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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

CHARACTERIZATION AND HPLC QUANTIFICATION OF PIPERINE IN VARIOUS PARTS OF PIPER GUINEENSE

SUBMITTED BY

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CHARACTERIZATION AND HPLC QUANTIFICATION OF PIPERINE IN VARIOUS PARTS OF PIPER GUINEENSE

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KUMASI, GHANA

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DECLARATION

I declare that the experimental work described in this thesis was carried out at the Department of Pharmaceutical Chemistry, KNUST. Any assistance obtained has been duly acknowledged. I declare to the best of my knowledge, this work has not been previously submitted for any other degree.

HEAD OF DEPARTMENT SUPERVISOR

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CANDIDATE

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ISAAC YAW ATTAH

DEDICATION

I dedicate this work to my parents and siblings.

ACKNOWLEDGEMENT

…… this is the Lord's doing, it is marvelous in our eyes… (Matthew 21:42)

My foremost and greatest appreciation goes to the Almighty God for all the favours and grace granted me during this project. Indeed He has set this cornerstone in place, may His name be glorified. Amen

My heartfelt gratitude goes to my supervisor, Prof. R. K. Adosraku for the solid support and kindness: his patience and guidance through the difficult times have greatly contributed in making this work a success.

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ABSTRACT

Medicinal plants have been used since medieval times for treating ailments. The extended use have led to high market value and adulteration of their herbal products.

Piper guineense, commonly known as West African Black Pepper, is a climbing plant up to 12m high by its adventitious rootlets. It has a corky lower stem and simple leaves which have five principal nerves. The fruits which are aromatic are red- brown when ripe and black when dry. Various parts of the plant have medicinal uses such as antibacterial, anticancer, etc. This project seeks to develop an HPLC method to standardize the various parts of *Piper guineense* using isolated piperine as a biomarker and a secondary reference. All samples of *P. guineense* parts were obtained and authenticated from the Physique garden of Pharmacognosy Department, KNUST.

Piperine was isolated from the dried fruits of *P. guineense* with ethanolic KOH and recrystallized from acetone: hexane 3:2. The melting point was determined to be 128ºC - 130ºC and the crude yield was 2.07%w/w. TLC gave R_f values of 0.285 ± 0.01291 for hexane: ethylactate: glacial acetic acid 3: 1: 0.3 and 0.70 ± 0.009574 for chloroform: ethylacetate 1: 1. It was characterized by melting point as well as Ultraviolet, Nuclear Magnetic Resonance and Mass Spectroscopy.

A validated reverse phase HPLC with methanol: water 80: 20 at a flow rate of 1.40ml/min on a Phenomenex Kromosil 5 C_8 (250mm x 4.6mm 5 micron id) column with detection at 343nm gave a retention time of 3.78 ± 0.05656 min.

Piperine was found to be 5.44 $x10^{-3}$ ± 9.24 x 10^{-5}%w/w in the dried leaves (DL), 0.0437 ± 0.000816%w/w for fresh leaves (FL), 0.115 ± 0.00228%w/w for dried stem (DS) and 3.345 ± 0.0339%w/w for dried fruits (DF).

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INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Medicinal plants have been used since medieval times as a source of medicine for the treatment of all kinds of ailments. Traditional medicine and medicinal plants continue to play a role in the primary healthcare systems of most cultures.

About 25% of the drugs prescribed worldwide and found in modern pharmacopoeias come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. Examples of important drugs obtained from plants are digoxin from *Digitalis* spp. used for treating heart failure , quinine (antimalarials) and quinidine (antiarrythmic) from *Cinchona* spp., vincristrine and vinblastine (anticancer agents) from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin. (**Rates, 2001)**

The World Health Organization (WHO) estimated that 80% of the populations of developing countries in Asia and Africa rely on traditional medicines, mostly plant drugs, for their primary health care needs. **(WHO, 2008)**

Again there has recently also been an increasing reliance on the use of medicinal plants in the industrialized societies and this has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used herbal remedies.

The medicinal properties of these plants could be based on the antioxidant, antimicrobial, antipyretic effects etc, of the phytochemicals or secondary metabolites in them. **(Soetan and Aiyelaagbe, May, 2009)**

This resurgence of interest in medicinal plants and traditional medicine as a whole is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in side effects and other problems, a large percentage of the world's population does not have access to conventional pharmacological treatment perhaps due to inaccessibility of healthcare centers and the ever increasing cost of synthetic drugs. Finally folk medicine and ecological awareness suggest that "natural" products are harmless. **(Rates, 2001)**

Contrary however to the view that medicinal plants and their products are harmless, it is rather fair to state that they are relatively safer to the human somatic system and environmentally compared to their orthodox counterparts because some medicinal plants have been established to have some toxicity. For example, cryptolepiene (an antimalarial) from *Cryptolepis sanguinolenta* has been shown to be cytotoxic, ergot alkaloids could also lead to hallucination in significant doses, digoxin overdose could lead to cardiac problems. This indicates that it is important to know the amount of phytochemicals especially the bioactive moiety in medicinal plants hence standardization.

With the ever-increasing use of herbal medicines worldwide and the rapid expansion of the global market for these products, the safety and quality of medicinal plant materials and finished

herbal medicinal products have become a major concern for health authorities, pharmaceutical industries and the public.

From time to time, undesirable and/or undeclared substances i.e. adulterants have been present or have been purported to be present in herbal medicines or medicinal plants in many parts of the world. This therefore necessitates a need for quality control methods for the standardization of such medicinal plant materials especially for their local and international trade and to ensure safety in consumption.

According to the WHO, these methods need to be validated for the material that is to be tested, e.g. roots, seeds, leaves, plant species and also for each type of instrument, e.g. for gas chromatography (GC), high-performance liquid chromatography (HPLC) or atomic absorption (AA) spectrophotometry. This process should include validation of detection limits, as these may depend on the instrument and type of sample.**(WHO, 2007)**

International trade of medicinal plants and herbal products is a very lucrative venture yielding US$ 5 billion in Western Europe alone from 2003- 2004. China alone profited US$ 14 billion in 2005. **(WHO, 2008)**

An estimated 951 tons of crude herbal medicine were sold at Ghana's herbal markets in 2010, with a total value of around US$ 7.8 million. The booming trade provides employment and sustainable income for cultivators, collectors from the wild and exporters of the herbs.

Among the herbal plants sold, seeds and fruits that doubled as spice and medicine (*Xylopia aethiopica, Monodora myristica, Aframomum melegueta, Piper guineense*) were in highest demand. **(van Andel *et al.*, 2012)**

Spices and their uses are woven deep into the civilization and in the history of nations. Their delightful flavour and pungency makes for the preparation of palatable dishes. They are also

mostly indispensable in the preparation of a number of herbal preparations because of their several medicinal and pharmacological properties.

World trade in spices in 2004 consisted of 1.547 million tons, valued at US$2.97 billion with black pepper contributing about US$ 394million and potential for increase. The marketing and export potential for standardized and value added products are even greater. **(Parthasarathy *et al.*, 2008)**

1.2 LITERATURE REVIEW

1.2.1 PIPER GUINEENSIS

Botanical name: *Piper guineese* Schum & Thonn.

Synonyms: *Piper leonense* C. D. C.; *Piper famechonii* C. D. C.

Common names: Ashanti pepper, West African black pepper, Guinea cubebs

Vernacular names: Ewe – kale, kukuabe; Twi – Soro wisa, Sesaa, Asonsa; Hausa – masoro; Ga-Adangbe - Gbowie

Family: Piperaceae

Phytochemical Constituents: Volatile oils. The alkaloids present includes piperine, wisamine, dihydrowisamine, dihydropiperine and other related alkaloids. It also has resins. The lignans in the plant includes dihydrocubebin, sesamin, aschantin and others.

Botanical description and distribution: It is a climber up to 12m high on trees, climbing by means of its adventitious rootlets. It has a corky lower stem. It has simple leaves which are opposite, ovate, acuminate at the apex and cordate at the base with five principal nerves. The plant has solitary spikes. The fruits are red- brown when ripe and black when dry. The fruit are

aromatic. The plant occurs in closed forests on trees in forests clearings. It is widely distributed in Africa from Guinea to Uganda.

Figure 1 : Leaves, fresh and dried seeds of *Piper guineense*.

Indications of *Piper guineense* : for boils, bronchitis, catarrh, chest pains, coughs, dyspepsia, impotence, insect repellant, lumbago and rheumatism. It is also used for treating uterine fibroids and wounds. **(Busia, 2007)**

1.2.2 FAMILY PIPERACEAE

The family Piperaceae has over 2000 species classified under four major genera namely, *Piper*, *Peperomia*, *Sarchorhachis*, and *Ottonia* and has been classified among the basal Angiosperms. Species of *Piper* are located in all types of vegetation but mostly as components of pioneer vegetation. **(Kato and Furlan, 2007)**

Most members of the genus *Piper* are climbers although some are shrubs and a few are trees which are distributed mainly in the tropics of the world. **(Chaveerach *et al.*, 2006)**

Some of the members of this family include *P. tuberculatum, P. longum, P. nigrum,* etc.

The occurrence of bioactive amides in *Piper* species is quite common and has been proven to be responsible for the insectidal, pesticidal and antimicrobial activity. The most significant compound of *Piper* species is the piperamide piperine which constitutes the active pungent principle of black pepper.

Other compounds such as chromenes, prenylated benzoic acid, hydroquinones, and polyketides with significant antifungal activities have also been isolated from various *Piper* species.

A preliminary study of the structure–activity relationships indicated that the antifungal activity against *C. cladosporioides* is associated directly with the lipophilic properties of the molecules such as that occasioned by methylation of chromene I, or by the introduction of a second isoprenyl moiety as in chromene III. It is also worth mentioning that benzoic acid derivatives from several *Piper* species are active against microorganisms and that, as with the amides, this class of compound appears to be characteristic of *Piper* species. These compounds together show significant evidence of activity relating to their toxicity, synergistic effect, and specificity against insects.

Hydroquinones have also been found in the Piper species, and their biological activities have been demonstrated to include inhibition of mitosis as well as antitumor, antileukemic, analgesic, relaxant, and antioxidant effects. Prenylated hydroquinones have been shown to inhibit lipoperoxidation compared to hydroquinone itself due to the presence of the lipophilic prenyl group that facilitates insertion of the molecule into the liposome in the model assay.

Piper species also contains alkylated phenolic compounds as lead for promising compounds with important antioxidant properties hence are been exploited in the cosmetic industry to alleviate the symptoms of aging. **(Kato and Furlan, 2007)**

1.2.3 PIPERINE

Piperine is the major alkaloid of black and long pepper of commerce and other Piper species. It is a trans – trans isomer of 1 - piperoyl piperidine. It is also known as piperoylpiperidide. It occurs in several peppers and was first isolated from the fruits of *Piper nigrum,* which furnish the black and the white peppers of commerce. Since then it has been isolated from *P. longum* and *P. officinarum, P. clusii, P. farnechoni* by different people.

The amount of piperine varies from 1 to 2 per cent, in long pepper, to from 5 to 9 per cent, in the white and the black peppers of commerce.

It may be prepared by treating the solvent-free residue from an alcoholic extract of black pepper, with a solution of sodium hydroxide to remove resin (this resin contain *chavicine,* an isomeride of piperine) and the solution of the washed, insoluble residue in warm alcohol, from which the alkaloid crystallises on cooling.

Piperine forms yellowish monoclinic needles. Its molecular formular is $C_{17}H_{19}0_3N$. It has a melting point (m.p.) of 128-129.5°C, is slightly soluble in water but completely soluble in alcohol, ether or chloroform. The alcoholic solution has a pepper-like taste. It yields salts only with strong acids. **(Henry, 1949)**

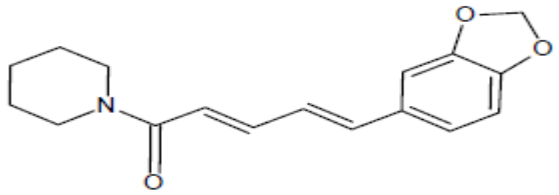


Figure 2: Structure of Piperine [(E,E)1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine], $C_{17}H_{19}0_3N$

1.2.4 RESEARCH UPDATES ON PIPER GUINEENSIS AND PIPERINE

Piper guineense

The results of a preliminary antimicrobial screening of the methanol extracts of some medicinal plants specifically spices in Ghana reveals that *P. guineense* has antibacterial activity against both Gram +ve and –ve bacteria and also has pronounced antifungal activity **(Konning *et al.*, 2004)**. Research have shown that *Piper guineense* could be important sources of bactericidal compounds against *M. tuberculosis* and could probably be promising candidates that can be further investigated **(Tekwu *et al.*, 2012).**

Piper guineense have also been shown to have significant antioxidant and anti-atherogenic effect against atherogenic diet intoxication in hamsters. They also prevented LDL oxidation by increasing the time (lag time) for its oxidation. They prevented the collapse of the antioxidant system and the increase of plasma parameters maintaining them towards normality **(Agbor *et al.*, 2012).** Through their antioxidant activity, inhibition of key enzymes in type 2 diabetes and sodium nitroprusside (SNP)-induced lipid peroxidation in rat pancreas - in vitro, *Piper guineese* and other spices have exhibited some antidiabetic activity with promising potential **(Adefegha and Oboh, 2012)**. Fruit extract of *P. guineense* have shown significant molluscicidal effect

Biomphalaria pfeifferi, the snail intermediate host of *Schistosoma mansoni*, which causes intestinal schistosomiasis **(Ukwandu *et al.*, 2011).**

Following electrical stimulation, the leaf and seed extracts of *Piper guineense* shown to possess among other pharmacological properties, a depolarizing neuromuscular blocking action on the skeletal muscle activity of rats and frogs **(Udoh *et al.*, 1999)**. Further studies on the uterine muscle of rats indicate the leaf and seed extracts of *Piper guineense* possess oestrogenic and oxytocic properties which might justify their usage in Nigerian traditional medicine **(Udoh, 1999).** Using parameters such as penile erection index, copulatory behaviour and orientation activities towards themselves (genital grooming) and female rats (ano-genital sniffing, mounting), aqueous extracts of *P. guineense* modified the sexual behaviour of male rats by increasing sexual arousal **(Kamtchouing *et al.*, 2002)**. A water extract of the West African black pepper *Piper guineense L.* was tested for activity against seizures induced in mice indicates the extract has prolonged anticonvulsant activity at doses which do not cause significant CNS depression **(Abila *et al.*, 1993).** *P. guineense* happens to be among the top listed plants in an ethnobotanical of plants and plant recipes for the treatment of inflammatory diseases such as rheumatoid- arthritis and asthma **(Ogbole *et al.*, 2010).**

Piperine

A study using reserpine, chlorpromazine, verapamil and carbonyl cyanide m-chlorophenylhydrazone as positive controls demonstrated that piperine significantly enhanced accumulation and decreased the efflux of ethidium bromide in *M. smegmatis*, which suggests that it has the ability to inhibit mycobacterial efflux pumps **(Jin *et al.*, 2011)**. Piperine isolated from the ethanolic extract of *Piper longum L.* has exhibited appreciable antihyperlipidemic activity in vivo, which is comparable to that of the commercial antihyperlipidemic drug,

simvastatin **(Jin *et al.*, 2009)**. Piperine has also been shown to possess a blood pressure-lowering effect mediated possibly through calcium channel blockade associated with varying vasomodulator effects **(Taqvi *et al.*, 2008)**. Piperine has also been shown to inhibit testosterone 5-alpha-reductase indicating their potential use as anti-androgenic agents in cosmetic hair-care agents **(Hirata *et al.*, 2007).**

It has been reported that piperine shows antitumor activity against Agrobacterium tumefaciens-induced crown gall tumor formation in potato disc. Again piperine has shown significant antibacterial activity against both Gram +ve and Gram –ve bacteria **(Das *et al.*, 2007).** In vivo studies performed in female Swiss mice indicates piperine inhibits development of sarcoma 180 significantly at both low and high doses. However treatment is accompanied by some level of liver toxicity.**(Bezerra *et al.*, 2006).** Kinetic analyses by a Lineweaver-Burk plot clearly indicated that piperine competitively inhibited MAO-A and MAO-B with the immobility times in the tail suspension test significantly similar to that of the reference antidepressant fluoxetine suggesting piperine possesses potent antidepressant-like properties **(Lee *et al.*, 2008).**

Piperine has been demonstrated to exhibit an immunoregulatory properties by either reducing or completely iinhibiting inflammatory agents such as nuclear factor-kappaB (NF-kappaB), c-Fos, CREB, ATF-2 and proinflammatory cytokine gene expression in B16F-10 melanoma cells **(Pradeep and Kuttan, 2004).** Subacute treatment of diabetes-induced oxidative stress in 30-day streptozotocin-induced diabetic Sprague-Dawley rats with piperine for 14 days indicates piperine is only partially effective as an antioxidant therapy in diabetes **(Rauscher *et al.*, 2000).** Experiments conducted to determine the Protective action of piperine against gastric ulcer induced by stress, indomethacin, HCl and pyloric litigation indicates piperine has a potent protective activity against all three forms of gastric ulceration by inhibiting the volume of gastric

juice, gastric acidity, and pepsin A. activity **(Bai and Xu, 2000)**. The plant principle piperine has been shown to have exerted a significant protection against tert-butyl hydroperoxide and carbon tetrachloride hepatotoxicity by reducing both in vitro and in vivo lipid peroxidation, enzymatic leakage of GPT and AP, and by preventing the depletion of GSH and total thiols in intoxicated mice. However it showed lower hepatoprotective potency than silymarin, a known hepatoprotective drug **(Koul and Kapil, 1993).** Piperine has also been demonstrated to enhance the bioavailability of curcumin by 2000% when administered concomitantly in animals and human volunteers. The study shows that in the dosages used, piperine enhances the serum concentration, extent of absorption and bioavailability of curcumin in both rats and humans with no adverse effects. Piperine has therefore to be a potent bioenhancer **(Shoba *et al.*, 1998).**

Before the advent of hplc, various methods of analysis were used to quantify piperine in Piper species. These methods include colorimetric, titrimetric methods, thin layer chromatography (tlc), a direct uv spectrophotometric, a tlc - uv spectrophotometric method and a tlc - densitometric method **(Jansz *et al.*, 1983).**

An isocratic HPLC method has been developed for analysis and isolation of the following four possible piperine-derived photoinduced isomers: piperine, isopiperine, chavicine, and isochavicine, using acetonitrile/ 0.5% formic acid (30/70 v/v), flow rate 0.8ml/min at 25 °C with diode array UV detection-mass spectrometry (LC-DAD/MS) **(Kozukue *et al.*, 2007).** Piperine and guggulsterones in compound Unani tablets and in a novel approach of drug release via oil-in-water (o/w) nanoemulsion formulation system were estimated simultaneously by a validated developed HPLC method. The mobile phase used consisted of acetonitrile and water in gradient flow starting with the organic phase from 10 to 80% in 30.0 min. Separated constituents

were identified by running their individual standards under identical HPLC conditions and comparing their retention times at 240nm wavelength using a UV – vis detector **(Kamal *et al.*, 2011).**

Using Photodiode array detection (PAD) to simultaneously monitor piperine at 340 nm and ketoconazole at 231 nm in a single sample, a simple and rapid HPLC method has been developed for the simultaneous analysis of ketoconazole and piperine in rat plasma and hepatocyte culture. Analysis was performed using a Symmetry C18 column (150x4.6 mm, 5 microm) and isocratic elution with 25 mM KH_2PO_4 (pH 4.5) - acetonitrile (50:50) with a flow-rate of 1 ml/min **(Bajad *et al.*, 2002).** A HPLC method was developed for the estimation of piperine in laboratory sample and marketed formulations of Pancasama churna (PSC) on a C-18 column with a mobile phase of methanol: water (69:31) with a 1.5ml/min flow rate at 343 nm using a UV-visible detector **(Gupta and Jain, 2011).**

In order to determine piperine in pepper (*Piper nigrum L.*) and its oleoresins, a HPLC method employing a C18 column and a mobile phase of acetonitrile / 1% aqueous acetic acid 50/50 $^{v}/_{v}$ was used. A UV- visible detector was used with 1.5ml/min flow rate. The wavelength of choice was 343 nm **(Wood *et al.*, 1988).**

1.3 JUSTIFICATION

Piper guineense is used in many folklore medicine and has a number of verified pharmacological activities. Piperine has been largely investigated to be the active moiety in the plant responsible for most of its activity and toxicity as shown by the above research updates. Spices like *P. guineense* are on demand on the international market. Piperine has been used as a biomarker to standardize other species of the pepper family such as *P. nigrum, P. longum* giving them higher

market premiums. However no such standardization has been done for *P. guineense*. Standardization of *P. guineense* would largely help to re-evaluate its market premium to boost the trade in Ghana since various parts of the plant are being sold and exported from West Africa. Various parts of *P. guineense* are used in folklore medicine and in many herbal preparations on the market without knowledge of the exact amount of piperine in such products. Piperine has certain serious antifertility toxicities in swiss mice by decreasing the activity of the antioxidant enzymes and sialic acid and hampering the epididymal environment where sperm maturation takes place **(Daware *et al.*, 2000**). A study on mice, rats, and hamsters show that piperine causes death in 3 – 17 minutes in the female rats due to respiratory paralysis at an LD_{50} value of 33.5 mg/kg body weight. Histopathologic changes observed included severe hemorrhagic necrosis and edema in gastrointestinal tract, urinary bladder and adrenal glands inferring that death in these animals could be to multiple organ damage **(Piyachaturawat *et al.*, 1983)**. Again doses of piperine doses of 2.25 and 4.5 mg/kg body weight in swiss male mice have been shown to induce immunotoxicological effects (**Dogra *et al.*, 2004)**. Standardization would however help reduce such toxic bouts.

