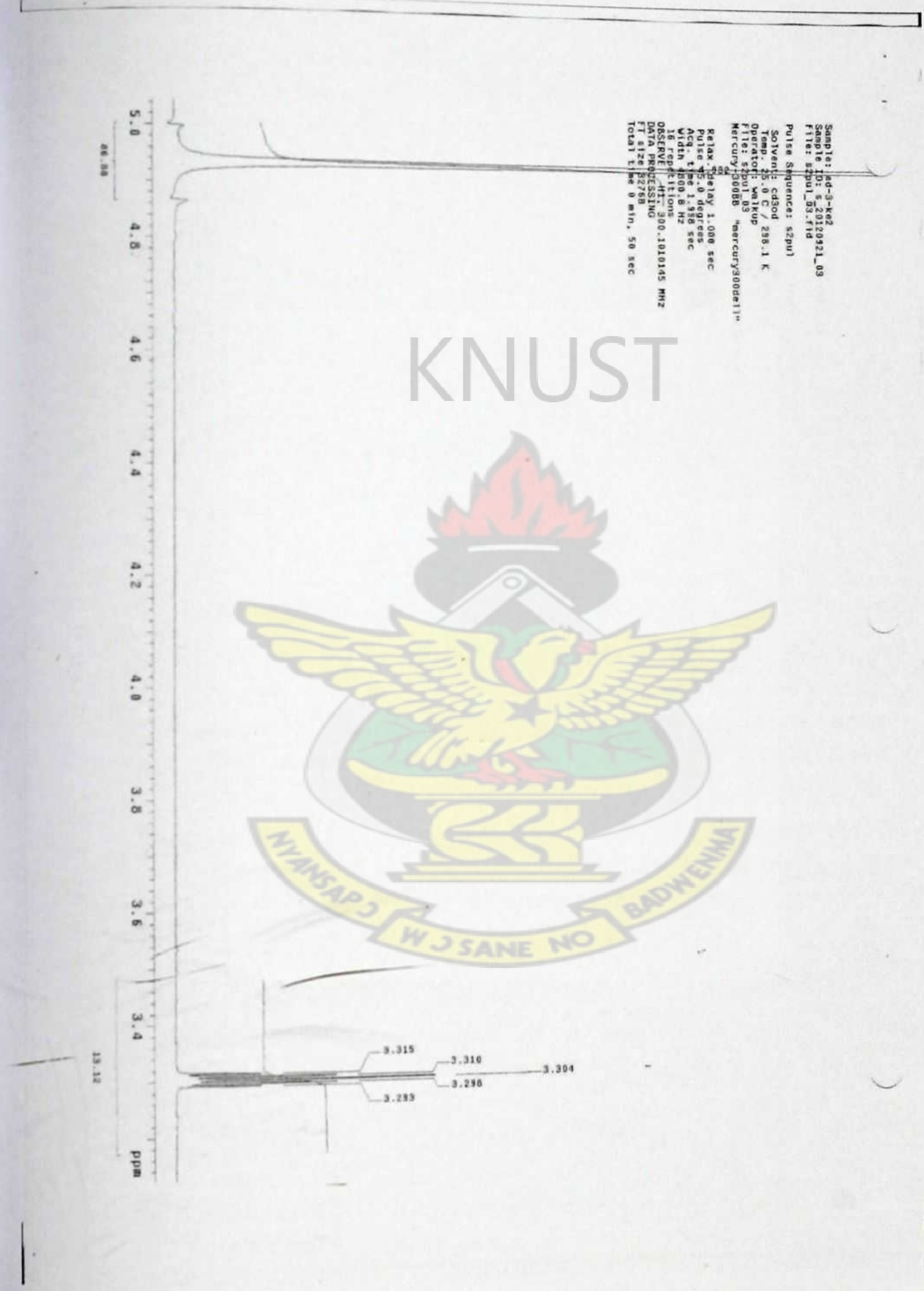


Figure 26 – Contracted 1H-NMR spectrum of Compound 2

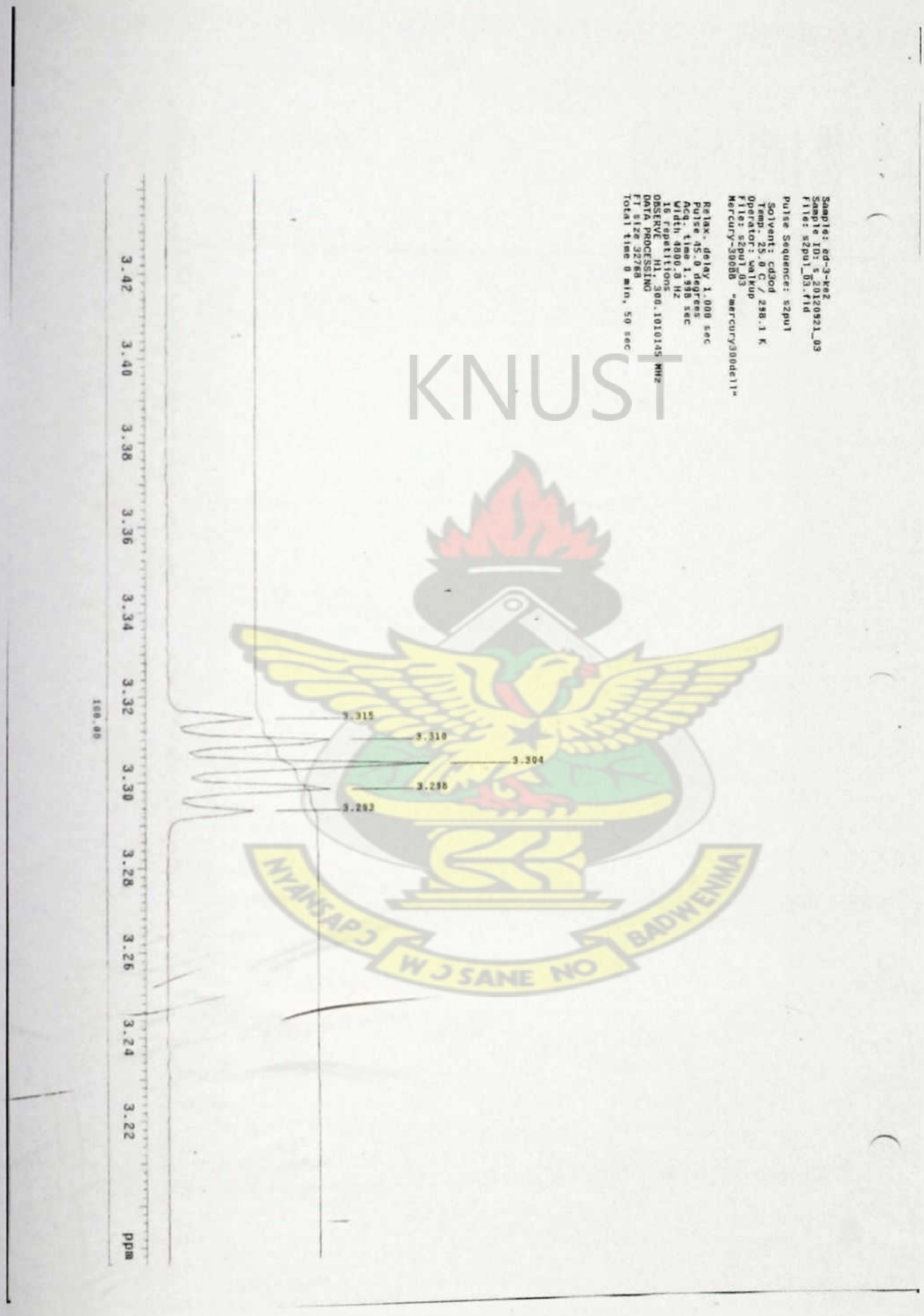


Figure 27 – Extended 1H-NMR spectrum of Compound 2