This project tends to develop a RP-HPLC method to quantify the amount of piperine in the fruits, leaves and stem of *P. guineense*. This method can then be used to standardize herbal preparations containing *P. guineense* and the plant parts for international trade.

1.4 OBJECTIVES

- To isolate, purify and characterize piperine from the dried fruits of *Piper guineense* to be used as a reference standard.
- To develop and validate an HPLC method for quantifying piperine.

- To use the validated HPLC method to determine the percentage amount by weight of piperine in the dried fruits, leaves and stem of *Piper guineense*.

1.5 THEORY OF EXPERIMENTAL WORK

1.5.1 ISOLATION AND PURIFICATION TECHNIQUES

Frequently after a chemical reaction, the desired product would have to be isolated from the reaction mixture which may contain impurities such as the solvent used as the reaction medium, excess reactants or reagents, unwanted reaction products (by - products) resulting from alternative reaction pathways and many more.

The physical state at ambient temperature of the crude mixture resulting from the reaction, i.e. whether it is a *one-phase* (either *solid* or *liquid)* or a *two-phase (solid/liquid* or *liquid/liquid)* system to a large extent determines the particular isolation procedure to be used for a particular product of interest and not just on its physical and chemical properties.

In the case of a solid organic product if the organic product is neutral and insoluble in water, washing with water may be used to remove soluble impurities such as inorganic salts. Alternatively the crude solid may be extracted with a suitable organic solvent, filtered, and the extract washed with water. Further washing, successively with dilute aqueous acid and dilute aqueous alkali removes basic and acidic impurities. Removal of solvent after drying leads to the recovery of the purified solid for recrystallisation from a suitable solvent. Continuous extraction of the solid (e.g. in a Soxhlet apparatus) may be necessary if the required product is only sparingly soluble in convenient organic solvents.

If the crude solid product contains the required product in the form of a salt (e.g. the alkali metal salt of a phenol) and is therefore water soluble, acidification of the aqueous solution (or basification in the case, for example, of amine salts) liberates the free acidic compound (or base) which may be recovered by filtration or solvent extraction as appropriate.

The *one-phase liquid system* is more frequently encountered since many organic reactions are carried out in solution. Direct fractional distillation may separate the product, if it is a liquid, from the solvent and other liquid reagents, or concentration or cooling may lead to direct crystallisation of the product if this is a solid. However, it is often more appropriate, whether the required product is a liquid or solid, to subject the solution to the acid/base extraction procedure outlined above. This acid/base extraction procedure can be done directly if the product is in solution in a water-immiscible solvent. Knowledge of the acid-base nature of the product and of its water solubility is necessary to ensure that the appropriate fraction is retained for product recovery.

In those cases where the reaction solvent is water miscible (e.g. methanol, ethanol, dimethylsulphoxide, etc.) it is necessary to remove all or most of the solvent by distillation and to dissolve the residue in an excess of a water-immiscible solvent before commencing the extraction procedure. The removal of solvent from fractions obtained by these extraction procedures is these days readily effected by the use of a rotary evaporator and this obviates the prolong removal of large volumes of solvent by conventional distillation. A crude reaction mixture consisting of *two phases* is very common. In the case of a *solid/liquid* system, it will of course be necessary to make certain in which phase the required product resides. A simple example is where the product may have crystallised out from the reaction solvent; the mixture therefore only requires to be cooled and filtered for the bulk of the product to be isolated. The filtrate should then routinely be subjected to suitable concentration or extraction procedures to obtain the maximum yield of product. Direct filtration would also be employed when the solid consists of unwanted reaction products, in which case the filtrate would be treated as the single-phase liquid system above. Where it is evident that the product has crystallised out admixed with

contaminating solid material a separation might be effected if the mixture is reheated and filtered hot.

Liquid/liquid two-phase systems are often encountered; for example, they result from the frequent practice of quenching a reaction carried out in an organic solvent by pouring it on to ice or into dilute acid. A further instance of a liquid/liquid system arises from the use of steam distillation as a preliminary isolation procedure. This is particularly suitable for the separation of relatively high-boiling liquids and steam volatile solids from inorganic contaminants, involatile tars, etc. The subsequent work-up procedure normally presents no additional problems since the phases are usually readily separable and can be treated in a manner appropriate to the chemical or physical properties of the required product by procedures already outlined.

All these preliminary procedures give solid or liquid products which are rarely of high purity; the degree of purity may be checked by chromatographic and spectroscopic methods. Purification may often be successfully accomplished by recrystallisation or sublimation for solids; fractional distillation under atmospheric or reduced pressure for liquids or low melting solids; molecular distillation for high-boiling liquids. In those cases where the use of these traditional methods does not yield product of adequate purity, resort must be made to preparative chromatographic procedures. Here knowledge of the chromatographic behaviour obtained from small-scale trial experiments will be particularly valuable.

The final assessment of the purity of a known product is made on the basis of its physical constants in comparison with those cited in the literature. In the case of a new compound the purity should be assessed and the structural identity established by appropriate chromatographic and spectroscopic methods **(Furniss *et al.*, 1989).**

1.5.2 FILTERATION TECHNIQUES

Filtration is the process of removing material, often but not always a solid, from a substrate in which it is suspended. This process is a physical one therefore any chemical reaction is inadvertent and normally unwanted. Filtration is accomplished by passing the mixture to be processed through one of the many available sieves called filter media. These are of two kinds: surface filters and depth filters. With the surface filter, filtration is essentially an exclusion process. Particles larger than the filter's pore or mesh dimensions are retained on the surface of the filter; all other matter passes through. Examples are filter papers, membranes, mesh sieves, and the like. These are frequently used when the solid is to be collected and the filtrate is to be discarded. Depth filters, however, retain particles both on their surface and throughout their thickness; they are more likely to be used in industrial processes to clarify liquids for purification.

In the laboratory, filtration is generally used to separate solid impurities from a liquid or a solution or to collect a solid substance from the liquid or solution from which it was precipitated or recrystallized. This process can be accomplished with the help of gravity alone or it can be accelerated by using vacuum techniques.

Filtration efficiency depends on the correct selection of the method to be used, the various pieces of apparatus available, the utilization of the filter medium most appropriate for the particular process, and the use of the correct laboratory technique in performing the manipulations involved. Although the carrier liquid is usually relatively nonreactive, it is sometimes necessary to filter materials from high alkaline or acidic carrier liquids or to perform filtration under other highly corrosive conditions. A variety of filter media exists from which it is possible to select whichever medium best fits the particular objectives and conditions of a given process**.**

Filter Media The most commonly used surface filter media in the analytical lab are mostly filter papers and membrane filters.

Filter papers come in various grades as a filter media. There are qualitative grades, low-ash or ashless quantitative grades, hardened grades, and glass-fiber papers. The proper filter paper must be selected with regard to porosity and residue (or ash). All grades of filter paper are manufactured in a variety of sizes and in several degrees of porosity. Selection of the proper porosity for a given precipitate is always important. If too coarse a paper is used, very small crystals may pass through, while use of too fine a paper will make filtration unduly slow. The main objective is to carry out the filtration as rapidly as possible while retaining the precipitate on the paper with a minimum loss.

Membrane filters are thin polymeric (plastic) structures with extraordinarily fine pores. Membrane filters are available in a wide variety of pore sizes in a number of different polymeric materials. There are two types of membrane filters—surface and depth. Surface membrane filters are used for final filtration or prefiltration, whereas a depth membrane filter is generally used in clarifying applications where quantitative retention is not required or as a prefilter to prolong the life of a downstream surface membrane filter

Filter Aids. During filtration certain gummy, gelatinous, flocculent, semicolloidal, or extremely fine particulates often quickly clog the pores of a filter paper. Filter aids consist of diatomaceous earth and are sold under the trade names of Celite or FilterAid. They are pure and inert powderlike materials that form a porous film or cake on the filter medium. In use, they are slurried or mixed with the solvent to form a thin paste and then filtered through the paper. An alternative procedure involves the addition of the filter aid directly to the problem slurry with thorough mixing.

Filter aids cannot be used when the object of the filtration is to collect a solid product because the precipitate collected also contains the filter aid. For quantitative purposes, filtration of gelatinous materials can be assisted by adding shredded ashless paper tables to the solution before filtration. The dispersed fibers form passages through the precipitate for flow of liquid.

Manipulations Associated with the Filtration Process

Decantation. When a solid readily settles to the bottom of a liquid and shows little or no tendency to remain suspended, it can be separated easily from the liquid by carefully pouring off the liquid so that no solid is carried along.

Washing. The objective of washing is to remove the excess liquid phase and any soluble impurities that may be present in the precipitate. Use a solvent that is miscible with the liquid phase but does not dissolve the precipitate. Solids can also be washed in the beaker by adding alittle of the solvent, mixing well with a glass rod and decanting repeatedly.

Gravity Filtration. During gravity filtration the filtrate passes through the filter medium under the forces of gravity and capillary attraction between the liquid and the funnel stem. The most common procedure involves the use of filter paper and a conical funnel. The procedure is slow, but it is highly favored for gravimetric analysis over the more rapid vacuum filtration because there is better retention of fine particles of precipitate and less rupturing or tearing of the paper. Fluting the paper permits free circulation of air and is advantageous when filtering hot solutions.

Vacuum filtration. This is a very convenient way to speed up the filtration process, but the filter medium must retain the very fine particles without clogging. Because of the inherent dangers of flask collapse from the reduced pressure, thick-walled filter flasks should be used. Vacuum filtration is advantageous when the precipitate is crystalline. It should not be employed for

gelatinous precipitates, as clogging will occur. When performing vacuum filtration with filter paper, the folded paper is inserted into a small porous metal liner in the apex of the funnel.

Solutions of very volatile liquids and hot solutions are not filtered conveniently with suction. The suction may cause excessive evaporation of the solvent, which cools the solution enough to cause precipitation of the solute.

Buchner funnels are often used for vacuum filtration. They have a flat, perforated bottom. A filter-paper circle of a diameter sufficient to cover the perforations is placed on the flat bottom, moistened, and tightly sealed against the bottom by applying a slight vacuum. In use, the precipitate is allowed to settle and the liquid phase is first decanted by pouring it down a stirring rod aimed at the center of the filter paper, applying only a light vacuum until sufficient solid has built up over the paper to protect it from breaking. The vacuum is then increased, and the remainder of the precipitate is added. The precipitate is washed by adding small amounts of wash liquid over the surface of the precipitate, allowing the liquid to be drawn through the solid slowly with the vacuum. Precipitates cannot be ignited and weighed in Buchner funnels. Precipitates can be air-dried by allowing them to stand in the funnel and drawing a current of air from the room through the precipitate with the source of vacuum **(Patnaik, 2004).**

1.5.3 SOLVENT EXTRACTION

Solutes have different solubilities in different solvents, and the process of selectively removing a solute from a mixture with a solvent is called *extraction.* The solute to be extracted may be in a solid or in a liquid medium, and the solvent used for the extraction process may be water, a water-miscible solvent, or a water-immiscible solvent. The selection of the solvent to be used depends upon the solute and upon the requirements of the experimental procedure. An ideal

extraction method should be rapid, simple, and inexpensive to perform; should yield quantitative recovery of target analytes without loss or degradation; and should yield a sample that is immediately ready for analysis without additional concentration or class fractionation steps.

Extraction procedures based on the distribution of solutes among immiscible solvents are carried out for two purposes. *Exhaustive extraction* involves the quantitative removal of one solute; *selective extraction* involves the separation of two solutes **(Patnaik, 2004).**

In this process the necessity of assessing the chemical and physical nature of the components of a particular reaction mixture with regard to their solubilities in solvents, and to their acidic, basic or neutral characteristics is very crucial.

The solvent selected will depend upon the solubility of the substance to be extracted in that solvent and upon the ease with which the solvent can be separated from the solute. If prior information is not available, solvent selection should be based on some small-scale trials. In the isolation of organic compounds from aqueous solutions, use is frequently made of the fact that the solubility of many organic substances in water is considerably decreased by the presence of dissolved inorganic salts (sodium chloride, calcium chloride, ammonium sulphate, etc.). This is the so-called *salting-out effect.* A further advantage is that the solubility of partially miscible organic solvents, such as ether, is considerably less in the salt solution, thus reducing the loss of solvent in extractions.

The process of extraction is concerned with the *distribution law* or *partition law* which states that if to a system of two liquid layers, made up of two immiscible or slightly miscible components, is added a quantity of a third substance soluble in both layers, then the substance distributes itself between the two layers so that the ratio of the concentration in one solvent to the concentration in the second solvent remains constant at constant temperature. It is assumed that the molecular

state of the substance is the same in both solvents. If CA and CB are concentrations in the layers A and B, then, at constant temperature:

CA/CB = constant = *K*

The constant *K* is termed the *distribution* or *partition coefficient.* As a very rough approximation the distribution coefficient may be assumed equal to the ratio of the solubilities in the two solvents.

Organic compounds are usually relatively more soluble in organic solvents than in water, hence they may be extracted from aqueous solutions. If electrolytes, e. g. sodium chloride, are added to the aqueous solution, the solubility of the organic substance is lowered, i.e. it will be *salted out*: this will assist the extraction of the organic compound **(Furniss *et al.*, 1989).**

1.5.4 RECRYSTALLIZATION TECHNIQUES

Organic compounds whether isolated from plants or synthetic reactions are seldom pure. Such impure crystalline solids are purified by crystallization from a solvent or mixture of solvents.

The purification of solids by crystallization is based upon differences in their solubility in a given solvent or mixture of solvents. Recrystallization in its simplest form consists of the following process:

- dissolving the impure substance in a minimum amount of some suitable solvent at or near the boiling point;
- filtering the hot solution from particles of insoluble material and dust;
- allowing the hot solution to cool thus causing the dissolved substance to crystallize out; and

- separating the crystals from the supernatant solution (or mother-liquor).

After washing and drying, the solid formed is tested for purity (usually by a melting point determination, spectroscopic methods, or by thin-layer chromatography). Recrystallised may be repeated with fresh solvent if the crystals are found to be impure. The process is repeated until the pure compound is obtained (i.e. the melting point is unchanged). However, confirmation by the other methods specified above is desirable. In recrystallization the general assumption is that the impurities are usually less than 5 per cent of the whole solid.

An ideal solvent for recrystallization should have the following characteristics;

- ✓ A high solvent power for the substance to be purified at elevated temperature and a comparatively low solvent power at the laboratory temperature below.
- ✓ It should dissolve the impurities readily or to a very small extent.
- ✓ It should yield well-formed crystals of the purified compound.
- ✓ It must be capable of easy removal from the crystals of the purified compound i.e. possess a relatively low boiling point.

There is also the assumption that the solvent does not react chemically with the substance to be purified. Factors such as ease of manipulation, toxicity, flammability and cost would determine which solvent combination is to be selected if two or more solvents appear to be equally suitable for recrystallisation **(Furniss *et al.*, 1989).**

Some of the common solvents used for recrystallisation, in increasing order of polarity, are hexane, benzene, chloroform, ethylacetate, acetone, ethanol, methanol, acetic acid and water.

The choice of solvent cannot be made on the basis of theoretical considerations alone, but must be experimentally determined, if no information is already available.

If the substance is found to be too soluble in another solvent to allow for satisfactory recrystallisation, mixed solvents may be used. The two solvents must however be completely miscible. The compound is first dissolve in the solvent in which it is very soluble and the solvent in which it is sparingly solube is added cautiously till slight turbidity appears. It is then heated until it is clear and the mixture is allowed to cool to room temperature.

Alcohol and water, acetic acid and water, benzene and petroleum ether, alcohol and benzene and methanol and ether are some of the solvent pairs that can be used **(Olaniyi and Ogungbamila, 1991).**

The use of ether as a solvent for recrystallisation should be avoided wherever possible, partly owing to its great flammability and partly owing to its tendency to creep up walls of the containing vessel, thus depositing solid matter by complete evaporation instead of preferential crystallisation. Carbon disulphide should also be avoided as much as possible because it has dangerously low flash point and forms very explosive mixtures with air.

When choosing a solvent for recrystallisation, the general rules below may be used though they might not completely hold as polarity of the solvents decrease:

1. A substance is likely to be most soluble in a solvent to which it is most closely related in chemical and physical characteristics.

2. In ascending a homologous series, the solubilities of the members tend to become more and more like that of the hydrocarbon from which they may be regarded as being derived.

3. A polar substance is more soluble in polar solvents and less soluble in nonpolar solvents **(Furniss *et al.*, 1989).**

Generation of crystals from a supersaturated solution may sometimes be very difficult, this may be overcome by any of the methods below.

- By scratching the inside of the vessel with a glass rod. The effect is attributed to breaking off of small particles of glass which may act as crystal nuclei, or to the roughening of the surface, which facilitates more rapid orientation of the crystals on the surface.
- By inoculating (seeding) the solution with some of the solid material or with isomorphous crystals, crystallization frequently commences and continues until equilibrium is reached.
- By cooling the solution in a freezing mixture (ice and salt, ice and calcium chloride, or solid carbon dioxide and acetone). By adding a few lumps of solid carbon dioxide; this produces a number of cold spots here and there, and assists the formation of crystals.
- If all the above methods fail, the solution should be left in an ice chest (or a refrigerator) for a prolonged period.
- Occasionally, conversion into a simple crystalline derivative is applicable; subsequent regeneration of the original compound will usually yield a pure, crystalline solid.

Decolorisation by Animal Charcoal (activated or decolorising charcoal) : It sometimes happens (particularly with aromatic and heterocyclic compounds) that a crude product may contain a coloured impurity, which on recrystallisation dissolves in the boiling solvent, but is then partly occluded by crystals as they form and grow in the cooling solution. Sometimes a very tenacious

occlusion may thus occur, and repeated and very wasteful recrystallisation may be necessary to eliminate the impurity. Moreover, the amount of the impurity present may be so small that the melting-point and analytical values of the compound are not sensibly affected, yet the appearance of the sample is ruined. Such impurities can usually be readily removed by boiling the substance in solution with a small quantity of finely powdered animal charcoal for a short time, and then filtering the solution while hot. The animal charcoal adsorbs the coloured impurity, and the filtrate is usually almost free from extraneous colour and deposits therefore pure crystals.

This decolorisation by animal charcoal occurs most readily in aqueous solution, but can be performed in almost any organic solvent. Care should be taken not to use an excessive quantity of charcoal, however, as it tends to adsorb some of the solute as well as .the coloured impurity **(Mann and Saunders, 1960).**

1.5.5 CHROMATOGRAPHY

Chromatography is a separation mechanism based on the differential distribution of the components of a mixture between a mobile bulk phase and an essentially thin film stationary phase. The thin film stationary phase may be either liquid or a solid, and the mobile phase a liquid or a gas.

The stationary phase may be either in the form of a packed column (column chromatography) through which a mobile phase is allowed to flow, or in the form of a thin layer adhering to a suitable form of backing material (thin layer chromatography) over which the mobile phase is allowed to ascend by capillary. Various combinations of these phase then give rise to the principal chromatographic techniques in general use **(Furniss *et al.*, 1989).**

There are several different types of chromatography currently in use – i.e. paper chromatography; thin layer chromatography (TLC); gas chromatography (GC); liquid chromatography (LC); high performance liquid chromatography (HPLC); ion exchange chromatography; and gel permeation or gel filtration chromatography.

The techniques used in all the types of chromatography depend on one of the following phenomena: adsorption; partition; ion exchange; or molecular exclusion.

Adsorption chromatography was developed first. It has a solid stationary phase and a liquid or gaseous mobile phase. (Plant pigments were separated at the turn of the 20th century by using a calcium carbonate stationary phase and a liquid hydrocarbon mobile phase. The different solutes travelled different distances through the solid, carried along by the solvent.) Each solute has its own equilibrium between adsorption onto the surface of the solid and solubility in the solvent, the least soluble or best adsorbed ones travel more slowly. The result is a separation into bands containing different solutes. Liquid chromatography using a column containing silica gel or alumina is an example of adsorption chromatography.

The solvent that is put into a column is called the eluent, and the liquid that flows out of the end of the column is called the eluate.

Partition chromatography has the stationary phase as a non-volatile liquid which is held as a thin layer (or film) on the surface of an inert solid. The mixture to be separated is carried by a gas or a liquid as the mobile phase. The solutes distribute themselves between the moving and the stationary phases, with the more soluble component in the mobile phase reaching the end of the chromatography column first.

Paper chromatography is an example of partition chromatography.

Ion exchange chromatography is similar to partition chromatography in that it has a coated solid as the stationary phase. The coating is referred to as a resin, and has ions (either cations or anions, depending on the resin) covalently bonded to it and ions of the opposite charge are electrostatically bound to the surface. When the mobile phase (always a liquid) is eluted through the resin the electrostatically bound ions are released as other ions are bonded preferentially. Domestic water softeners work on this principle.

Molecular exclusion differs from other types of chromatography in that no equilibrium state is established between the solute and the stationary phase. Instead, the mixture passes as a gas or a liquid through a porous gel. The pore size is designed to allow the large solute particles to pass through uninhibited. The small particles, however, permeate the gel and are slowed down so the smaller the particles, the longer it takes for them to get through the column. Thus separation is according to particle size.

THIN LAYER CHROMATOGRAPHY (TLC)

TLC is similar to paper chromatography, but the stationary phase is a thin layer of a solid such as alumina or silica supported on an inert base such as glass, aluminum foil or insoluble plastic. The mixture is 'spotted' at the bottom of the TLC plate and allowed to dry. The plate is placed in a closed vessel containing solvent (the mobile phase) so that the liquid level is below the spot. TLC has advantages over paper chromatography in that its results are more reproducible, and that separations are very efficient because of the much smaller particle size of the stationary phase. The solvent ascends the plate by capillary action, the liquid filling the spaces between the solid particles. This technique is usually done in a closed vessel to ensure that the atmosphere is saturated with solvent vapour and that evaporation from the plate is minimised before the run is

complete. The plate is removed when the solvent front approaches the top of the plate and the position of the solvent front recorded before it is dried (this allows the Rf value to be calculated).

Table 1: Types of chromatographic techniques (Furniss *et al.*, 1989)

Technique	Stationary phase	Mobile phase	Format	Principal sorption mechanism
Paper chromatography (PC)	Paper (cellulose)	Liquid	Planar	Partition (adsorption, ion-exchange, exclusion)
Thin-layer chromatography (TLC)	Silica, cellulose, ion-exchange resin, controlled porosity solid	Liquid	Planar	Adsorption (partition, ion-exchange, exclusion)
Gas chromatography (GC)				
Gas-liquid chromatography (GLC)	Liquid	Gas	Column	Partition
Gas-solid chromatography (GSC)	Solid	Gas	Column	Adsorption
Liquid chromatography (LC)				
High-performance liquid chromatography (HPLC)	Solid or bonded-phase	Liquid	Column	Modified partition (adsorption)
Size-exclusion chromatography (SEC)	Controlled porosity solid	Liquid	Column	Exclusion
Ion-exchange chromatography (IEC) Ion chromatography (IC)	Ion-exchange resin or bonded-phase	Liquid	Column	Ion-exchange
Chiral chromatography (CC)	Solid chiral selector	Liquid	Column	Selective adsorption

As each solute distributes itself (equilibrates) between the stationary and the mobile phase, the distance a solute moves is always the same fraction of the distance moved by the solvent. This fraction is variously called the retardation factor or the retention ratio, and is given the symbol **R** or $\mathbf{R_f}$.

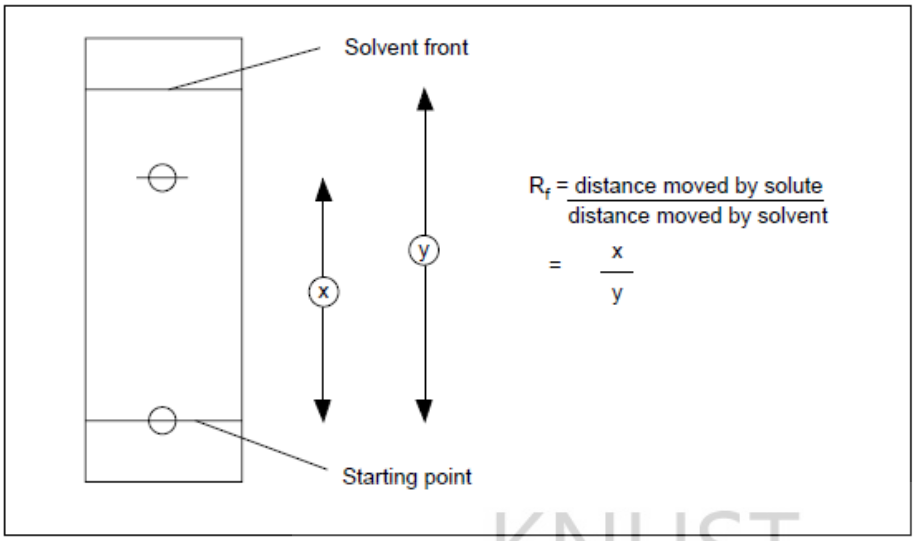


Figure 3: A representative chromatogram of TLC and determination of Retention factor, R_f (BLOCH, 2006)

So as long as the correct solvent and type of chromatography paper are used, a component can be identified from its retention ratio.

TLC has applications in industry in determining the progress of a reaction by studying the components present; and in separating reaction intermediates. In the latter case a line of the reaction mixture is 'painted' across the TLC plate instead of a single spot, and the line of product after separation is cut out of the plate and dissolved in an appropriate solvent.

Many spots are not visible without the plates being 'developed'. This usually involves spraying with a solution that is reversibly adsorbed or reacts in some way with the solutes. Two examples of developing solutions are iodine in petroleum ether (useful for identifying aromatic compounds, especially those with electron donating groups – eg $C_6H_5NH_2$) and ninhydrin (useful for identifying amino acids). Iodine vapour is also used to develop plates in some cases. Alternatively, specially prepared plates can be used that fluoresce in ultraviolet light. The plates are used in the normal manner, but once dried they are placed under an ultraviolet lamp. Solute spots mask fluorescence on the surface of the plate – i.e. a dark spot is observed. Some

compounds have their own fluorescence which can be used for identification, or retardation factors can be used to identify known solutes.

Table 2: Some examples of TLC developing reagents (Furniss *et al.*, 1989)

Method of detection	Color of solute spots	Application
General reagents		
Phosphomolybdic acid + heat	Dark blue	Many organics
Conc. sulphuric acid + heat	Charred brown-black	All organics
Iodine vapor	Brown	Many organics
Selective reagents		
Ninhydrin	Pink to purple	Amino acids and amines
2,4-Dinitrophenylhydrazone	Orange/red	Carbonyl compounds
Bromocresol green/ blue	Yellow	Organic acids
2,7-Fluorescein	Yellow-green	Most organics
Vanillin/ sulphuric acid	Blue, green, pink	Alcohols, ketones
Rhodamine-B	Red fluorescence	Lipids
Anisaldehyde/antimony trichloride	Various	Steroids
Diphenylamine/zinc	Various	Pesticides

High performance liquid chromatography (HPLC)

HPLC within the past decade has become perhaps the primary analytical tool for compounds which are non-volatile or thermally unstable so that they are amenable to gas- liquid chromatography (GLC) analysis. These compounds may include natural and synthetic products. In principle however HPLC arose from the conventional liquid chromatography with the use of prepacked columns with modified surfaces of the conventional silica or alumina as packing materials making them more efficient for adsorption, normal and reverse phase partition, ion exchange, gel permeation, and more recently affinity chromatography **(Furniss *et al.*, 1989).**

The essential components for HPLC instrumentation include the following:

1. A pump or pumps to force the mobile phase through the system. Suitable pressure gauges and flow meters are placed in the system.

2. Sampling valves and loops to inject the sample into the mobile phase just at the head of the separation column.
3. A separation column in which the sample components are separated into individual peaks before elution.
4. A detector and readout device to detect the presence of solutes in the mobile phase and record the resulting chromatogram. To collect, store, and analyze the chromatographic data, computers, integrators, and other data-processing equipment are being used more frequently in conjunction with the strip chart recorder **(Patnaik, 2004).**

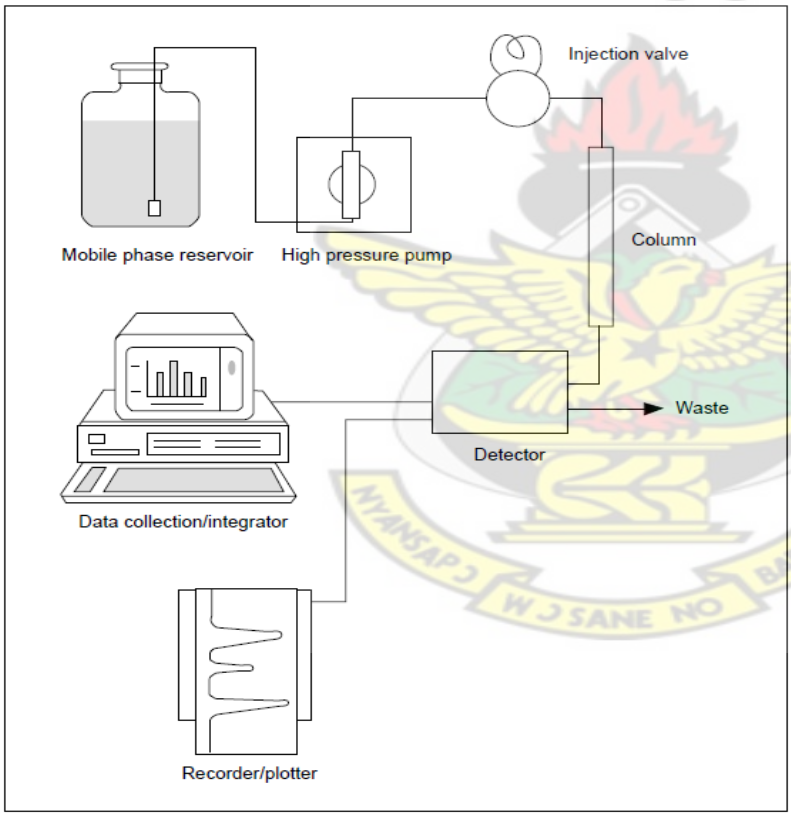
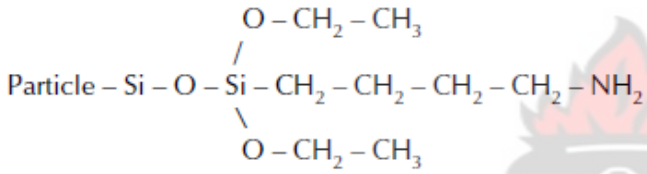


Figure 4: Representative diagram of a complete chromatograph of HPLC (Patnaik, 2004)

The efficiency of a separation increases if the particles in the stationary phase are made smaller. This is because the solute can equilibrate more rapidly between the two phases. However, if the

particles are made smaller, capillary action increases and it becomes more difficult to drain the column under gravity. Consequently, a high pressure has to be applied to the solvent to force it through the column.

The stationary phase normally consists of uniform porous silica particles of diameter 10^{-6} m, the surface pores having a diameter of 10^{-8}–10^{-9}m. (This gives the solid a very high surface area.) The particles can be bonded with a non-volatile liquid that allows interactions of solutes with different polarities. These liquids are held on the silica particles by covalent bonds – eg the surface of one polar resin has the structure below.



Interaction is then possible between the lone pair of electrons on the nitrogen atom and the solute molecule. The stationary phase particles are packed into the HPLC column and are held in place by glass fibres coated with inert alkyl silane molecules. The separation in HPLC is normally so efficient that a long column is not necessary. (If the column was too long the pressure needed would be excessive.) Columns are typically 10–30 cm long, with an internal diameter of 4 mm.

Reproducibility is essential, and this is only possible if a constant flow rate is maintained. This means that the pump used must be capable of generating a uniform pressure; twin cylinder reciprocating pumps are typical. This type of pump has two chambers with pistons 180^{o} out of phase, and can generate pressures up to 10 MPa/100 atmospheres. The high pressures involved mean that the instrumentation has to be very strong, and the 'plumbing' is usually constructed from stainless steel. The pump and the piping must be inert to the solvent and solutes being passed through them.

The flow rates of HPLC columns are slow – often in the range 0.5–5 mil/min. The volumes of the columns are very small, and this means that the injection of the sample must be very precise and it must be quick without disturbing the solvent flow. Sample volumes are small, about 5–20 mm^3 is usually sufficient.

Table 3: Stationary Phases for HPLC (Patnaik, 2004)

Stationary phase	Sorption mechanism	Characteristics
Unmodified silica, SiO_2	Adsorption, normal-phase	Polar, retention times variable due to adsorbed water
Bonded phases		
Octadecyl silica, -$C_{18}H_{37}$ (ODS or C18) Octyl silica, -C_8H_{17} Propyl silica, -C_3H_7	Modified partition, reversed-phase	Nonpolar, but unreacted silanol groups cause polar solutes, especially bases, to tail, pH range limited to 2.5–7.5
		All separate a very wide range of solutes
Aminopropyl, -$C_3H_6NH_2$	Modified partition, normal or reversed phase	Polar, separates carbohydrates pH range limited to 2.5–7.5
Sulphonic acid, -$(CH_2)_nSO_3H$	Cation-exchange	Slow mass transfer broadens peaks, limited sample capacity, pH range limited to 2.5–7.5 for silica-based materials
Quaternary amine, -$(CH_2)_nNR_3OH$	Anion-exchange	
Controlled-porosity silicas (some with –$Si(CH_3)_3$ groups)	Size exclusion	Compatible with both organic and aqueous solvents, pH range limited to 2.5–10
α-, β-, γ-cyclodextrin silicas	Chiral selectivity based on adsorptive interactions	Expensive, limited life, resolution sensitive to mobile phase composition
Polymer phases		
Cross-linked styrene/divinyl benzene co-polymers, unmodified or with ion-exchange groups	Modified partition, exclusion or ion-exchange	Nonpolar if unmodified, stable over pH range 1–13

In HPLC, both isocratic and gradient elution can be achieved during the separation. The amounts passing through the column are usually too small to extract from the solvent before identification, so the solutes in solution are analysed as they leave the column. Most compounds separated by HPLC absorb ultraviolet light. The eluate is passed along a small cell so that ultraviolet radiation can be passed through the liquid. The absorption thus read can be used to

both identify and quantify the amount of separated compound in the mixture. The absorption is integrated and recorded as a chromatogram as simplified below

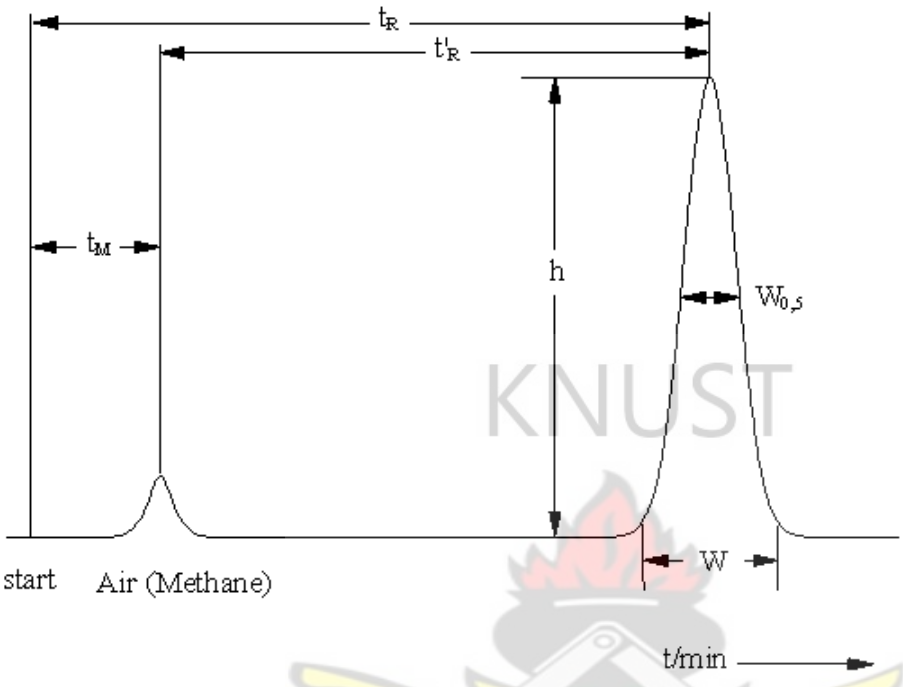


Figure 5: Scheme of a chromatogram

Where $\mathbf{t_R}$ is total retention time of the compound (in the whole chromatographic system), $\mathbf{t'_R}$ is adjusted retention time of the compound (retention time in the stationary phase), $\mathbf{t_M}$ is "Dead time" (retention time in the mobile phase), $\mathbf{W_{0,5}}$ is peak width at half height and **h** is the height of a signal.

In chromatography, a "theoretical plate" is a part of the column, where a thermodynamic equilibrium of the analytes in the stationary and the mobile phase appears. In the "ideal" chromatography, this is a reversible process. But in reality ("not-ideal" chromatography), you have to take the effects of diffusion into consideration, which causes at least a peak form like a *Gauss' error* curve.

The peak sharpness is given by the number (**n)** of these theoretical plates in the column. The lower the Height Equivalent of a Theoretical Plate **H (HETP),** the sharper the peaks:

$$HETP = \frac{L}{n}$$

$$n = 5.54 \times \left(\frac{t_R}{W_{0.5}} \right)^2$$

Where *L* is the length of the column used.

QUANTIFICATION

The basic theory for quantification involves the measurement of peak height or peak area. To determine the concentration of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration. Three different calibration methods, each with its own benefits and limitations, can be utilised in quantitative analysis, external standard, internal standard and the standard addition method.

The external standard method is the simplest of the three methods. The accuracy of this method is dependent on the reproducibility of the injection of the sample volume. To perform this method, a standard solution of known concentration of the compound of interest is prepared. A fixed amount, which should be similar in concentration to the unknown, is injected. Peak height or area is plotted versus the concentration for each compound. The plot should be linear and go through the origin. The concentration of the unknown is then determined according to equation below.

$$\text{Conc.}_{\text{unknown}} = \left(\frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}}\right) \times \text{Conc.}_{\text{known}}$$

The calibrator concentrations should cover the range of the likely concentration in the unknown sample. Only concentrations read within the highest and lowest calibration levels are acceptable. Concentrations read from an extrapolated regression line may not be accurate. This applies to all of the quantification methods

HYPHENATED HPLC METHODS

Interfacing of HPLC with other instruments, for the purpose of identifying the structure of an unknown compound in a mixture has become a major development over the years. These methods are called Hyphenated HPLC methods. Therefore methods like HPLC coupled with mass spectrophotometer (HPLC-MS), HPLC- IR, HPLC- NMR, HPLC-MS-MS, etc are common. These techniques usually involve chromatographic separation followed by peak identification with a traditional detector such as UV, combined with further identification of the compound with the MS, IR or NMR spectrometer. These hyphenated methods can also be used for quantification of the amount of solute separated.

Whichever method is used it is vital that the volume of liquid used is very small, otherwise the sharpness of the separation peaks will disappear and the resolution of the final chromatogram will be lost. Once the retention time of a solute has been established for a column using a set of operating conditions, that solute can be identified in a mixture from its retention time (assuming that another component with the same retention time is not also present). **(Faust, 1997).**

1.5.6 METHOD VALIDATION PARAMETERS

In the method validation process there are several parameters that are considered. The parameters outlined in the WHO Technical Report Series are explained below

Accuracy is the degree of agreement of test results with the true value, or the closeness of the results obtained by the procedure to the true value. It is normally established on samples of the material to be examined that have been prepared to quantitative accuracy. Accuracy should be established across the specified range of the analytical procedure.

It is acceptable to use a "spiked" placebo where a known quantity or concentration of a reference material is used.

Precision is the degree of agreement among individual results. The complete procedure should be applied repeatedly to separate, identical samples drawn from the same homogeneous batch of material. It should be measured by the scatter of individual results from the mean (good grouping) and expressed as the relative standard deviation (RSD)

Robustness (or ruggedness) is the ability of the procedure to provide analytical results of acceptable accuracy and precision under a variety of conditions. The results from separate samples are influenced by changes in the operational or environmental conditions. Robustness should be considered during the development phase, and should show the reliability of an analysis when deliberate variations are made in method parameters.

Factors that can have an effect on robustness when performing chromatographic analysis include:

— stability of test and standard samples and solutions;

— reagents (e.g. different suppliers);

— different columns (e.g. different lots and/or suppliers);

— extraction time;

— variations of pH of a mobile phase;

— variations in mobile phase composition;

— temperature; and

— flow rate

Linearity indicates the ability to produce results that are directly proportional to the concentration of the analyte in samples. A series of samples should be prepared in which the analyte concentrations span the claimed range of the procedure. If there is a linear relationship, test results should be evaluated by appropriate statistical methods. A minimum of five concentrations should be used

Range is an expression of the lowest and highest levels of analyte that have been demonstrated to be determinable for the product. The specified range is normally derived from linearity studies.

Specificity (selectivity) is the ability to measure unequivocally the desired analyte in the presence of components such as excipients and impurities that may also be expected to be present. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and assay

Detection limit (limit of detection, LOD) is the smallest quantity of an analyte that can be detected, and not necessarily determined, in a quantitative fashion.

Quantitation limit (limit of quantitation, LOQ) is the lowest concentration of an analyte in a sample that may be determined with acceptable accuracy and precision.