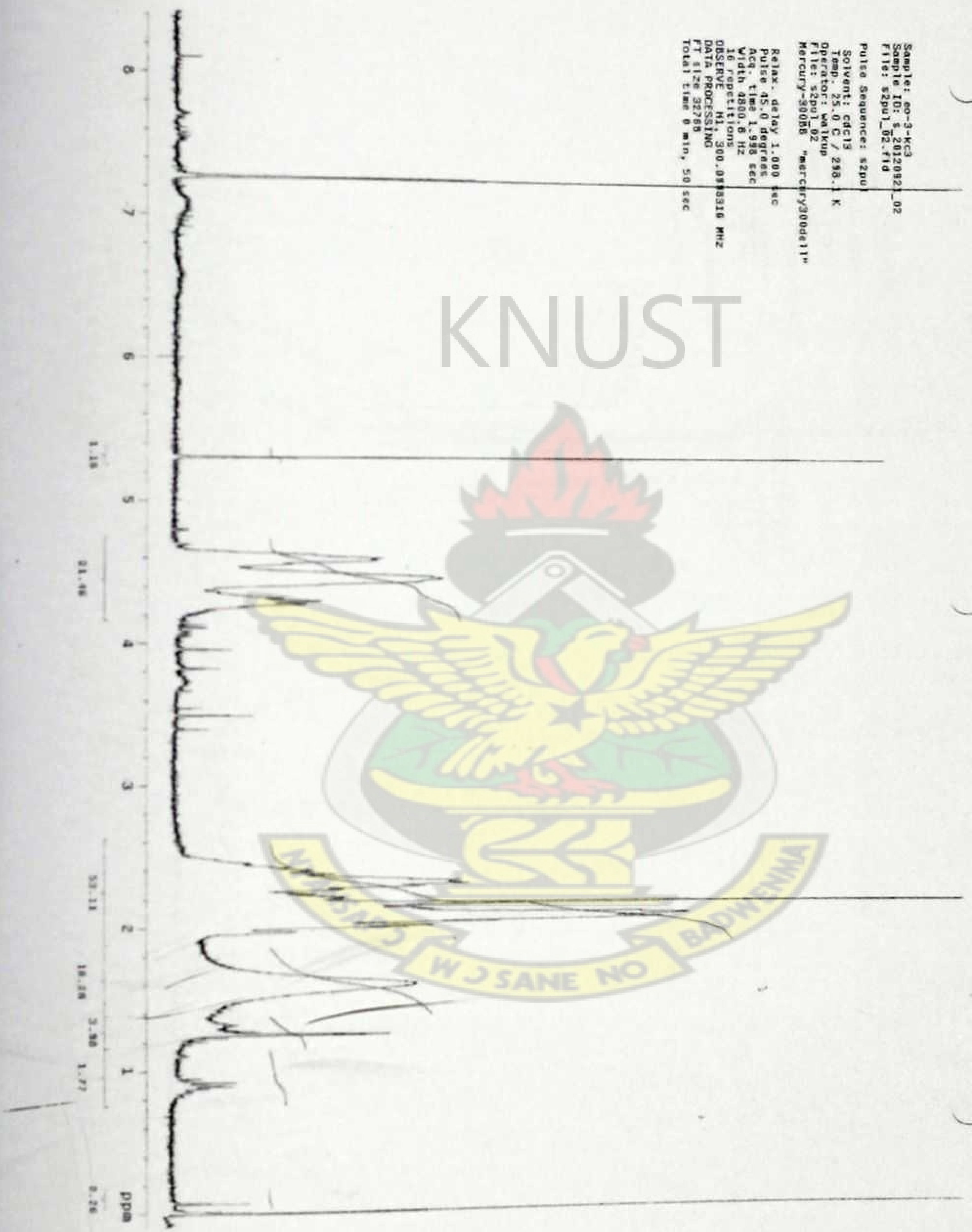


Figure 28 - Contracted 1H-NMR spectrum of Compound 3

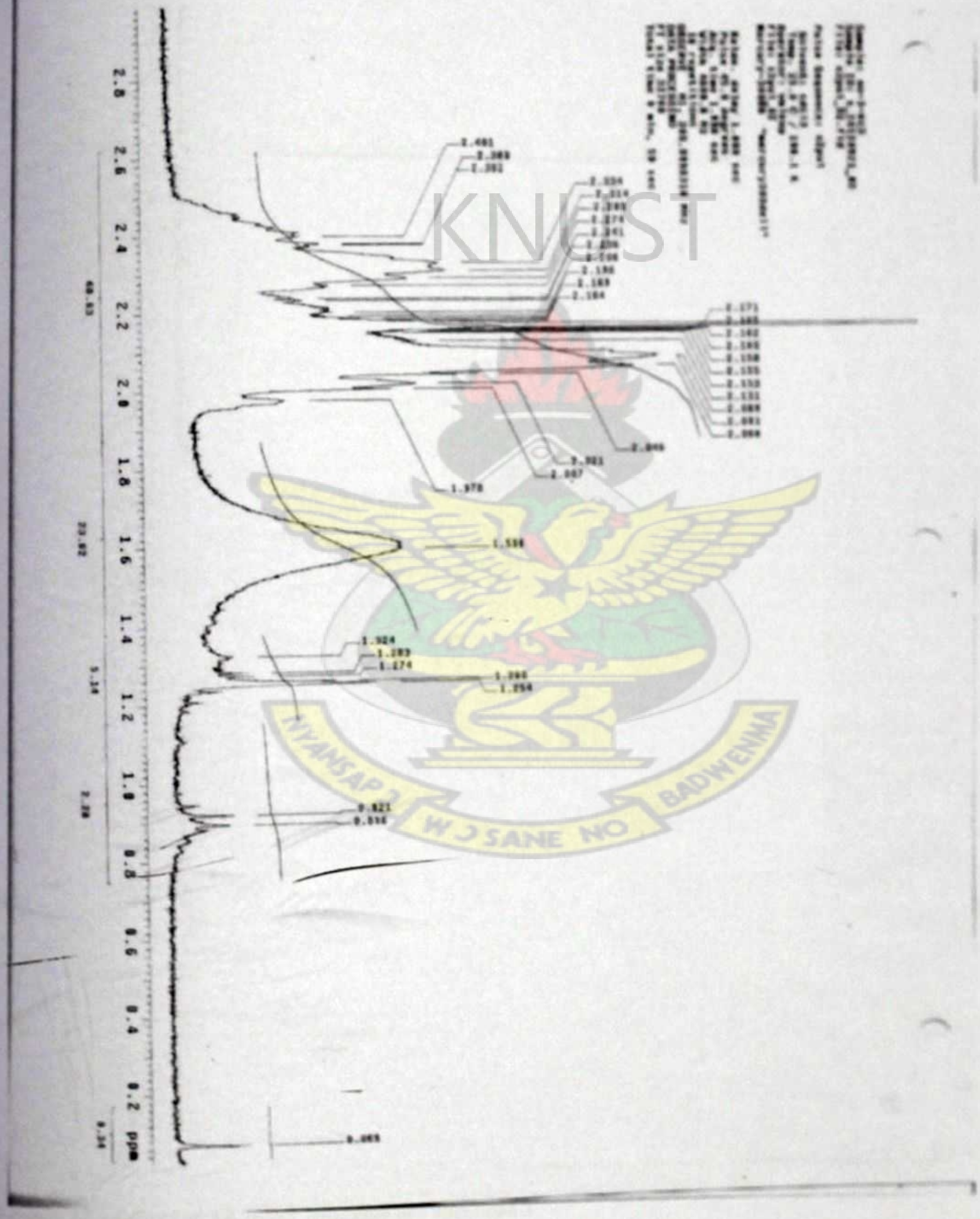


Figure 29 - Extended 1H-NMR spectrum of Compound 3