It is to be noted that for both LOD and LOQ, approaches for determination may include instrumental or non-instrumental procedures and could include those based on:

— visual evaluation;

— signal to noise ratio;

— standard deviation of the response and the slope;

— standard deviation of the blank; and

— calibration curve.

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. System suitability test parameters that need to be established for a particular procedure depend on the type of procedure being evaluated, for instance, a resolution test for an HPLC procedure **(WHO, 2006).**

1.5.7 SPECTROPHOTOMETRY

Spectroscopy is the study of the interaction of electromagnetic radiation with matter. Electromagnetic radiation can be described as a wave traveling at the speed of light. This radiation makes up the electromagnetic spectrum.

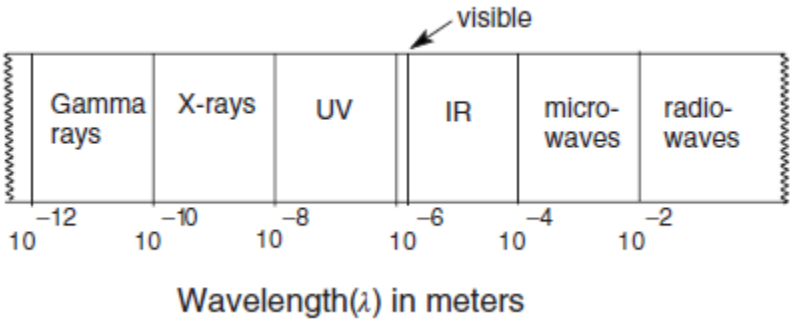


Figure 6: Electromagnetic spectrum (BLOCH, 2006)

Energy can be transmitted by electromagnetic waves. They are characterized by their frequency *V,* the number of waves passing a fixed point per second, and their wavelength λ, which is the distance between the peaks of any two consecutive waves. As the frequency increases, the wavelength decreases and, conversely, as the frequency decreases, the wavelength increases.

In any electromagnetic wave, wavelength and frequency are related to the energy of a photon, *E*, by Planck's constant *h* (6.62 *10^{-34}Js) and *c* (2.998 *$10^{10}$$cms^{-1}$), the velocity of radiant energy in a vacuum by

$$E = h\nu = \frac{hc}{\lambda}$$

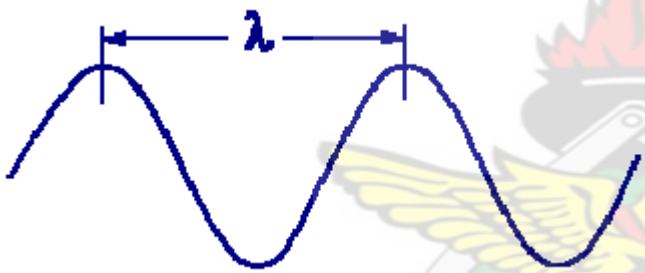


Figure 7: The wavelength λ of an electromagnetic radiation (Owen, 2000)

ULTRAVIOLET - VISIBLE SPECTROSCOPY

When radiation interacts with matter, a number of processes can occur, including reflection, scattering, absorbance, fluorescence/phosphorescence (absorption and reemission), and photochemical reaction (absorbance and bond breaking). In general, when measuring UV-visible spectra, we want only absorbance to occur.

Because light is a form of energy, absorption of light by matter causes the energy content of the molecules (or atoms) to increase. The total potential energy of a molecule generally is represented as the sum of its electronic, vibrational, and rotational energies:

$E_{total} = E_{electronic} + E_{vibrational} + E_{rotational}$

The amount of energy a molecule possesses in each form is not a continuum but a series of discrete levels or states. The differences in energy among the different states are in the order:

$E_{electronic} > E_{vibrational} > E_{rotational}$

In some molecules and atoms, photons of UV and visible light have enough energy to cause transitions between the different electronic energy levels. The wavelength of light absorbed is that having the energy required to move an electron from a lower energy level to a higher energy level.

A molecule or ion will exhibit absorption in the visible or ultraviolet region when radiation causes an electronic transition within its structure. Thus, the absorption of light by a sample in the ultraviolet or visible region is accompanied by a change in the electronic state of the molecules in the sample.

The energy supplied by the light will promote electrons from their ground state orbitals to higher energy, excited state orbitals or antibonding orbitals.

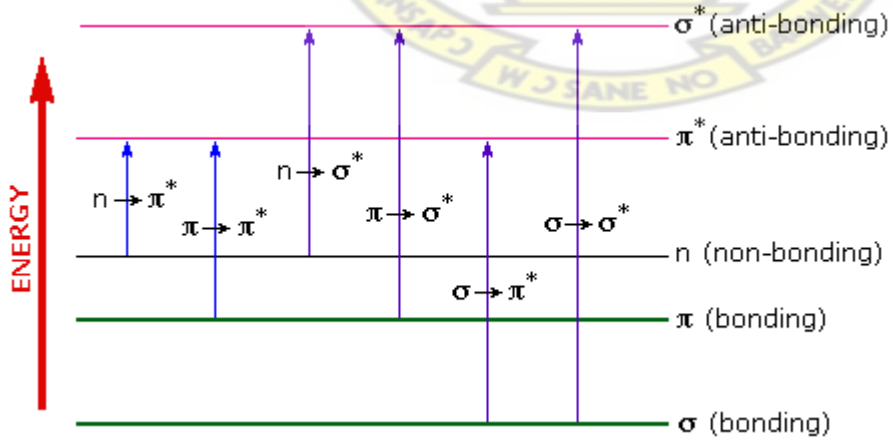


Figure 8: Energy and molecular transitions **(Owen, 2000)**

After the absorption of UV- Vis light the following electronic transitions can occur : σ to σ*,n to σ*,n to π* and π to π*. Both σ to σ* and n to σ* transitions require a great deal of energy and therefore occur in the far ultraviolet region or weakly in the region 180-240nm. Consequently, saturated groups do not exhibit strong absorption in the ordinary ultraviolet region. Transitions of the n to π* and π to π* type occur in molecules with unsaturated centers; they require less energy and occur at longer wavelengths than transitions to σ* antibonding orbitals. Transitions to π* antibonding orbitals which occur in the ultraviolet region for a particular molecule may well take place in the visible region if the molecular structure is modified. Many inorganic compounds in solution also show absorption in the visible region. π to π * transitions, when occurring in isolated groups in a molecule, give rise to absorptions of fairly low intensity. However, conjugation of unsaturated groups in a molecule produces a remarkable effect upon the absorption spectrum. The wavelength of maximum absorption moves to a longer wavelength and the absorption intensity may often increase.

Chromophores and auxochromes

A chromophore (literally color-bearing) group is a functional group, not conjugated with another group, which exhibits a characteristic absorption spectrum in the ultraviolet or visible region.

If any of the simple chromophores is conjugated with another (of the same type or different type) a multiple chromophore is formed having a new absorption band which is more intense and at a longer wavelength that the strong bands of the simple chromophores.

This displacement of an absorption maximum towards a longer wavelength (i.e. from blue to red) is termed a bathochromic shift. The displacement of an absorption maximum from the red to blue ultraviolet is termed a hypsochromic shift.

The color of a molecule may be intensified by groups called auxochromes which generally do not absorb significantly in the 200 - 800nm region, but will affect the spectrum of the chromophore to which it is attached. The most important auxochromic groups are OH, NH_2, CH_3 and NO_2 and their properties are acidic (phenolic) or basic. The actual effect of an auxochrome on a chromophore depends on the polarity of the auxochrome **(Owen, 2000).**

The Spectrometer

The UV-Vis spectrometer essentially consists of the following parts

1. Sources (UV and visible)
2. Wavelength selector (monochromator)
3. Sample cell (some additional reference cells)
4. Detector
5. Signal processor and chart recorder

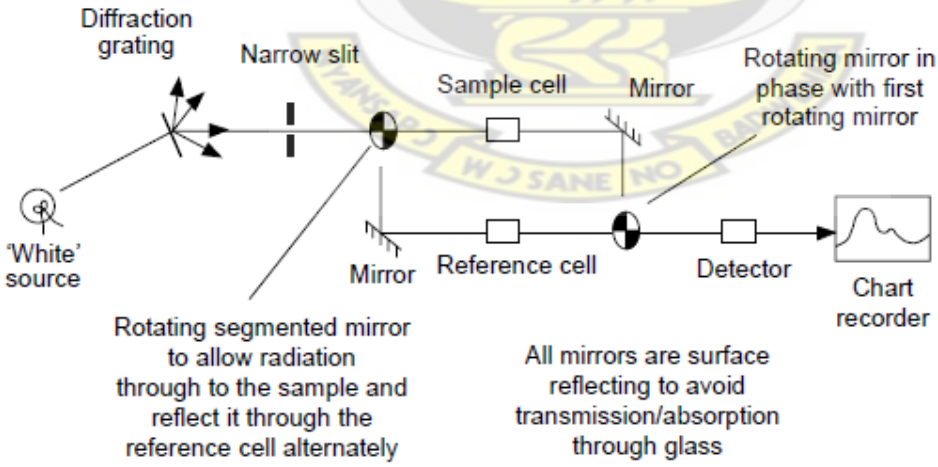


Figure 9: Diagram showing how the ultraviolet/visible spectrometer works (Faust, 1997)

Quantification

UV-Vis Spectrophotometry is used for both qualitative and quantitative investigations of samples. The wavelength at the maximum of the absorption band give information about the structure of the molecule or ion and the extent of the absorption is proportional with the amount of the species absorbing the light.

Quantitative measurements are based on Beer's Law (also known as "Lambert- Beer Law" or even "Bouguer-Lambert-Beer Law") which is described as follows:

$A = ec\,l$

where A = absorbance [no units, because it is calculated as $A = \log_{10}(I_0/I)$, where I_0 is the incident light's intensity and I is the light intensity after it passes through the sample];

e = molar absorbance or absorption coefficient [in $dm^3\ mol^{-1}\ cm^{-1}$units]; It is specific absorbance if concentration is in $\%^{w}/_{w}$

c = concentration (molarity) of the compound in the solution [in $moldm^{-3}$ units];

l = path length of light in the sample [in cm units]. **(Owen, 2000).**

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance (NMR) spectroscopy is a powerful method for elucidating the structure of a molecule. It gives information about the number, type, and connectivity of hydrogen and carbon atoms. The technique involves the interaction (*resonance*) of energy from an external energy source with the *magnetic* properties of a *nucleus*. This does not involve a nuclear reaction or a chemical reaction. The material being tested can be recovered unchanged.

The nuclei of certain isotopes have an intrinsic spinning motion around their axes that generates a magnetic moment along the axis of spin. The simultaneous application of a strong external

magnetic field H_0 and the energy from a second and weaker radio-frequency source (H_1) (applied perpendicular to H_0) to the nuclei results in the rotation of the macroscopic nuclear magnetization away from its equilibrium position parallel to the applied magnetic field. Absorption occurs when these nuclei undergo transition from one alignment in the applied field to an opposite one. The energy needed to excite these transitions can be measured.

The resonance frequency, *n*, that causes the transitions between energy levels is given by

$$\Delta E = h\nu = \frac{\mu H_0}{I}$$

where μ = magnetic moment of the nucleus, I = spin quantum number, h / π and h = Planck's constant.

Nuclei with I = 1/2 give the best resolved spectra because their electric quadrupole moment is zero. These nuclei include ^{1}H, ^{13}C, ^{19}F, ^{29}Si, and ^{31}P.

Relaxation Processes

Energy absorbed and stored in the higher-energy state must be dissipated and the nuclei returned to the lower-energy state. Otherwise, the radio frequency (rf) system equalizes the populations of the energy states, the spin system becomes saturated, and the absorption signal disappears. The *spin–lattice relaxation* involves interaction of the spin with the fluctuating magnetic fields produced by the random motions of neighboring nuclei (called the *lattice*). Nuclei lose their excess energy as thermal energy to the lattice. The relaxation process is first order and decreases exponentially with time. **(Patnaik, 2004).**

Instrumentation

NMR instrumentation involves these basic units:

1. A magnet to separate the nuclear spin energy states.

2. (a) One rf channel for field or frequency stabilization, which produces stability for long-term operation, (b) one radio frequency, rf, channel to furnish irradiating energy to the sample, and (c) a third channel that may be added for decoupling nuclei.

3. A sample probe that houses the sample and also coils for coupling the sample with the rf transmitter and the phase-sensitive detector. It is inserted between the pole faces of the magnet.

4. A detector to collect and process the NMR signals.

5. A sweep generator for sweeping the rf field through the resonance frequencies of the sample. Alternatively, the magnetic field may be swept and the rf field held constant.

6. A recorder to display the spectrum **(Patnaik, 2004).**

Sample Preparation

The sample to be tested is put in a thin glass tube, about the size of a pencil, and placed inside the coils (that supply radiowaves) in the core of the magnet. Only a few milligrams of sample are required. About 0.5 mL of solvent is used. The solvent used does not contain atoms (nuclei) that absorb radiowave energy in the range that protons absorb radiation. Solvents used include carbon tetrachloride (CCl_4), deuterated chloroform ($CDCl_3$), and deuterated water (D_2O). Deuterium (D or ^2H), an isotope of hydrogen, does not absorb radiation in the same range as does "normal" ^1H. The same solvents are used to dissolve samples when the ^{13}C spectrum is desired. **(Bloch, 2006).**

^{1}H NMR Spectroscopy

^{1}H NMR spectroscopy is referred to as proton NMR spectroscopy. A proton, by definition, is a hydrogen atom without an electron, H^+. Proton NMR is a study of hydrogen atoms covalently bonded to another atom, usually carbon. The terms proton and hydrogen atom are interchangeably in NMR discussions.

Protons in *different* chemical environments are called *nonequivalent protons* and they absorb radiation at *slightly different frequencies* than those in an identical chemical environment called *equivalent protons.* The frequencies measured in megahertz, MHz, values are expressed relative to some standard mostly tetramethylsilane $(CH_3)_4Si$ (TMS). All the protons in TMS are equivalent protons. Chemically equivalent protons absorb radiation at the same frequency. The difference in absorption frequencies from this standard is in hertz (not megahertz) values.

Chemical Shifts

The exact position of each resonance frequency of a nucleus is referred to as its **chemical shift**, which is characteristic of the chemical nature of the particular nucleus.

Chemical shift is conventionally measured relative to the frequency of a reference compound, using a dimensionless parameter, δ, defined as the ratio of their chemical shift difference divided by the operating frequency of the spectrometer and multiplied by 10^6 to give more convenient numerical values.

$$\delta = \frac{(\nu - \nu_{ref})}{\nu_{spectrometer}} \cdot 10^6 \text{ ppm}$$

where υ_{ref} and $\upsilon_{spectrometer}$ are the resonance and operating frequencies of the reference compound and the spectrometer respectively.

Chemical shifts are expressed in parts per million (ppm). The chemical shift value in ppm (or δ) units is independent of the magnetic field strength of the instrument used. TMS is arbitrarily given a value of zero (0) on the ppm (or δ) scale and other absorptions are relative to TMS. Most protons absorb at values to the left (0 to 10) of TMS on this scale. The vertical lines represent proton absorptions. The difference between the absorption of a proton and TMS is called a *chemical shift*. If a chemical shift occurs at 5 ppm, it is said to be *downfield* relative to TMS and

TMS absorbs *upfield* relative to the absorption at 5 ppm. The terms upfield and downfield are commonly used in describing a spectrum.

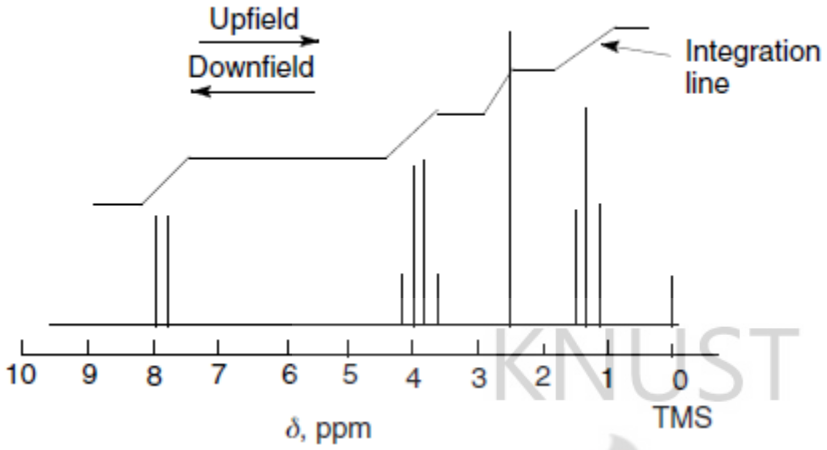


Figure 10: ^{1}H NMR spectrum (Bloch, 2006)

A decrease in electron density around an atom is called deshielding. Deshielding causes a downfield chemical shift. A downfield chemical shift means that absorption is shifted further to the left from TMS. Increasing electron density around a proton is called shielding and causes an upfield chemical shift **(Bloch, 2006).**

Spin- Spin Coupling and Multiplicity

The number of lines in multiplets from protons is n +1 lines, where n is the number of nuclei producing the splitting. Thus, one neighboring proton splits the observed resonance of a proton on an adjacent group into a doublet, the intensities of which are in the ratio 1:1, two produce a triplet (1:2:1), three a quartet (1:3:3:1), four a quintet (1:4:6:4:1), and so on. The peaks within a multiplet are proportional to the coefficients of the binomial expansion. The relative intensity of each of the multiplets, integrated over the whole multiplet, is proportional to the number of nuclei in the group—useful for quantitative work and for structural assignments. **(Patnaik, 2004).**

^{13}C NMR Spectroscopy

In a general way, the chemical shifts of carbon-13 atoms follow the same shielding and deshielding characteristics that are found in proton NMR spectra; the major exception is that the ring current effects observed for aromatic protons are not observed for aromatic carbons. The hybridization of a carbon determines to a great extent the range within which its ^{13}C NMR signal is found. The ^{13}C resonances of sp^3 carbon atoms absorb at highest field (20 to 100 ppm), followed by *sp* carbon atoms (70 to 130 ppm), and while sp^2 hybridized centers are shifted farthest (120 to 240 ppm) in organic compound.

Decoupling

A complex splitting pattern can be simplified by spin-spin decoupling. A technique called *broadband decoupling* eliminates all hydrogen-carbon spin-spin coupling interactions. When a spectrum is decoupled, the multiplet is collapsed into a single peak, enhancing the signal-to-noise ratio. It is more difficult to integrate the areas of ^{13}C peaks but newer NMR spectrometers use techniques that give the relative number of carbon atoms in each peak as is done in proton NMR.

Distortionless Enhancement by Polarization Transfer (DEPT)

This is another technique for analyzing ^{13}C spectra. In this process, the spectrometer varies the signal sent to the sample. Carbon atoms that are not bonded to any hydrogen atoms (quarternary carbon atoms and carbonyl, C=O, carbon atoms) do not appear in DEPT spectra. A comparison of these spectra to a completely decoupled spectrum run at standard conditions enables easy identification of primary, secondary, tertiary, and quaternary carbon atoms.

MASS SPECTROMETRY

Mass spectrometry (MS) is a method for determining the molecular weight of a compound. It provides the most structural information about even the least amount of sample. It provides qualitative and quantitative information about the atomic and molecular composition of inorganic and organic materials and their chemical structures. It is also used with other characterization techniques to identify the total structure of a compound. Despite it's numerous advantages, it cannot as an analytical tool be used to differentiate between optical and geometric isomers and *o*-, *m*- and *p*- substituent positions on an aromatic ring. Again, it is limited to identifying hydrocarbons that produce similar fragmented ions in terms of scope.

As an analytical tool, it's principle of operation is based on formation of gaseous ions from a molecule or atoms present in a sample and separating them in space or time and the separated ions are detected according to their **mass-to-charge ratio**, *m/z*. The quantities of ions of each mass detected constitute a **mass spectrum**, which may be represented graphically or tabulated. Peak intensities are expressed as a percentage of that of the most abundant ion (*m/z* 31 for methanol), which is designated the **base peak**. The spectrum provides structural information and often an accurate relative molecular mass from which an unknown compound can be identified or a structure confirmed. Quantitative analysis is based on measuring the numbers of a particular ion present under closely controlled conditions **(Kealey and Haines, 2002).**

Mass spectrometer

All mass spectrometers consists of these basic functional components

1. inlet sample system,

- which facilitates the controlled introduction of gaseous or vaporized liquid samples via a **molecular leak** (pinhole aperture) and solids via a **heated probe** inserted through a vacuum lock

2. ion source,
   - to generate ions from the sample vapor
3. ion acceleration system,
   - ions generated in the source are accelerated into the analyzer chamber by applying increasingly negative potentials to a series of metal slits through which they pass
4. mass (ion) analyzer,
   - to separate the ions produced in the ion source according to their different mass–charge ratios
5. ion-collection system, usually an electron multiplier detector,
6. data-handling system, and
7. vacuum system connected to components (1) through (5). To provide a collision-free path for ions once they are formed, the pressure in the spectrometer must be less than 10^{-6} torr.

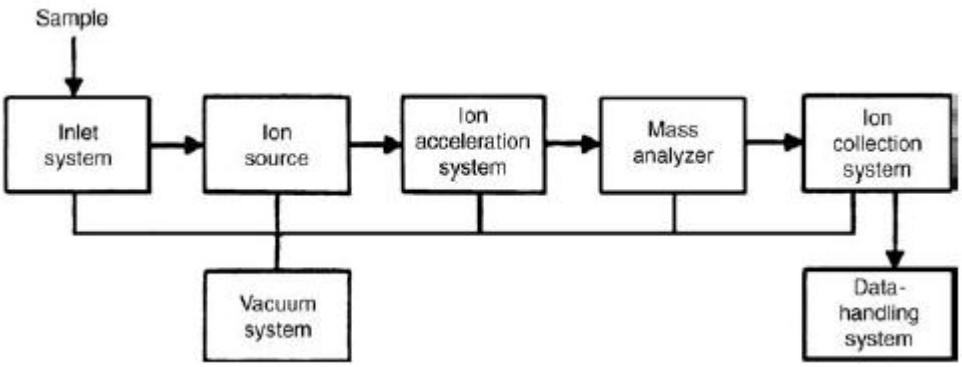
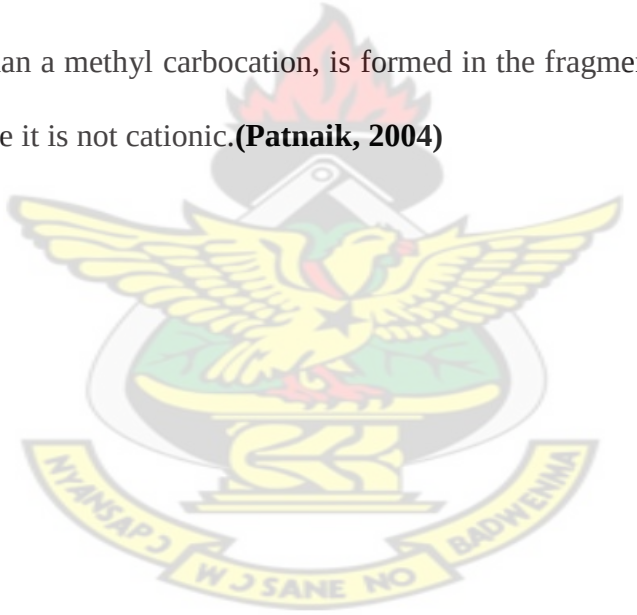


Figure 11: Components of a mass spectrometer (Patnaik, 2004)

Fragmentation

Molecular ions when formed are usually dissociated or **fragmented** into ions and neutral species of lower mass, which in turn may dissociate further. *Fragmentation patterns* give additional information about the structure of a molecule since they are distinctive for a particular molecular structure and are indicative of the specific functional groups present, therefore giving data on the structure and identity of the analyte.

The individual bond strengths in a molecule determine the points of cleavage throughout the structure. Molecular rearrangements and recombinations may also occur. Note the methyl carbocation is not formed. Radicals are somewhat more stable than carbocations. A more stable methyl radical, rather than a methyl carbocation, is formed in the fragmentation process but it is not detected by MS since it is not cationic.**(Patnaik, 2004)**



Chapter 2
EXPERIMENTAL

2.1 INSTRUMENTS AND MATERIALS

- T90+ UV/VIS Spectrometer (PG instruments Ltd.) with UVWin Spectrophotometer Software Version 5.2.0
- Buchi water bath B-419
- Buchi rotavapor R- 210
- Buchi recirculator chiller B- 741
- Adam – analytical weighing balance PW124
- Stuart Melting Point Apparatus SMP10
- No. 1 Whatmann filter papers
- Kontron instruments HPLC pump 422
- Power Chrom integrator
- Perkin Elmer UV/ visible detector
- Phenomenex Kromosil 5 C_8 250mm x 4.6mm 5 micron column
- Precoated plates-silica gel Gf 254, 0.25mm Merck W. (Germany)
- UV viewing system (CHROMATO-VUE® C-70G)
- Reflux apparatus
- Fruits, leaves (fresh and air dried) and stem of Piper guineense collected in the first week of March, 2012 from the Physique garden of the Pharmacognosy Department, Faculty of Pharmacy and authenticated by a Botanist at the Herbal Medicine Department, KNUST.

2.2 REAGENTS AND SAMPLES USED

- Potassium hydroxide (BDH, Poole, England)
- Ethanol (96%)
- Chloroform (BDH)
- Ethylacetate (BDH)
- n-Hexane (Park Scientific Ltd, UK)
- Acetone (Sigma- Aldrich, Germany)
- Distilled water
- 10% alcoholic potassium hydroxide (KOH) in 96% ethanol
- Distilled Methanol (BDH)
- Dragendorff's solution

2.3 METHOD

2.3.1 PHYTOCHEMICAL TESTS ON PIPER GUINEENSE

The fruits, leaves and stems were air dried and finely ground. Fresh leaves were also fine ground for the analysis.

Alkaloids

About 200mg of the powdered plant material (fruits, leaves and stem of *Piper guineense*) in a test tube was shaken with 5ml of methanol and filtered. The filtrate was then mixed with about 2ml of 10% HCl. A few drops (5 drops) of Dragendorff's reagents by the side of the test tube. A reddish-orange precipitate was observed in the test tube indicating the presence of alkaloids.

Glycosides

About 150mg of the powdered plant material (fruits, leaves and stem) in a test tube was shaken with 5ml of methanol and filtered. 1ml of concentrated HCl is added to the filtrate in a test tube and the mixture for about 25 minutes. 1ml each Fehlings solution A and B were added and the mixture heated for a further 5 minutes. A brick – red precipitate indicated the presence of glycosides.

Tannins and Phenolic compounds

About 200mg of the powdered plant material (fruits, leaves and stem) in a test tube was shaken with 5ml of methanol and filtered. 5 drops of neutral 5% ferric chloride was added. A greenish black coloration indicated the presence of tannins.

Saponins

About 200mg of the powdered plant material (fruits, leaves and stem) in a test tube was shaken with 5ml of water. The test tube was covered and vigorously shaken for about 6 min. Observation of a foam which does not disappear rapidly upon standing but persistent for about 5 min indicated the presence of saponins.

2.3.2 EXTRACTION AND ISOLATION OF PIPERINE FROM THE FRUITS OF PIPER GUINEENSE

120g of the finely ground plant material was placed in the boiling flask of the reflux apparatus and refluxed with about 350ml of ethanol for 3hrs. The mixture was then allowed to cool and the ethanol filtered off. For exhaustive extraction, the marc was refluxed again with another 350ml of ethanol for 3hrs. The combined ethanolic extract (about 700ml) was then concentrated under vacuum in a rotary evaporator at 70ºC to about 25ml of concentrate which is transferred into a

beaker. 50ml of 10% ethanolic KOH was then added, stirred well and the mixture allowed to stand for about 2hr. The mixture was then decanted leaving the insoluble residue settled below the beaker. The supernatant solution was then allowed to stand undisturbed for 48hrs for fine yellowish crystals of piperine to precipitate out.

2.3.4 RECRYSTALLIZATION OF PIPERINE

The precipitated yellow crystals were collected by filtration, washed with a minimum amount of the 10% ethanolic KOH and water. The crystals were recrystallized and purified from a mixture of acetone: hexane 3:2. The dried recrystallized piperine was then weighed.

2.3.5 MELTING POINT DETERMINATION

The melting point of the purified piperine was determined using the Stuart melting point apparatus.

2.3.6 SPECTROPHOTOMETRIC ANALYSIS OF ISOLATED PIPERINE

Calibration of Absorbance Scale of the UV/Vis Spectrophotometer

Potassium dichromate was. 60mg of the potassium dichromate after drying to a constant mass at 130 ° C was accurately weighed, dissolved in 0.005M sulphuric acid to 1 litre solution of dichromate with the same acid. Using the acid as reference, the UV/Vis absorption spectrum of the solution was determined over the wavelength range 225-450nm with a 1cm cell. Compared with the British Pharmacopoeia stipulations **(BP, 2007)**, the λ_{max} , λ_{min} and their A (1%,1cm) of the dichromate fell within the tolerable range.

Determination of Ultraviolet/visible absorption spectrum of isolated Piperine

Different concentrations of piperine ranging from 0.001%w/v – 0.00001%w/v were prepared with methanol and scanned over a wavelength range of 220 - 400nm using the calibrated T90+ UV/VIS Spectrometer (PG instruments Ltd.) with UVWin Spectrophotometer Software Version 5.2.0 in a 1cm cell using methanol as reference. This was to choose the wavelength of absorption for HPLC method development.

^{1}HNMR, ^{13}C NMR AND MASS SPECTROMETRIC ANALYSIS OF ISOLATED PIPERINE.

The Proton and Carbon-13 nuclear magnetic resonance spectra of the isolated piperine were obtained on Mercury-300BB Varian Spectrometer, the sample was dissolved in deuterated chloroform ($CDCl_3$).

2.3.7 EXTRACTION OF PIPER GUINEENSE SAMPLES PRIOR TO ISOCRATIC REVERSE PHASE HPLC ANALYSIS.

0.3000g of the powdered dried fruits (**DF**) of Piper guineense was weighed into a ceramic mortar and slowly triturated with 20ml of distilled methanol for about 5 minutes. The methanolic extract was filtered into a 100ml volumetric flask by decanting from the mortar unto a filter paper. 20ml of methanol was again added to the residue in the mortar and triturated again for 5 minutes and filtered by decanting as before into a 100ml flask. The procedure above was repeated for two more 20ml portions of methanol on the residue. The total filterate in 100ml volumetric flask was then made to the mark with distilled methanol.

The same procedure was used for 3.0g of the leaves (fresh and dried) and stem extracts of *Piper guineense* prior to HPLC analysis.

The samples were coded as **DL** (dried leaves), **FL** (fresh leaves), **DS** (dried stem) and **DF** (dried fruits)

2.3.8 TLC ANALYSIS OF PIPERINE AND SAMPLE EXTRACTS OF PIPER GUINEENSE

The isolated piperine was run alongside the fruits, leaves and stem extracts of *Piper guineense* on a precoated silica gel to confirm the presence of piperine in the extracts and to determine their retardation factors (R_f) for identification. The mobile phases used were chloroform: ethylacetate 1:1 and Hexane: ethylacetate: glacial acetic acid 3: 1: 0.3. The detection was under both UV light and by spraying with Dragendorff reagent.

2.3.9 INVESTIGATION FOR MOBILE PHASE FOR THE HPLC ANALYSIS.

Different mobile phases were explored in the determination of a suitable HPLC condition for the assay of piperine in the fruits, leaves and stem of the *Piper guineense* plant taking into consideration type of column, structure and pka of piperine. Different flow rates were also investigated to determine an appropriate retention time. The suitable conditions eventually chosen were methanol: water (80: 20) at a flow rate of 1.40ml/min under isocratic elution.

2.3.10 HPLC ANALYSIS OF PIPERINE IN THE PLANT PARTS OF PIPER GUINEENSE

The samples prepared above were analysed by a chromatograph consisting of Kontron instruments HPLC pump 422 with a programmable Perkin Elmer UV/ visible detector and a Power Chrom integrator. The column used was a Phenomenex Kromosil 5 C_8 250mm x 4.6mm 5 micron id. The mobile phase consisted of methanol: water 80: 20 pumped at a flow rate of 1.40ml/min. 100µl of the samples were injected unto the column of ambient temperature. A

maximum run time of 6.5min was allowed during analysis and the eluent was monitored at 343nm and an AUFS of 1.00.

The chromatograms were analysed by the integrator to estimate the peak areas as a function of the concentrations of piperine in the samples. Isolated piperine was used to draw a calibration curve from which the content of piperine in the samples was determined.

2.3.11 HPLC METHOD VALIDATION

LINEARITY, RANGE, LIMIT OF DETECTION (LOD) and LIMIT OF QUANTIFICATION (LOQ):

These were inferred from the calibration curve drawn using different concentrations of isolated piperine.

PRECISION:

Both intraday precision (repeatability) and intermediate (interday) precision were determined using 0.001%w/v of isolated piperine. Six (6) different injections were made for three (3) different days. The relative standard deviations (RSD) were determined.

ROBUSTNESS:

Using a 0.001%w/v of piperine and varying just one (1) parameter at a time while keeping all others constant, the effect of changes in absorbance wavelength, flow rate and changes in mobile phase composition with small limits were determined. The RSD of the resulting areas were determined. Statistical Analysis of Variance (ANOVA) was also used to determine whether there are significant differences between the results these variations from those of the actual HPLC conditions.

ACCURACY:

The plant samples were spiked with 0.001%w/v of isolated piperine and the percentage recoveries determined. The RSD of these values were determined.

Chapter 3
RESULTS AND CALCULATIONS

3.1 Phytochemical test

The table below summarizes the results for the analysis performed on the various parts of *Piper guineense*.

Table 4: Results of phytochemical tests on *Piper guineense*

Phytochemical Test	Seed	Leaves (both dried and fresh)	Stem
Alkaloid	Positive	Positive	Positive
Glycosides	Positive	Positive	Negative
Tannins	Positive	Positive	Positive
Saponins	Positive	Positive	Positive

3.2 Yield and Melting Point of isolated piperine

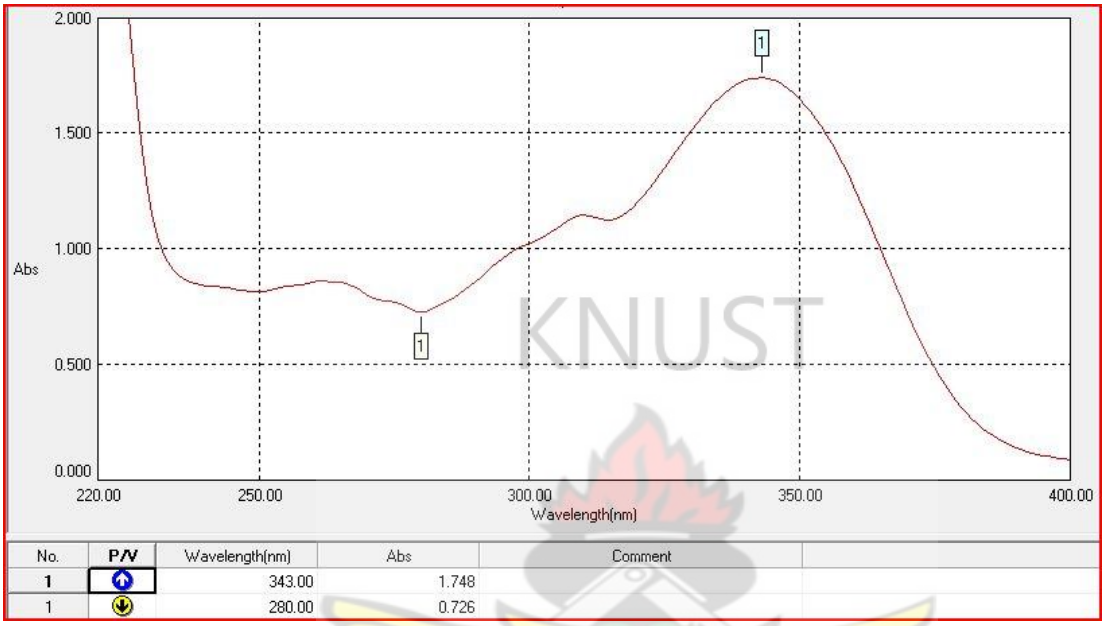
The yield of piperine after extraction and recrystallization was 2.2755g (2.07%w/w).

Table 5: Results of melting point

1st determination	128ºC - 130 ºC
2nd determination	128 ºC - 130 ºC
Average melting point	129 ºC

3.3 Determination of λmax from Ultraviolet/visible absorption spectrum of isolated Piperine

The λmax determined to be 343nm from the spectra.



No.	P/V	Wavelength(nm)	Abs	Comment
1	↑	343.00	1.748	
1	↓	280.00	0.726	

Figure 12: Sample UV/Vis spectrum of piperine

3.4 TLC of isolated piperine

Below are sample chromatograms for TLCs run

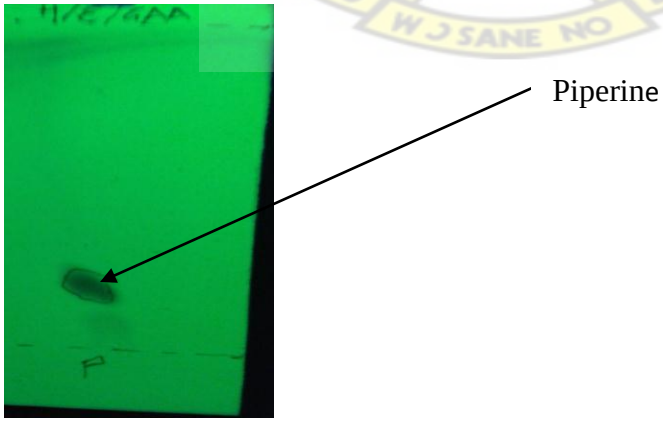


Figure 13: TLC Chromatogram of piperine in hexane: ethylacetate: glacial acetic acid 3: 1: 0.3 under 254nm UV light.

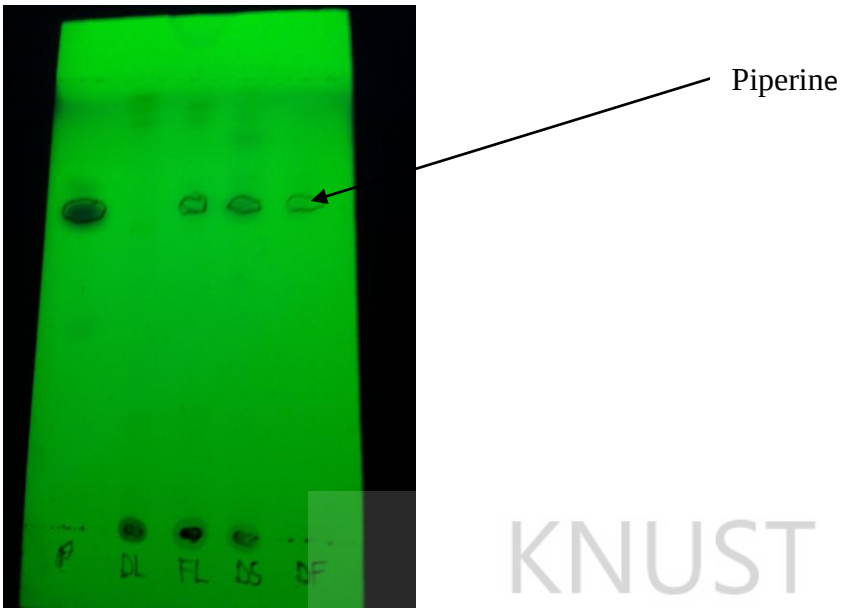


Figure 14: TLC chromatogram of isolated piperine run alongside plant extracts in chloroform: ethylacetate 1: 1 viewed under 254nm UV light

Table 6: R_f values of piperine in different mobile phases

Mobile phase	Average R_f value (n = 4)
Hexane: ethylacetate: glacial acetic acid 3: 1: 0.3	0.285 ± 0.01291
Chloroform: ethylacetate 1: 1	0.70 ± 0.009574

3.5 METHOD DEVELOPMENT

Table 7: Summary of investigation for Ideal HPLC method for Piperine analysis

Column type	Mobile phase composition	Flow rate (ml/min)	Retention time (R_t)/ min	Comments
C18	Methanol/water 70/30	1.0	6.10	Broad peak. Significant peak tailing (T > 2)

C18	Methanol/water 70/30	1.5	3.545	Broad peak. Significant peak tailing (T > 2)
C18	Methanol/water 80/20	1.0	4.957	Slightly sharp peak. Significant peak tailing (T> 2) at base.
C18	Methanol/water 90/10	1.0	3.658	Slightly sharp peak. Significant peak tailing (T ≈ 2) at base.
C18	acetate buffer (pH 5.5) / methanol 70/30	1.0	5.373	Significant tailing. Peak sharp at apex. T > 2
C18	Methanol /2% Acetic Acid 85/15	1.0	6.631	Sharp peak. Tailing factor, T ≈ 2
C18	Methanol/ KH_2PO_4 buffer (pH 4.5) 85/15	1.0	4.750	Sharp peak tailing at base (T ≈ 2)
C18	Methanol/ KH_2PO_4 (pH 10.5) 85/15	1.0		No peak showed up to 12.5 min
C8	Methanol/ water 70/30	1.0	4.954	Peak tailing (T≈ 2) with shoulders due to mobile phase
C8	Methanol/ water 80/20	1.0		Sharp peak. T < 2. Less resolution between mobile phase and peak
C8	Methanol/ water 80/20	1.4	3.69	Sharp peak. T < 2. Best resolved peak

3.6 HPLC METHOD VALIDATION

3.6.1 Linearity, Range, LOD and LOQ

Linearity, Range, LOD and LOQ could all be inferred from the calibration curve drawn for isolated piperine. The Range was **0.0002%w/v – 0.004%w/v**. The Coefficient of Correlation, R^2, was **0.998** for the equation of the line **y = 1535x – 0.034**.