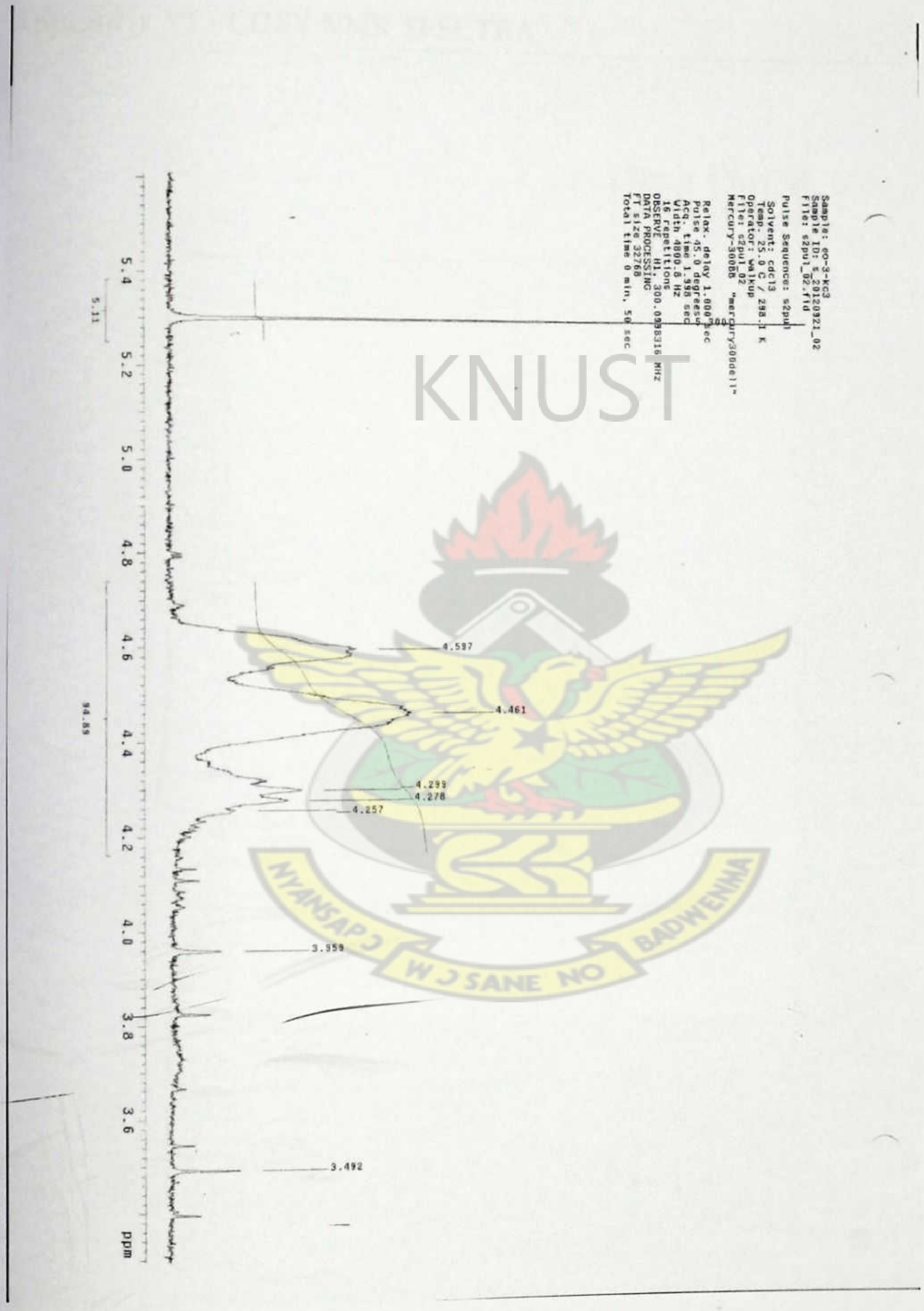


Figure 30 – Extended 1H-NMR spectrum of Compound 3

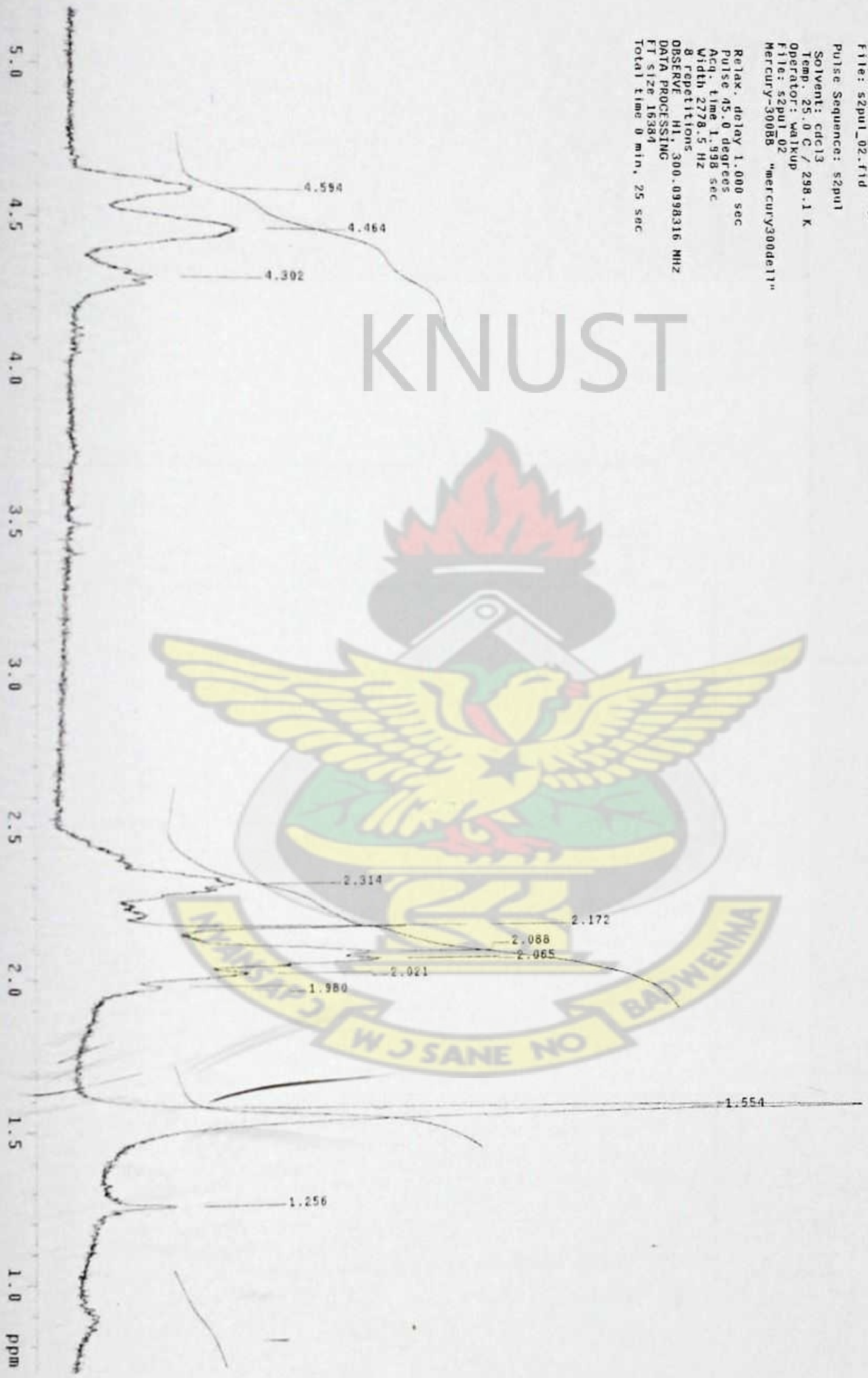
Appendix VI - COSY NMR SPECTRA

```
Sample: ed-3-ek1-cosy
Sample ID: s_20121031_02
File: s2pul_02.fid

Pulse Sequence: s2pul

Solvent: cdcl3
 Temp. 25.0 C / 298.1 K
Operator: walkup
File: s2pul_02
Mercury-300BB  "mercury300de11"

Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 1.998 sec
Width 2778.5 Hz
 8 repetitions
OBSERVE    H1, 300.0998316 MHz
DATA PROCESSING
FT size 16384
Total time 0 min, 25 sec
```