Table 8: Concentrations and their peak areas for isolated piperine

Conc. of Piperine (% w/v)	Peak Area
0.004	6.038
0.002	3.206
0.001	1.456
0.0008	1.207
0.0004	0.546
0.0002	0.241

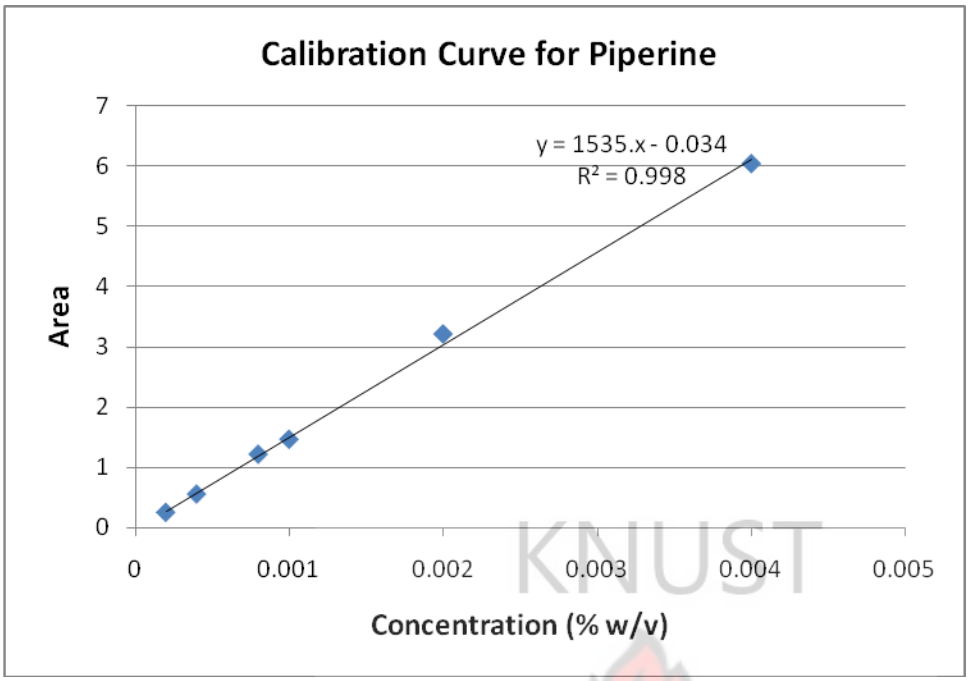


Figure 15: Calibration curve for isolated piperine

Table 9: Results for residual areas for LOD and LOQ determination

Conc. of Piperine (% w/v)	Actual Peak Area, APA	Estimated Peak Area, EPA	Residual Peak Area, RPA (APA – EPA)
0.004	6.038	6.106	- 0.068
0.002	3.206	3.036	0.170
0.001	1.456	1.501	- 0.045
0.0008	1.207	1.194	0.013

0.0004	0.546	0.580	- 0.034
0.0002	0.241	0.273	- 0.032
Standard Deviation of residual areas, σ			0.087058

Limit of Detection, LOD = 3.3σ / S

where σ is the standard deviation of the residual areas and S is the slope of the calibration curve.

Therefore LOD = (3.3 x 0.087058) / 1535

= 0.2873 / 1535

= 1.872×10^{-4}

Limit of Quantification, LOQ = 10σ / S

= (10 x 0.087058) / 1535

= 0.87058 / 1535

= 5.672×10^{-4}

3.6.2 Precision

Table 10: Values for intraday precision (repeatability)

Run	Peak Area	Retention time, R_T,(min)
1	1.48	3.70
2	1.47	3.74

3	1.45	3.78
4	1.50	3.86
5	1.46	3.82
6	1.46	3.78
Mean	**1.47**	**3.78**
Standard Deviation, SD	**0.017889**	**0.05656**
RSD (%)	**1.217**	1.496

Table 11: Results for intermediate (interday) precision

Day	Peak Area
One (1)	1.45
	1.46
	1.48
	1.47
	1.49
	1.45
Two (2)	1.46
	1.47
	1.44
	1.49
	1.43
	1.47

Three (3)	1.54	
	1.45	
	1.45	
	1.47	**Mean = 1.466**
	1.45	**Standard Dev., SD = 0.0249**
	1.46	**RSD = 1.704%**

3.6.3 Robustness

Table 12: Effect of variation of wavelength on the HPLC analysis of Piperine

λ nm	Area	Precision, (RSD%)	Nature of Peak
340	1.453 ± 0.015275	1.051049	Sharp peak, no tailing
342	1.4567 ± 0.015275	1.048644	Sharp peak, no tailing
344	1.4633 ± 0.015275	1.043867	Sharp peak, no tailing
346	1.46 ± 0.02	1.369863	Sharp peak, no tailing

Statistical ANOVA: Single Factor for wavelength variation (0.05 confidence limit)

F = 0.5606, *F crit* = 3.47805 and *P-value* = 0.69667

Table 13: Effect of variation of mobile phase composition on the HPLC analysis of Piperine

Composition	Area	Precision, (RSD%)	Nature of Peak
Methanol: water 78:22	1.476 ± 0.015275	1.034	Less sharp peak, no tailing
Methanol: water 82:18	1.463 ± 0.005774	0.395	No difference from normal

Statistical ANOVA: Single Factor for mobile phase composition variation(0.05 confidence limit)

F = 2.00, *F crit* = 7.7086 and *P-value* = 0.2302

Table 14: Effect of variation of flow rate on the HPLC analysis of Piperine

ml/min	Area	Precision, (RSD%)	Nature of Peak
1.20	1.66 ± 0.01	0.60241	Broad peak, prominent tailing
1.50	1.4533 ± 0.015275	1.051049	Sharp peak, no tailing
1.60	1.3733 ± 0.005774	0.420401	Sharp peak, no tailing

Statistical ANOVA: Single Factor for flow rate variation (0.05 confidence limit)

F = 537.0909, *F crit* = 5.14325 and *P-value* = 1.71×10^{-7}

3.7 Calculation of percentage content of piperine in the plant part of *Piper guineense*

Sample calculation for the fruits:

From the calibration curve (Figure 19), the equation of the regression line is given as

$y = 1535x - 0.034$

therefore,

$x = (y + 0.034) / 1535$

where y = peak area and x = piperine concentration (%w/v)

therefore for DF (2 times dilution) of peak area 7.68,

$x = (7.68 + 0.034) / 1535$

$x = 7.714 / 1535$

$x = 0.005025\%w/v$

but since the stock was diluted 2 times, concentration of stock = 2 x 0.005025

$= 0.0101\%w/v$

The above value implies 0.0101g of piperine is dissolved in 100ml of methanol. But this quantity of piperine was determined from 0.3g of grounded fruits dissolved in 100ml of methanol, therefore

Percentage by weight of piperine determined in the fruits = (0.0101g / 0.30g) x 100%

$= 3.35\%w/w$

The above method of calculation was applied to all the samples, their mean values, standard deviation and precision (RSD) was determined accordingly.

Table 15: Results for percentage content of piperine in various parts of *Piper guineense*.

Type of extract	Dilution of stock	Area	% w/w (%content) of piperine	Mean %w/w	Standard Deviation, SD (±)	Relative Standard Deviation (RSD), %
Dried leaves, DL (3g/100ml)	2 times	0.090	5.39×10^{-3}	5.44×10^{-3}	9.24×10^{-5}	1.697
		0.090	5.39×10^{-3}			
		0.094	5.55×10^{-3}			
Fresh leaves, FL (3g/100ml)	2 times	0.96	0.043	0.0437	0.000816	1.87
		0.96	0.043			
		0.98	0.044			
	4 times	0.46	0.043			
		0.47	0.044			
		0.48	0.045			
Dried Stem, DS (3g/100ml)	2 times	2.70	0.119	0.115	0.00228	1.98
		2.58	0.113			
		2.58	0.113			
	4 times	1.31	0.116			
		1.29	0.115			
		1.28	0.114			

Dried Fruits, DF(0.3g/100ml)	2 times	7.68	3.35	3.345	0.0339	1.0138
		7.55	3.29			
		7.75	3.38			
	4 times	3.83	3.36			
		3.79	3.32			
		3.85	3.37			

3.8 Calculation of percentage recoveries of piperine after spiking extracts

Sample Calculation for Dried Stem, DS (2 times dilution):

The extract was spiked 0.001%w/v of piperine resulting in a peak area of 4.13. However, from Table 16, initial peak area for same extract without spiking was 2.70.

Recovered area = 4.13 – 2.70

= 1.43

Therefore from, x = (y + 0.034) / 1535

Recovered concentration = (1.43 + 0.034) / 1535

= 1.464 / 1535

= 0.000954%w/v

Hence Percentage Recovery (% Recovery) = (0.000954 / 0.001) x 100

= 95.77%

This sample method of calculating the percentage recovery was applied to all the samples, their mean values, standard deviation and precision (RSD) was determined accordingly.

Table 16: Results for percentage recovery of spiked extracts of *Piper guineense.*

Type of Extract	Spiked piperine Conc. (%w/v)	Area	Recovered Conc. (%w/v)	% Recovery	Mean % recovery	Standard deviation (±)	Relative Standard Deviation (RSD), %
DL (2 times dil.)	0.001	1.55	9.73×10^{-4}	97.33	98.84	2.0724	2.097
		1.57	9.798×10^{-4}	97.98			
		1.60	1.012×10^{-3}	101.2			
FL (2 times dilution)	0.001	2.38	9.44×10^{-4}	94.42	96.14	1.527	1.589
		2.37	9.67×10^{-4}	96.68			
		2.35	9.73×10^{-4}	97.33			
DS (2 times dil.)	0.001	4.13	9.577×10^{-4}	95.77	97.27	1.861	1.912
		4.07	9.935×10^{-4}	99.35			
		4.03	9.67×10^{-4}	96.68			
DF (4 times dil.)	0.002	6.93	2.029×10^{-3}	101.43	100.36	1.853	1.847
		6.75	1.964×10^{-3}	98.22			
		6.87	2.029×10^{-3}	101.43			

3.9 Results for Spectroscopic Analysis

3.9.1 NMR Analysis

Refer to Appendix for the full NMR Spectra. The labeled structure of Piperine below is to aid analysis of the NMR Spectra and assignment of carbons and protons to their chemical shifts..

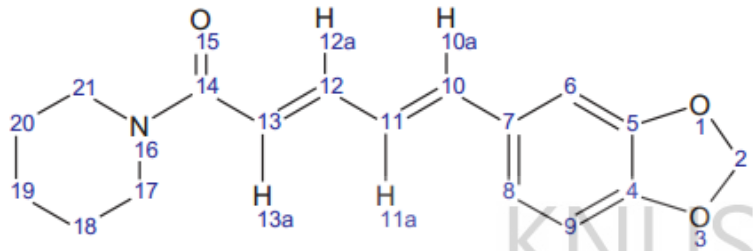


Figure 16: Labelled Structure of Piperine

Table 17: Values for Chemical Shifts and Assigned Carbons

δ(ppm)	Assignment
24.653	C19
25.621	18
26.720	20
30.969	Impurity
43.233	17
46.919	21
101.273	2
105.618	6
108.483	9
120.007	13
122.535	8
125.312	10
130.988	7
138.235	11

142.507	12
148.089	5
148.163	4
165.409	14
76 – 78	Solvent Peak

3.9.2 Mass Spectroscopy

For mass spectroscopic data of isolated piperine, refer to Figure 31 in Appendix.

Chapter 4
DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Phytochemical screening, extraction yield and melting point

Phytochemical sreeening

The preliminary screening of a plant materials reveals the kind of metabolites present are mostly responsible for its therapeutic activity and these are characteristic of the plant material hence can be used as a form of identification. The presence of alkaloids, glycosides, tannins and saponins as detected in the fruit was consistent with results of phytochemical screening on *Piper guineense* fruit from **(Gbadamosi *et al.*, 2011)** and **(Ejele *et al.*, 2012)**.

The leaves (both dried and fresh) of *Piper guineense* also tested positive for all the investigated metabolites and these results are comparable with those from **(Udoh *et al.*, 2012)** and **(Mensah *et al.*, 2008**).

(Tuani *et al.*, 1994) also reported results of phytochemical screening on the bark of *Piper guineense* similar to those reported in this project on the stem.

The above reports for the phytochemical screening of specific metabolites in *Piper guineense* compared to those in literature strongly indicates that the plant under investigations may be *Piper guineense.* The reports also indicated that alkaloids, alongside other secondary metabolites, were present in the various plant parts investigated – a strong indication that piperine, the major alkaloid in pepper species could be present.

Extraction Yield

The extraction and purification (recrystallization) process yielded **2.07%w/w** of piperine from the dried fruits.

However, a crude yield of 2.50%w/w of isolated piperine by a different method of extraction have been to be reported for dried fruits of *Piper longum.***(Deepthi et al., 2012)**

The disparity in terms of yield of piperine could be due to the different species involved and the difference in the method of extraction and isolation.

Despite the simplicity of the extraction process compared to other methods of extraction, it was still liable to random errors. Recrystallization may also contribute certain losses.

Melting Point

Melting point was determined to be an average of **128ºC - 130 ºC**. This value is consistent for those reported in literature from **(Kolhe *et al.*, 2011)** and **(Henry, 1949)**, indicative of a measure of purity and identity.

4.2 TLC of isolated piperine

The TLC of isolated piperine showed a single spot and the retardation factors determined in two different solvent systems for isolated piperine were 0.285 ± 0.01291 for hexane: ethylactate: glacial acetic acid 3: 1: 0.3 and 0.70 ± 0.009574 for chloroform: ethylacetate 1: 1 respectively. The extracts run along isolated piperine indicate except for the dried leaves **DL,** they all contain piperine. However since piperine was quantified in **DL** under HPLC, it only means that the quantities of piperine present in **DL** were below the detection limit.

The chromatogram of the extracts and the retardation factor of piperine could be used as a fingerprint in the identification of the extracts.

4.3 SPECTROPHOTOMETRIC ANALYSIS OF ISOLATED PIPERINE

4.3.1 UV/VIS SPECTROSCOPY

After the calibration of the absorbance scale, the spectrum (figure 12) obtained indicates a λmax of 343nm hence this wavelength was used for piperine detection during the HPLC analysis. This λmax is consistent with that used by **(Kolhe *et al.*, 2011)** in the HPLC quantification of piperine in *Piper nigrum.* Moreover, **(Wood *et al.*, 1988)** has reported that the 343nm λmax was the most sensitive and gives about three (3) times piperine response compared to 266nm, 280nm and the other possible wavelengths of absorption.

Compounds could also be identified by comparing their UV λmax and spectra with standards form literature. The UV spectrum of piperine when compared to that from literature **(Singh *et al.*, 2011**) over a wavelength range of 200nm – 400nm indicates the isolated compound may be piperine.

4.3.2 NUCLEAR MAGNETIC RESONANCE

All discussions relating to NMR are made with reference to the labeled structure of piperine in figure 16.

^{1}H NMR ANALYSIS

Since the available Proton NMR Spectrum did not show the specific chemical shift for the peaks appearing, the general pattern was compared to those in literature **(NOP, 2006)** and **(Hanson, 2012).** The comparison shows a similar pattern for Proton NMR hence the isolate could be piperine. Further discussions below only uses estimated proton chemical shift values from the spectra available.

The peak showing at about 2.2ppm could be from an impurity.

^{13}C NMR ANALYSIS

From Table 17, the chemical shifts for ^{13}C NMR spectra available when compared to those from literature **(NOP, 2006)** and **(Clark, 2007)** showed similar ppm values indicating the isolate may be piperine.

The peak at about 30.969ppm could be an impurity.

HSQC DEPT Analysis

For DEPT analysis, methylene (-CH_2-) carbons show negative (-ve) projections while methyl (-CH_3) and methine (-CH-) carbons show positive projections with the methyl carbons being more upfield.

From the HSQC-DEPT the methylene carbons 18, 19 and 20 (24.65ppm- 26.72ppm) in the negative projection correlated with multiplets of methylene protons (1.55ppm – 1.69ppm). Carbons 17 and 21, also methylene carbons attached to a hetero- atom nitrogen (-CH_2-N, 43.233ppm and 46.919ppm) also showed correlation to multiplets of methylene protons (3.527ppm – 3.636ppm) which are more downfield because of the attached electronegative nitrogen and the neighboring carbonyl.

The positive projection first showed the impurity carbon peak (30.969ppm) and a corresponding singlet proton at 2.165ppm – 2.187ppm.

From Figure 30, the positive projections of aromatic carbons 6 and 9 (105ppm and 108ppm respectively) correlates with protons in the aromatic region of the Proton NMR (7.12ppm multiplet and 6.86ppm doublet), however C6 is more downfield because it is in a more electronegative environment. C8 (122ppm) is also an aromatic carbon with protons at 6.930ppm.

Carbons 10 and 11 (125ppm and 138ppm respectively) correlates with multiplets protons around 6.80ppm due to their proximity to the shield effect of the aromatic ring. C13 (120ppm) also correlated to a doublet vinylic proton (about 6.5ppm) influenced by the neutralizing effect on the nearly carbonyl by the nitrogen atom and the pi electrons of the C13 double bond. C12 proton (about 7.40ppm, ddd) is however the most deshielded among the alternating double bonds because it is trans to the C13 proton and inductively experiences more the carbonyl electron withdrawing effect.

As observed carbons 4, 5, 7 and 14 did not show in the HSQC DEPT analysis because they are quaternary carbons. Carbon 2 however could not be accounted for in the dept analysis.

4.3.3 MASS SPECTROSCOPY

From Fig 31, the base peak from mass spectroscopic analysis of isolated piperine was determine to have a *m/z* value of 286.03 corresponding to 100% relative intensity. However mass spectrometric analysis employing Atmospheric Pressure Chemical Ionisation (APCI) generate protonated ionic species thus MH^+ instead of M^+ ions **(Van Bramer, 1998)**, hence 1amu had to be substracted 286.03 to give a true *m/z* value of the base peak to be 285.03. The charge on the base peak is +1 therefore the mass of the analyte is 285.03 which is consistent with the 285.34g/mol found in literature **(NIST, 2012).**

The peak at *m/z* 201.05 (28.08%) may be the piperoyl fragment of piperine after the piperidine (MH^+ = 86.20 *m/z*) **(MassBank, 2012)** portion has been broken off during ionization.

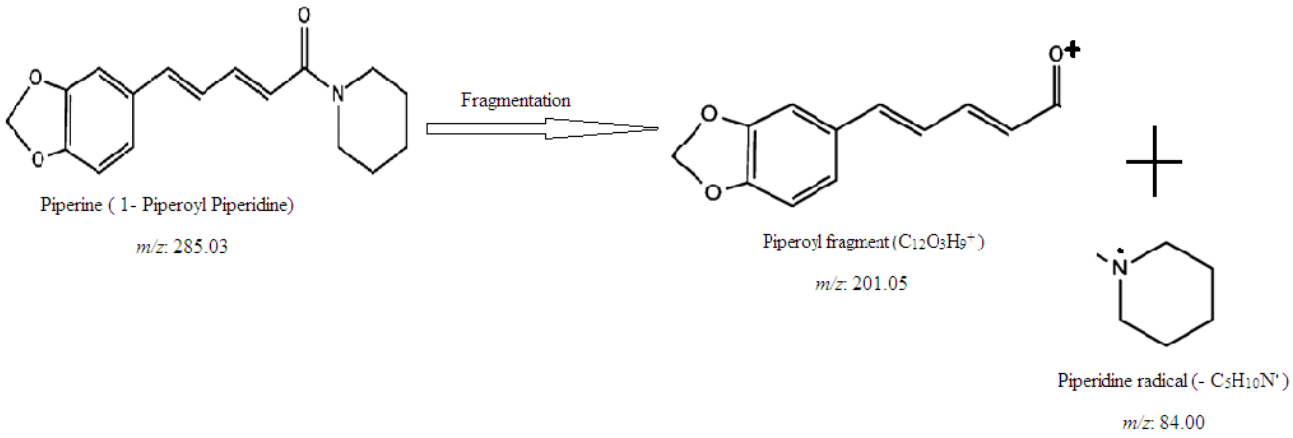


Figure 17: Fragmentation pattern of piperine (1- Piperoylpiperidine) to piperoyl cation and piperidine radical

4.4 HPLC METHOD DEVELOPMENT

Piperine has a pka of 12.20 and is neutral to litmus so should elute freely with an appropriate water/ methanol, however its bulky non polar nature makes it tail strongly on a non polar column such as a C18 column and this increases tailing and retention time. Increasing the methanol component of the mobile phase did not reduce the tailing due to the strong interaction between piperine and the stationary phase. Increasing the flow rate only reduced retention time a little but did not reduce the tailing. The use of pH modifiers and buffers were to protonate piperine and make it less adsorptive unto the C18 column. However piperine has a strong amide linkage which cannot easily be hydrolysed or protonated hence could not easily be affected by pH changes or buffers.

A C8 column gave a better chromatogram for isolated piperine with less tailing and at a satisfactory flow rate of **1.40ml/min** and a retention time of **3.78 ± 0.05656 min.** This is because there is less adsorption of piperine unto the stationary phase of the column.