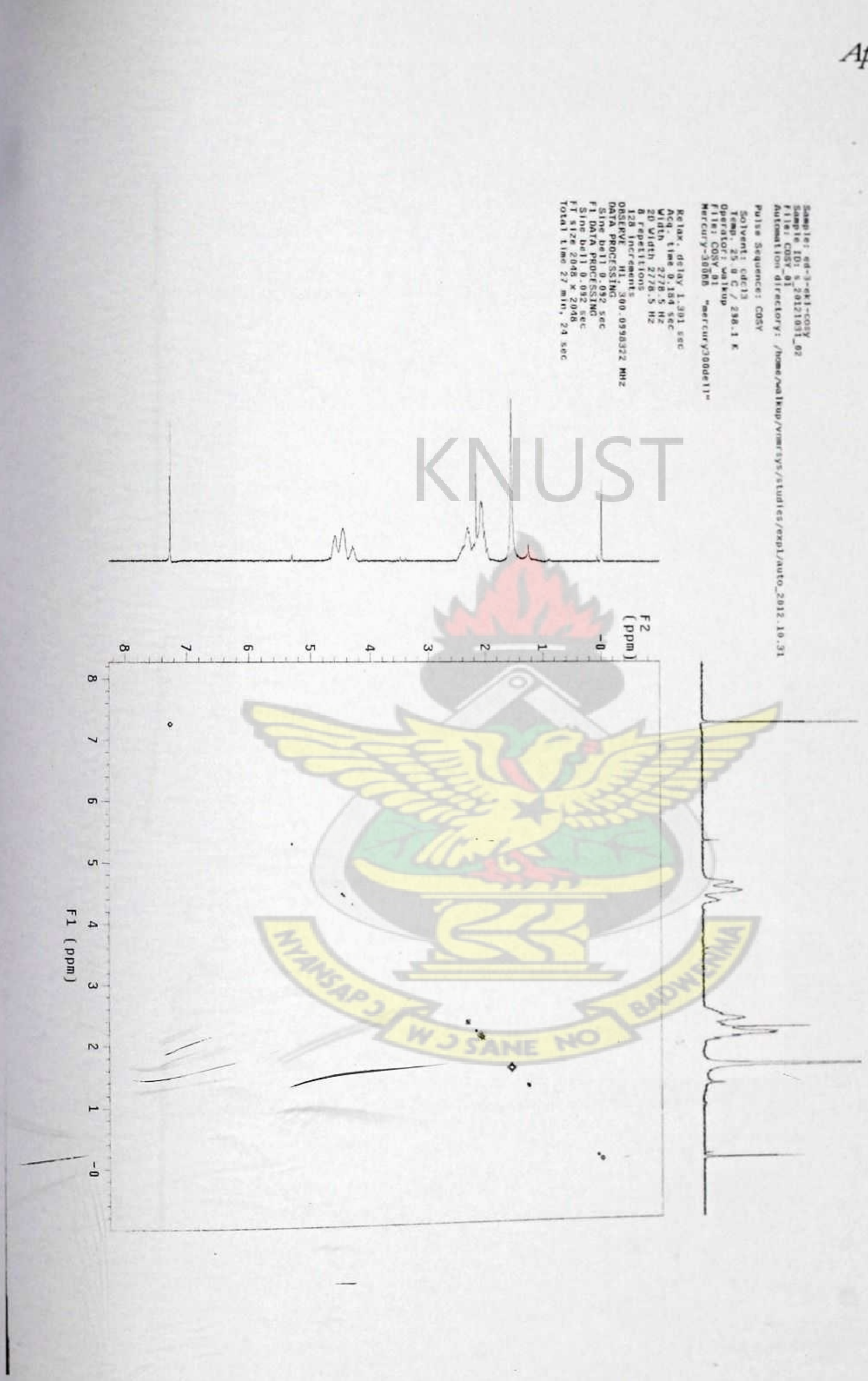
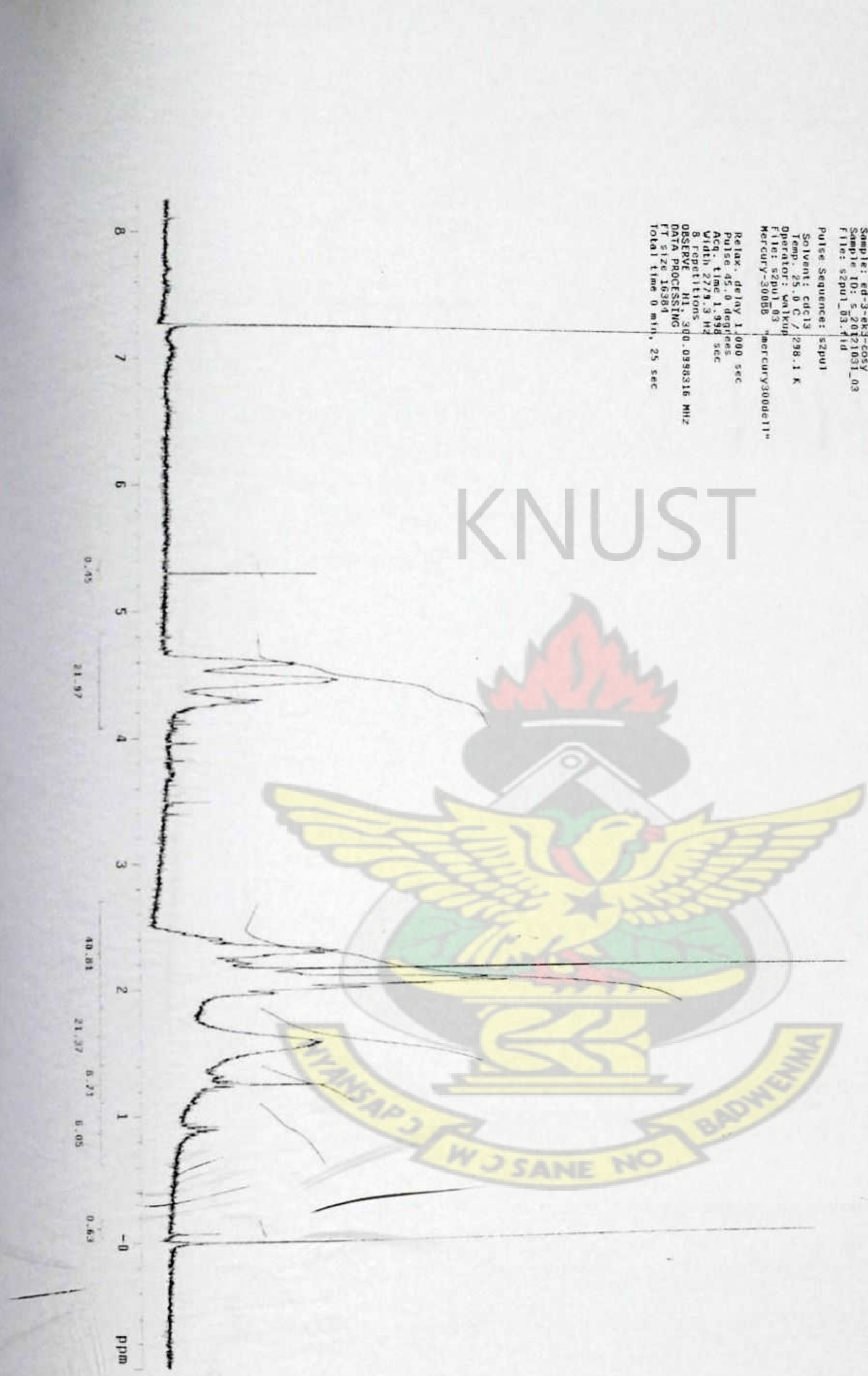