4.5 HPLC METHOD VALIDATION

4.5.1 Linearity and Range:

The analytical method was found to be linear within the concentration range **0.0002%w/v – 0.004%w/v** with the equation of the line being **y = 1535x – 0.034**. The coefficient of correlation, R^2 value, was **0.998** and this corresponds to the minimum stipulated by the ICH **(ICH, 2005)** indicating that there is a linear relationship between concentrations of piperine and their peak areas within the stated range.

4.5.2 LOD and LOQ:

The LOD which is the minimum sample concentration that can be detected by the method was **1.872×10^{-4}%w/v**. This concentration could reliably be differentiated by the HPLC analytical method from the background noise of the instrument and mobile phase.

The lowest concentration of piperine that can be quantified with acceptable precision and accuracy by the HPLC method, the LOQ, was **5.672×10^{-4}%w/v.**

4.5.3 Precision:

Intraday Precision (Repeatability)

The **(ICH, 2005)** stipulates a value ≤ 2% RSD for six replicate determinations for a method to be said to be repeatable. The results obtained for repeatability studies indicates an RSD of **1.217%,**

lower than the standard hence the developed HPLC method can give repeatable results within a short time or under same lab conditions.

Interday (intermediate) precision

An **(ICH, 2005)** stipulated value ≤2% RSD of six replicate determinations each for three different analyte concentrations on three consecutive days indicates the method is precise. An RSD of **1.704%** was obtained for the method over the three day period indicating it's precision.

4.5.4 Robustness

Wavelength Variation

In the statistical Analysis of Variance (ANOVA), for there to be a significant variation between results after varying one or more factors of a particular method, the calculated F value should be lesser than the F crit value for the set of results. The results for wavelength variation indicates the ***Fcrit value*** **(3.47805)** to be greater the ***F value*** **(0.5606)**. This implies that within the wavelength range 343 ± 3nm, the HPLC analytical method for piperine would produce similar and precise results.

Mobile Phase Composition Variation

Single factor ANOVA reflects a ***Fcrit value*** **(7.7086)** greater than the ***F value*** **(2.00)** indicating there is no significant difference between the the results for the variation of methanol: water composition from 78: 22 to 82: 18.

Flow Rate Variation

The ***Fcrit value*** **(5.14325)** for the single factor ANOVA was lesser than the the ***F value*** **(537.0909)** meaning there is a significant difference in result produced by the HPLC method with variation of the flow rate within the range 1.40 ± 0.20ml/min. However, from the results the flow rate of 1.20ml/min gave a broad tailing peak and may be responsible for the significant variation. The method is therefore robust for flow rates of 1.30 – 1.60ml/min.

4.5.5 Accuracy

Accuracy may be inferred once precision, linearity and specificity have been established and hence the analytical method developed was also accurate. In addition, the average percentage recoveries of piperine from the various samples were above 95.00% with RSDs lesser than 2.0% except for that of DL. This therefore implies accuracy.

4.6 Percentage Content (%w/w) of Piperine in plant parts of *Piper guineense*

Piperine was found to be $5.44 \times 10^{-3} \pm 9.24 \times 10^{-5}$%w/w in the dried leaves (DL) extract, meaning every 100g of the matured dried leaves of *Piper guineense* contains $5.44 \times 10^{-3} \pm 9.24 \times 10^{-5}$g. That for fresh leaves (FL) extract was 0.0437 ± 0.000816%w/w i.e. every 100g of the fresh leaves sold on the local market contains 0.0437 ± 0.000816g of piperine.

Piperine was found to be 0.115 ± 0.00228%w/w in dried stem (DS) extract thus every 100g of the dried stem of *Piper guineense* used for medicinal purposes contains 0.115 ± 0.00228g of piperine.

3.345 ± 0.0339%w/w was the percentage content of piperine in the dried fruits extract (DF), therefore each 100g of dried fruits exported contains 3.345 ± 0.0339g of piperine.

Pepper species have been reported to contain about 5-9% piperine. Piperine content is 3- 9% and 3-5% (on dry weight basis) in *Piper nigrum* and *Piper longum* respectively **(Madhavi *et al.*, 2009)** in the fruits.

This value of 3.345 ± 0.0339%w/w of piperine determine for *Piper guineense* fruits is comparable to those reported for *Piper nigrum* and *Piper longum* sold on the international market.

This HPLC method could therefore be used to standardize *Piper guineense* for export in order to increase its market premium.

These values of piperine content are however subject to influence of factors such as the environment of the plant and the time of harvest for analysis.

4.7 CONCLUSION

Phytochemical screening

Alkaloids were determined to be present in the various parts of piper guineense under investigation. Saponins, tannins and glycosides were also present. Glycosides were however not present in the stem.

Isolation and Yield

Piperine was successfully isolated from the dried fruits of *Piper guineense* and recrystallized from acetone: hexane 3:2 with a crude yield of 2.07%w/w.

Melting point

The melting point of piperine was determined to be 128 - 130ºC.

Spectroscopic analysis

Piperine was identified by UV spectroscopy by comparing the spaectrum and the λmax (343nm) to standards in literature. Piperine was also authenticated by 1H, 13C and HSQC – DEPT NMR. Mass Spectroscopy was used to determine the molecular weight of piperine to be 285g/mol.

TLC of isolated piperine:

A TLC R_f of 0.285 ± 0.01291 was determined for hexane: ethylactate: glacial acetic acid 3: 1: 0.3 and 0.70 ± 0.009574 for chloroform: ethylacetate 1: 1.

HPLC Method Development and Validation

A reverse phase HPLC was developed on a Phenomenex Kromosil 5 C_8 (250mm x 4.6mm 5 micron id) column at ambient temperature unto which 100µl of samples were injected and eluted with a mobile phase of methanol: water 80: 20 at a flow rate of 1.40ml/min and eluents detected at 343nm and AUFS 1.00.

The HPLC method developed was validated and found to be precise, accurate, robust, and linear within the concentration range of **0.0002%w/v – 0.004%w/v**. The Limit of detection(LOD) and Limt of Quantitation(LOQ) of the method were respectively found to be **1.872 x 10^{-4}%w/v** and **5.672 x 10^{-4}%w/v**. Hence, the method can be used to standardise herbal preparations containing extracts of *P. guineense*.

Percentage Content

The content of piperine in various parts of Piper guineense is as follows –

Dried leaves (**DL**): **5.44 x 10^{-3}± 9.24 x 10^{-5}%w/w**

Fresh leaves (**FL**): **0.0437 ± 0.000816%w/w**

Dried stem (**DS**): **0.115 ± 0.00228%w/w**

Dried fruits (**DF**): **3.345 ± 0.0339%w/w**

4.8 RECOMMENDATIONS

- The percentage content of piperine in the various parts of *Piper guineense* must be investigated seasonally to establish the appropriate period of harvest that produces the highest levels of piperine.
- The stability of piperine in herbal aqueous extracts must be investigated to aid in the standardisation of herbal preparations containing extracts *P. guineense.*
- Stability studies with challenges such as heat on piperine should be performed to know the decomposition pattern.
- Suitable methods have to be developed for detection of possible degradation products and acceptable limits set for any toxic components that are discovered.

APPENDIX

TLC CHROMATOGRAM

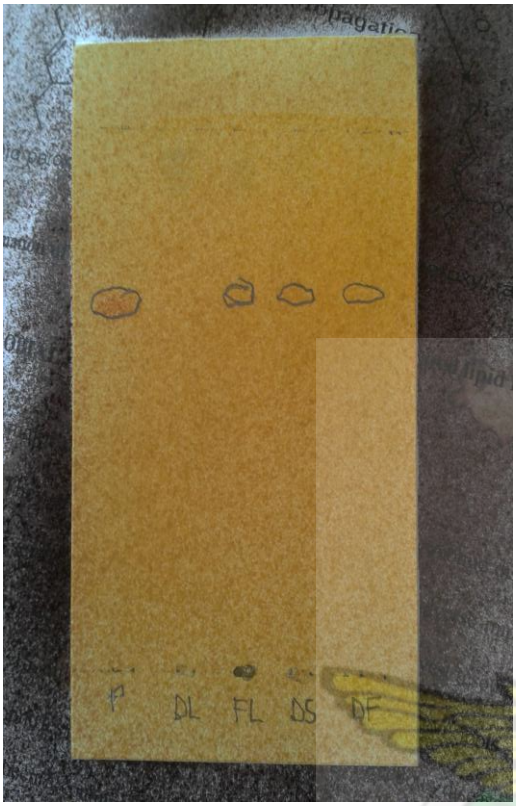
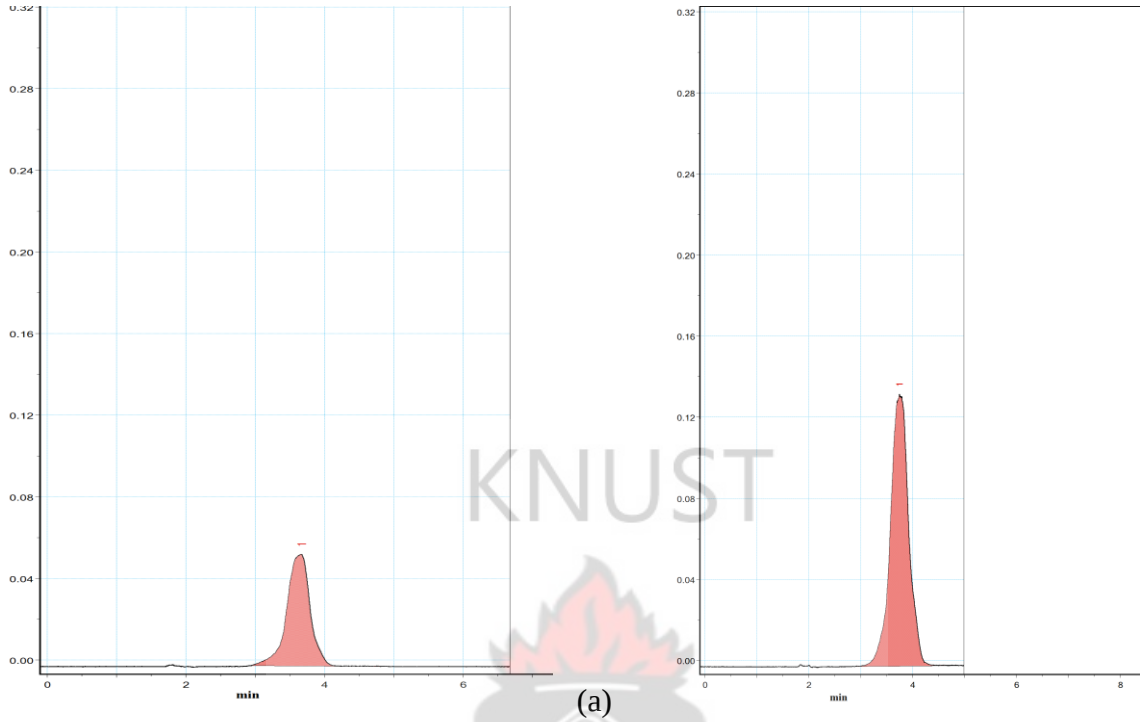


Figure 18: Chromatogram showing piperine run alongside sample extracts of *Piper guineense* in chloroform: ethylacetate 1: 1 and detected with Dragendoff reagent.

SAMPLE HPLC CHROMATOGRAMS



(a) (b)

Figure 19: Chromatogram of 0.001%w/v (a) and 0.002%w/v (b) of pure piperine

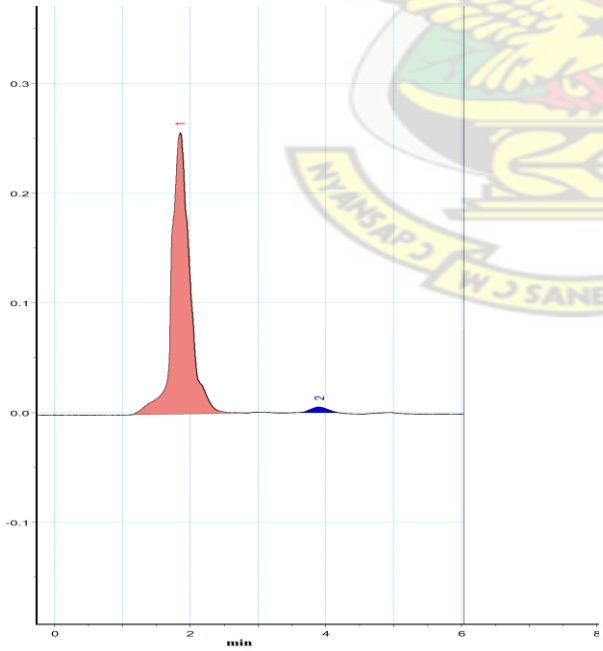


Figure 20: Chromatogram of DL extract (2 times dilution)

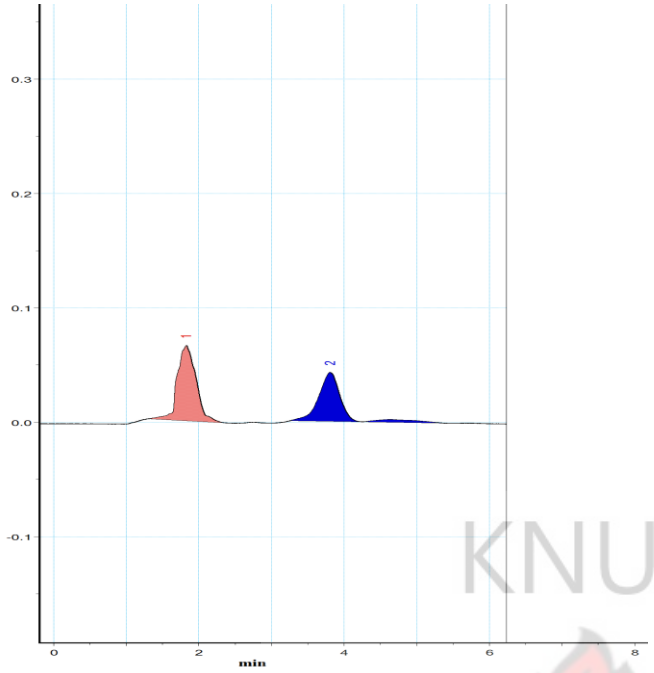


Figure 21: Chromatogram of FL extract (2 times dilution)

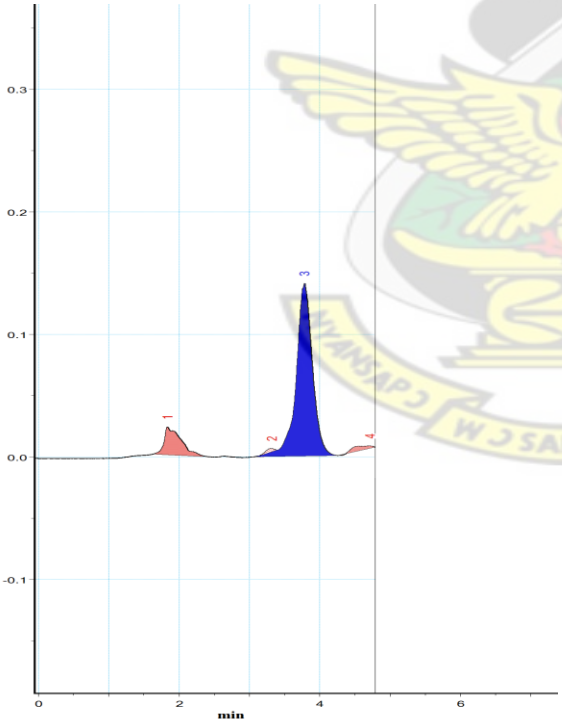


Figure 22: Chromatogram of DS extract (2 times dilution)

DATA FOR METHOD VALIDATION

Table 18: Values for wavelength variations

λ nm	Retention time R_T (min)	Area
340	1.44	3.67
	1.47	3.76
	1.45	3.68
342	1.46	3.67
	1.47	3.69
	1.44	3.59
344	1.46	3.69
	1.48	3.71
	1.45	3.69
346	1.46	3.83
	1.44	3.80
	1.48	3.75

Table 19: Values for flow rate variations

Flow rate(ml/min)	Retention time, R_T,(min)	Area
1.20	4.29	1.65
	4.39	1.67
	4.34	1.66
1.50	3.65	1.47
	3.59	1.44
	3.61	1.45
1.60	3.44	1.37
	3.38	1.38
	3.42	1.32

Table 20: Values for mobile phase variation

Composition	Retention time, R_T,(min)	Area
78/22	3.99	1.49
	3.98	1.48
	4.06	1.46
82/18	3.69	1.47
	3.70	1.46
	3.72	1.46