Figure 31 - COSY spectrum of Compound 1

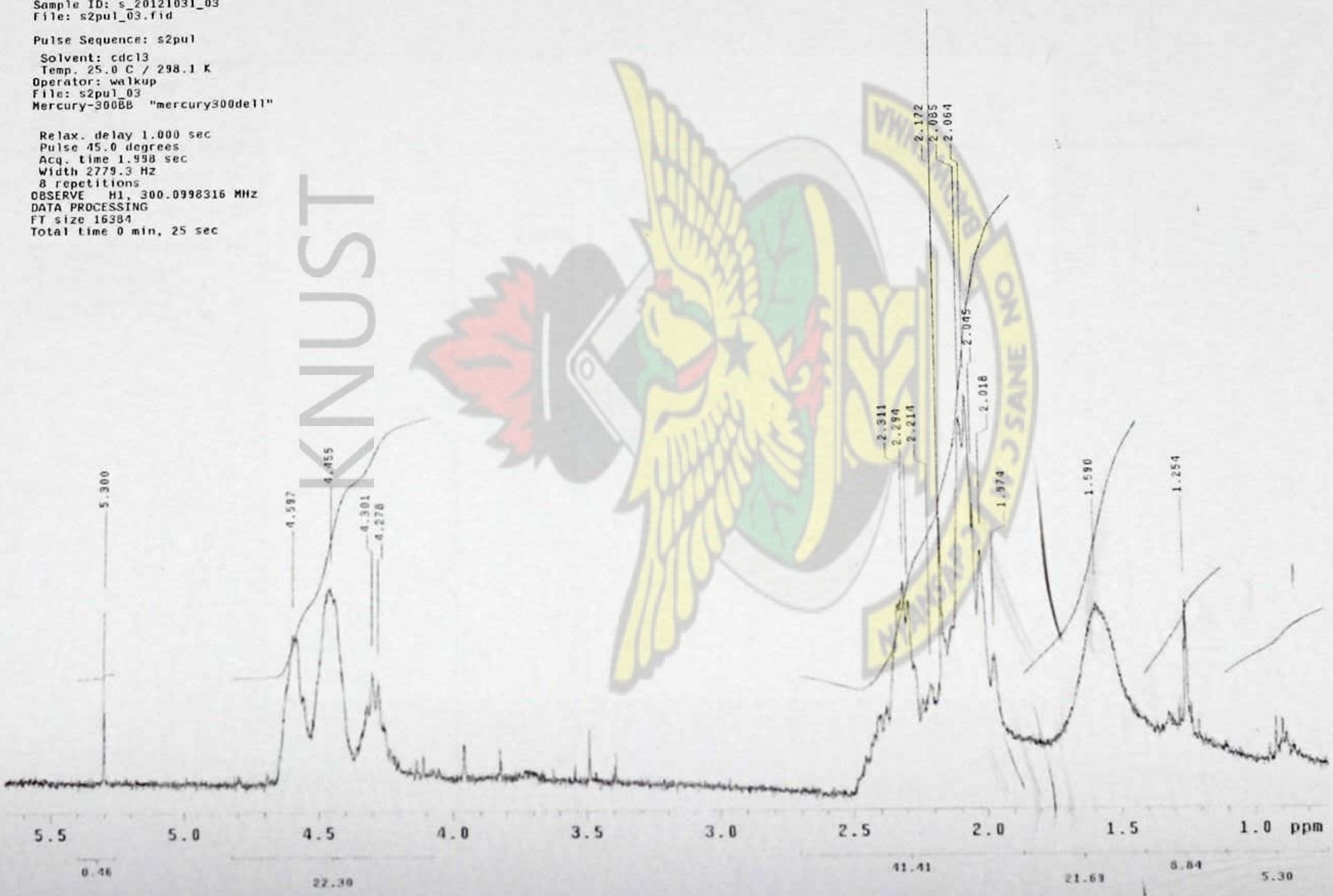

Sample: ed-3-ek3-cosy
Sample ID: s_20121031_03
File: s2pul_03.fid
Pulse Sequence: s2pul
Solvent: cdcl3
Temp. 25.0 C / 298.1 K
Operator: Walkup
File: s2pul_03
Mercury-300BB "mercury300de11"
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 1.998 sec
Width 2779.3 Hz
8 repetitions
OBSERVE H1, 300.0998316 MHz
DATA PROCESSING
FT size 16384
Total time 0 min, 25 sec
8
7
6
5
4
3
2
1
0
ppm
0.45
21.97
40.81
21.37
0.73
0.05
0.63

Sample: ed-3-ek3-cosy
Sample ID: s_20121031_03
File: s2pul_03.fid

Pulse Sequence: s2pul

Solvent: cdcl3
Temp. 25.0 C / 298.1 K
Operator: walkup
File: s2pul_03
Mercury-300BB "mercury300dell"

Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 1.998 sec
Width 2779.3 Hz
8 repetitions
OBSERVE H1, 300.0998316 MHz
DATA PROCESSING
FT size 16384
Total time 0 min, 25 sec



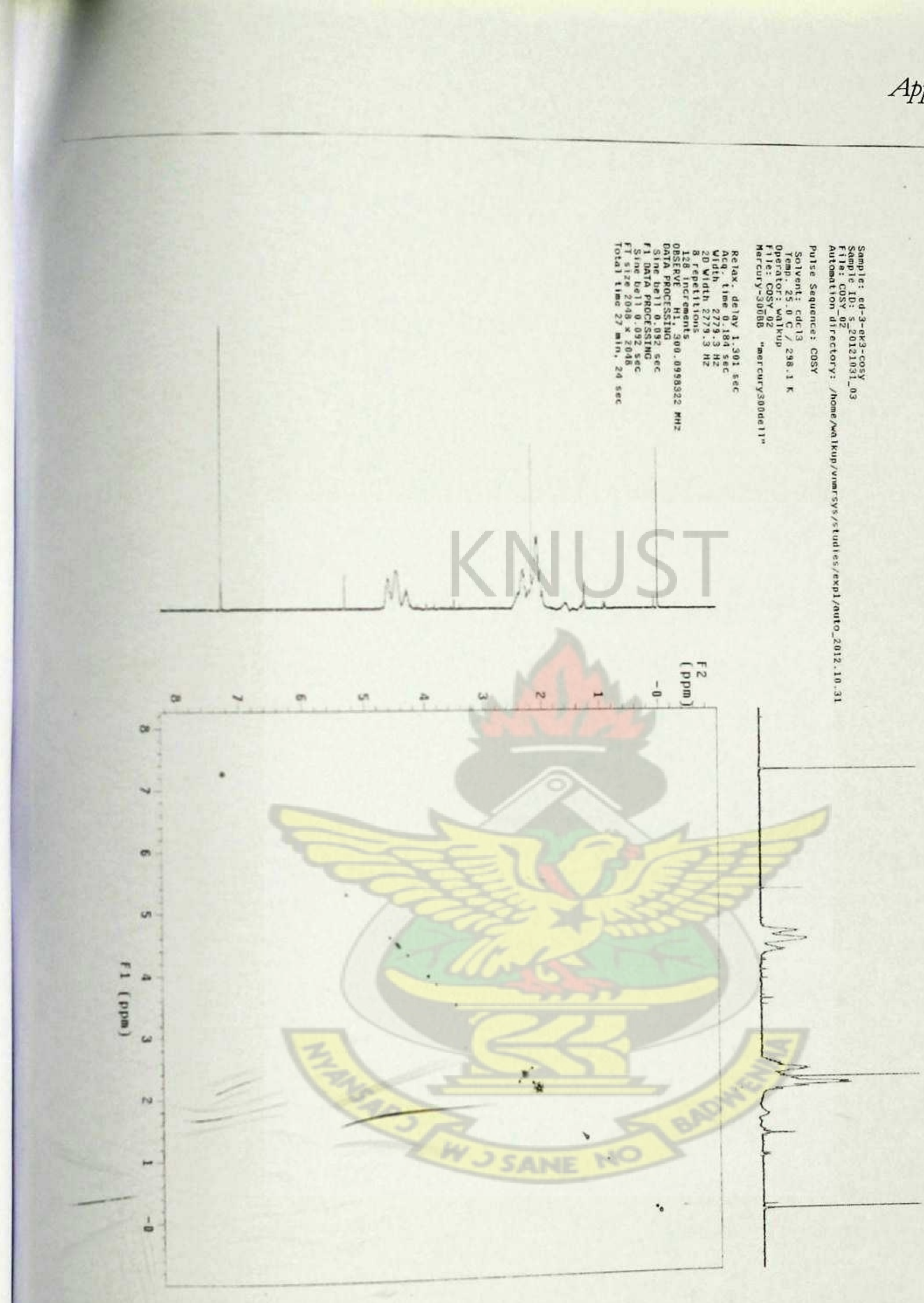


Figure 32 - COSY spectrum of Compound 3