NMR SPECTRA

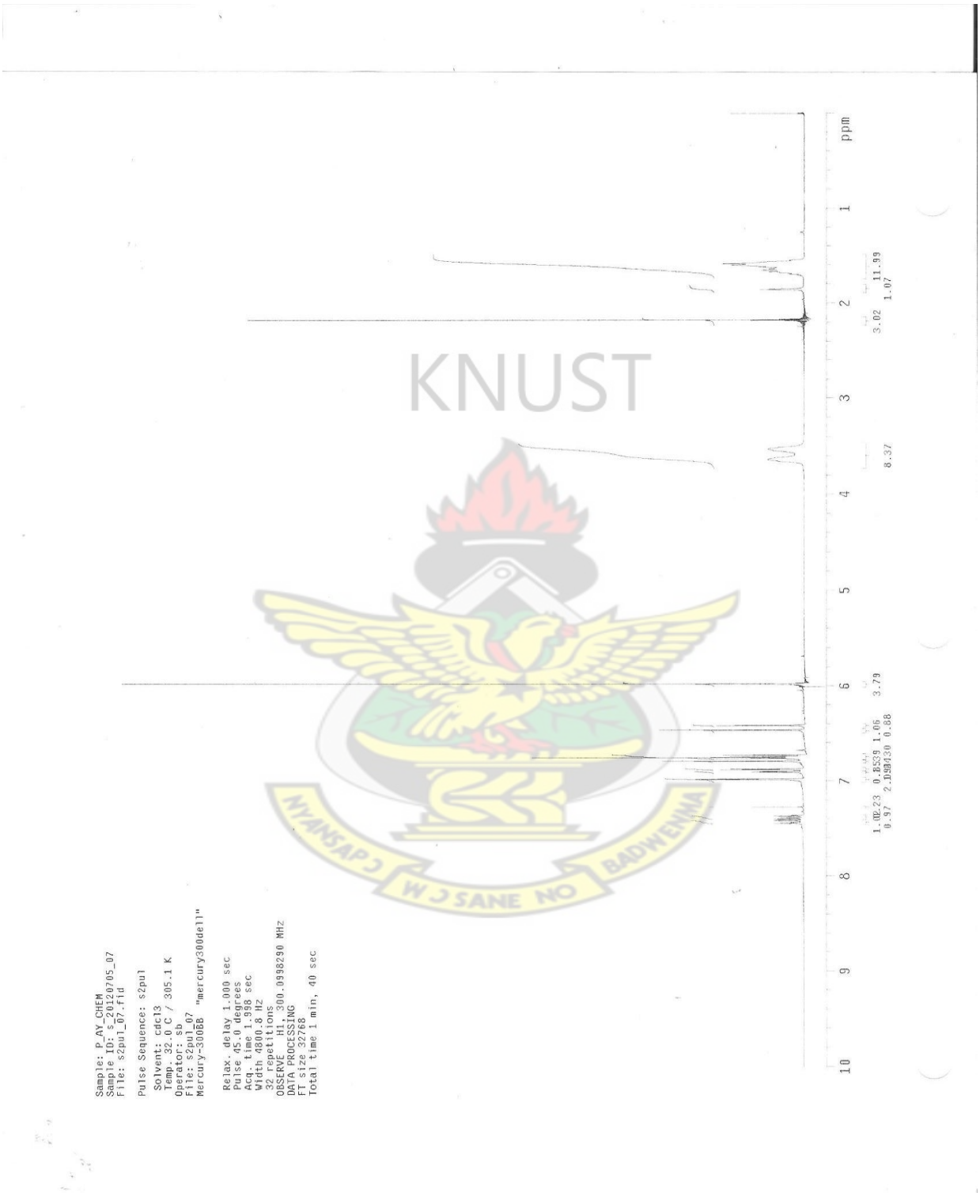


Figure 23: ^{1}H NMR of Isolated Piperine

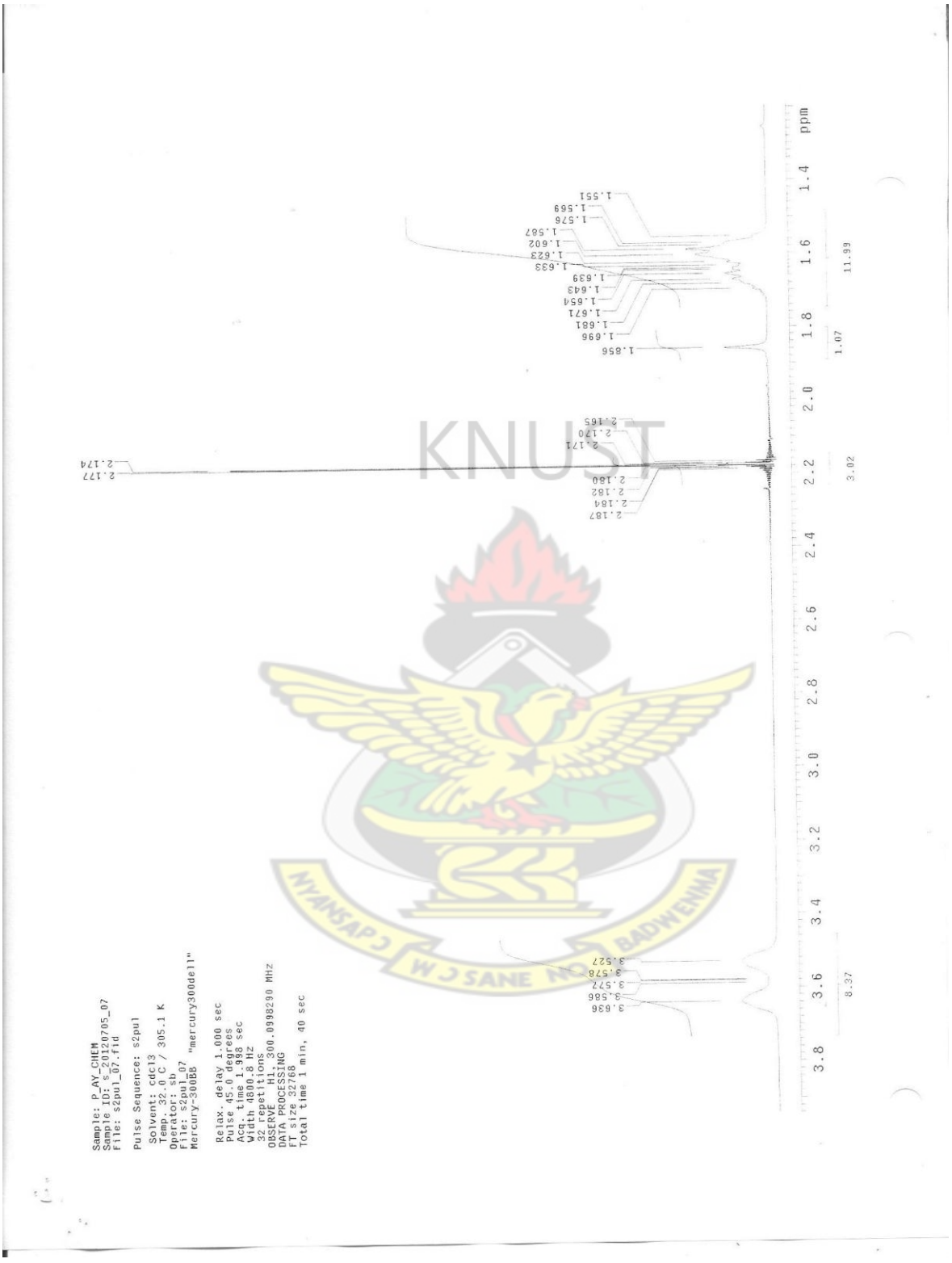


Figure 24: Expanded ^{1}H NMR of Isolated Piperine

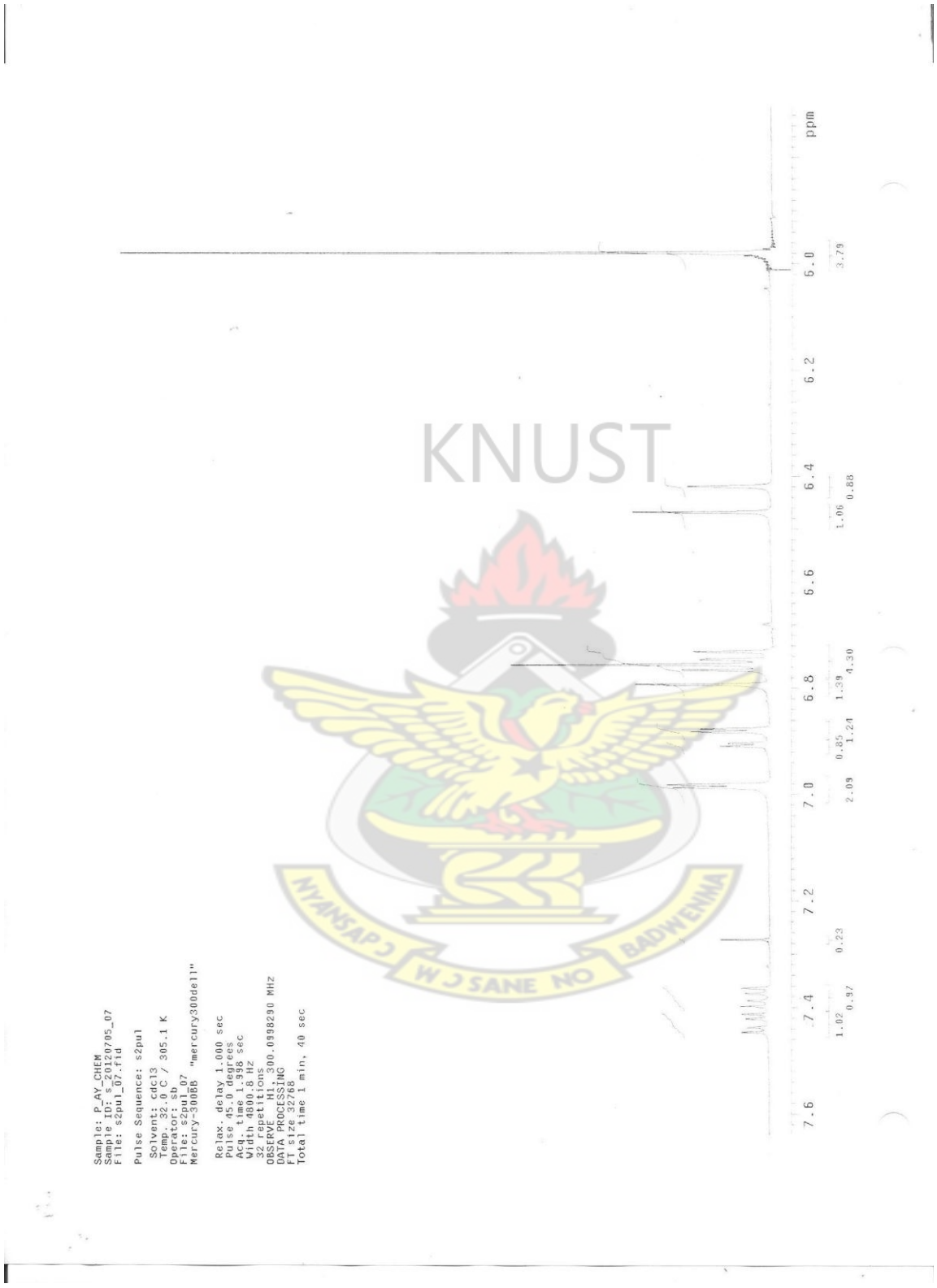
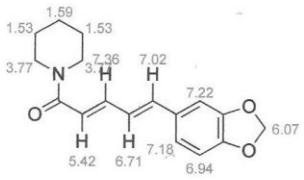
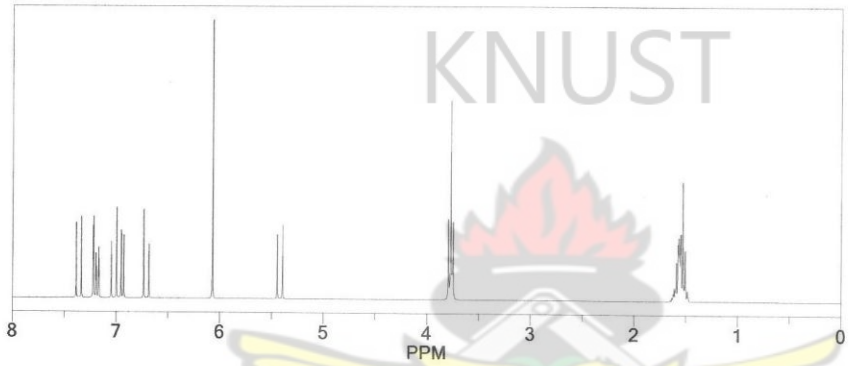


Figure 25: Expanded ^{1}H NMR of isolated Piperine

ChemNMR ^{1}H Estimation



Estimation quality is indicated by color: good, medium, rough



Protocol of the H-1 NMR Prediction:

Node	Shift	Base + Inc.	Comment (ppm rel. to TMS)
CH2	6.07	5.90	1,3-dioxole
		0.17	general corrections
CH2	3.77	2.74	piperidine
		0.60	1 -C(=O)R from N-CHx
		0.43	general corrections
CH2	3.77	2.74	piperidine
		0.60	1 -C(=O)R from N-CHx
		0.43	general corrections
CH	7.22	7.26	1-benzene
		-0.49	1 -O-C
		-0.11	1 -O-C
		0.04	1 -C=C
		0.52	general corrections
CH	6.94	7.26	1-benzene
		-0.11	1 -O-C
		-0.49	1 -O-C
		-0.05	1 -C=C
		0.33	general corrections
CH	7.18	7.26	1-benzene
		-0.44	1 -O-C
		-0.11	1 -O-C
		0.04	1 -C=C
		0.43	general corrections
CH2	1.53	1.50	piperidine
		0.03	general corrections
CH2	1.53	1.50	piperidine
		0.03	general corrections
CH2	1.59	1.50	piperidine
		0.09	general corrections

Figure 26: ^{1}H NMR Estimation of Piperine from ChemNMR

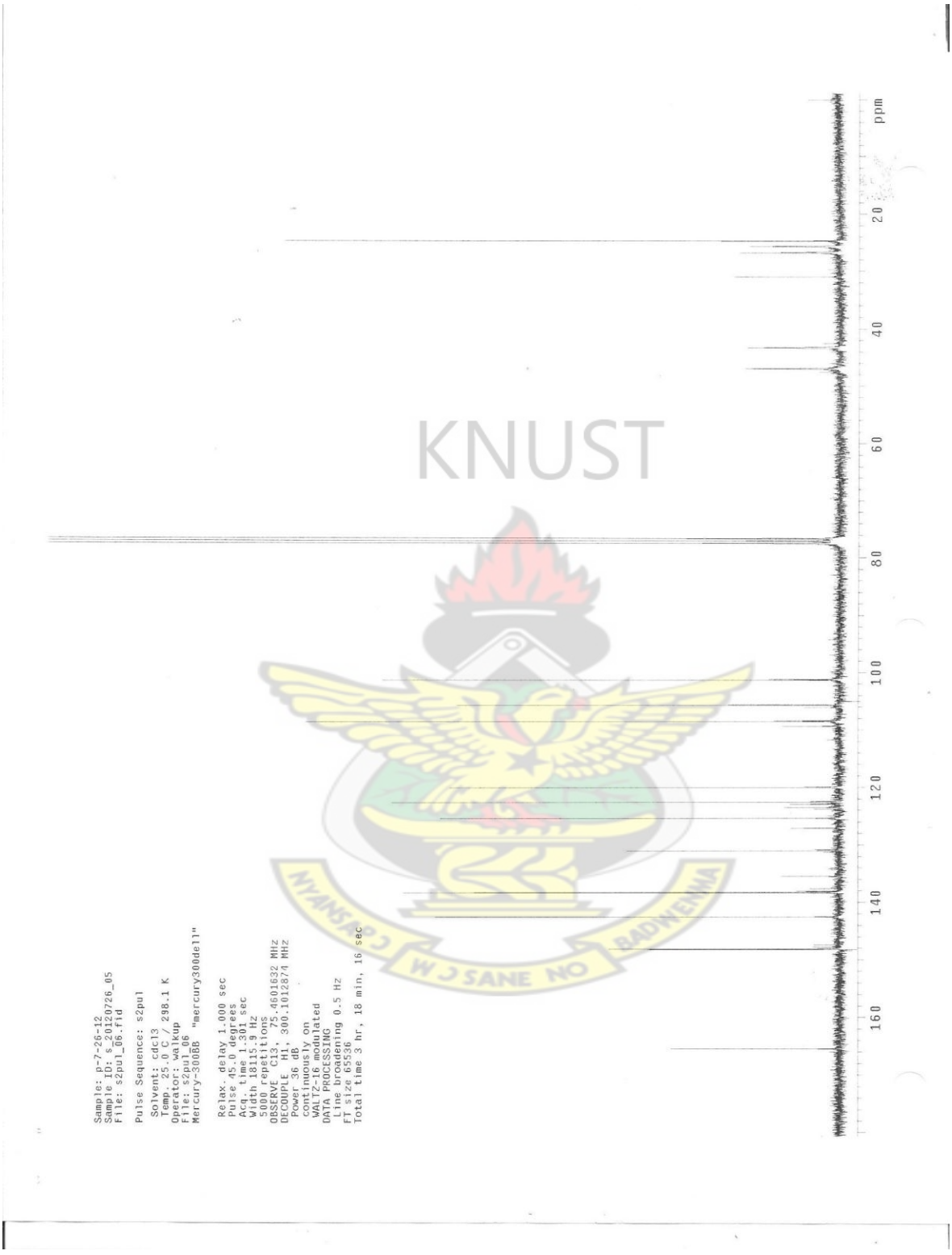


Figure 27: ^{13}C NMR of Isolated Piperine

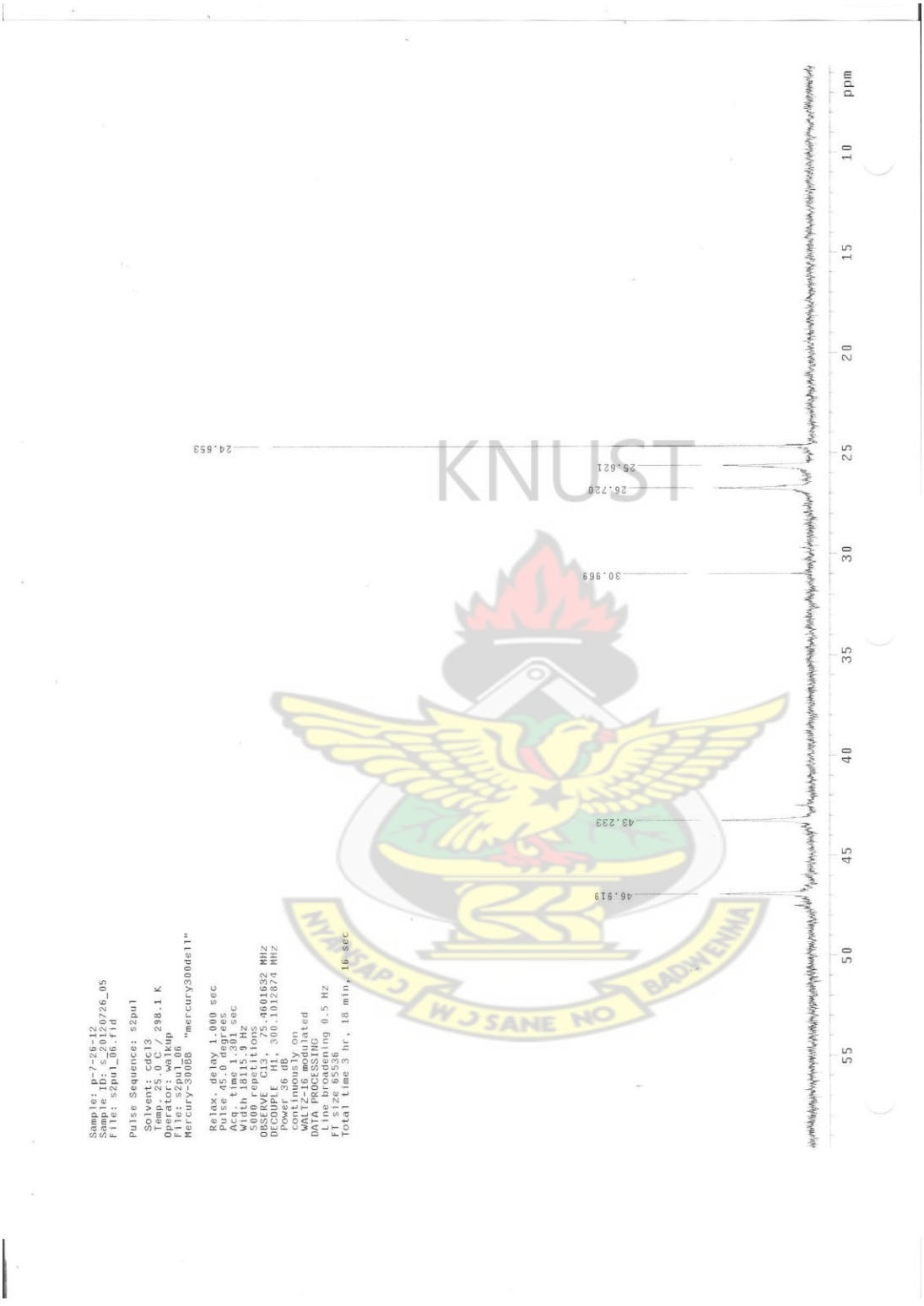


Figure 28: Expanded ^{13}C NMR Isolated Piperine

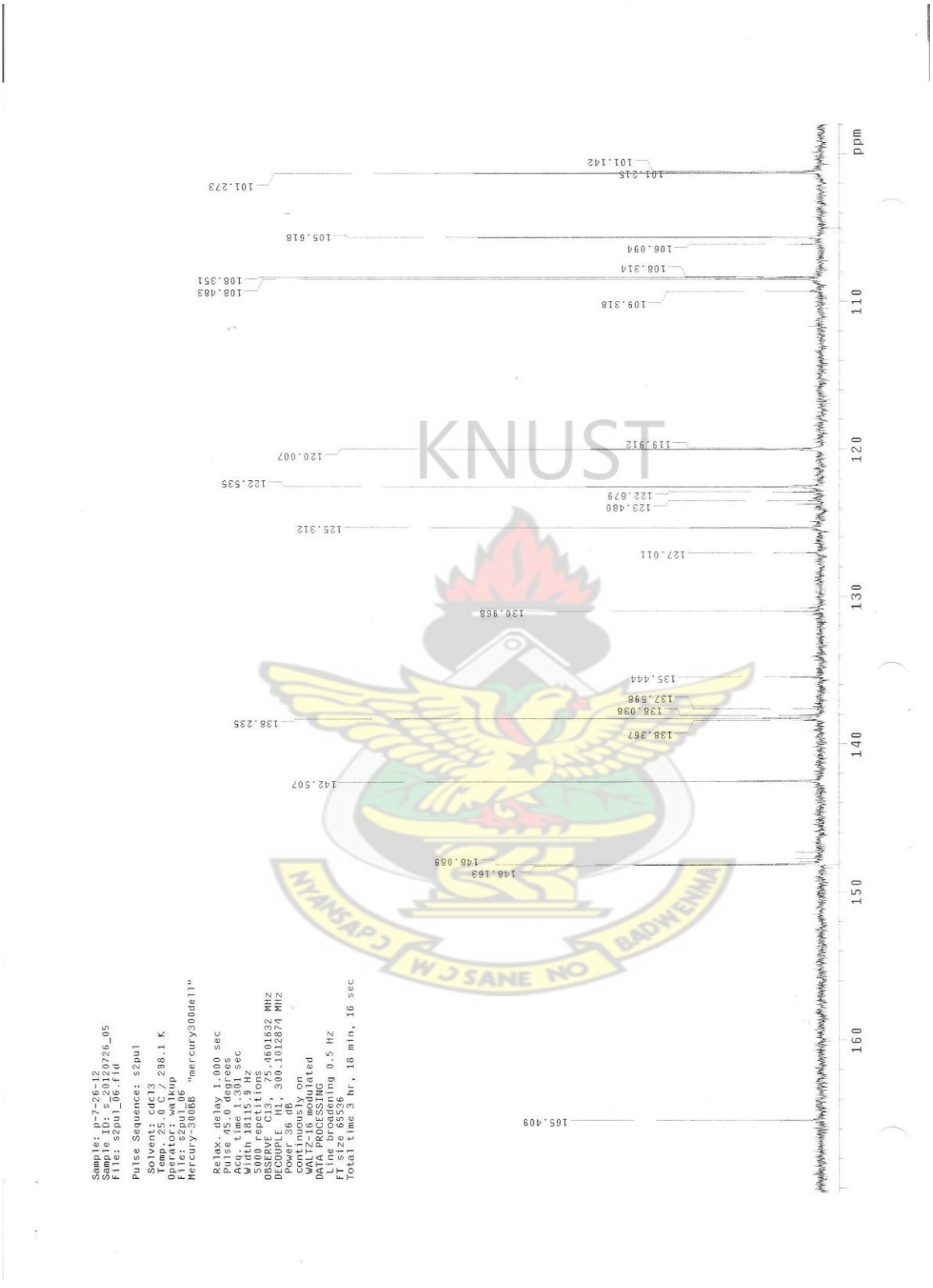


Figure 29: Expanded ^{13}C NMR of Isolated Piperine

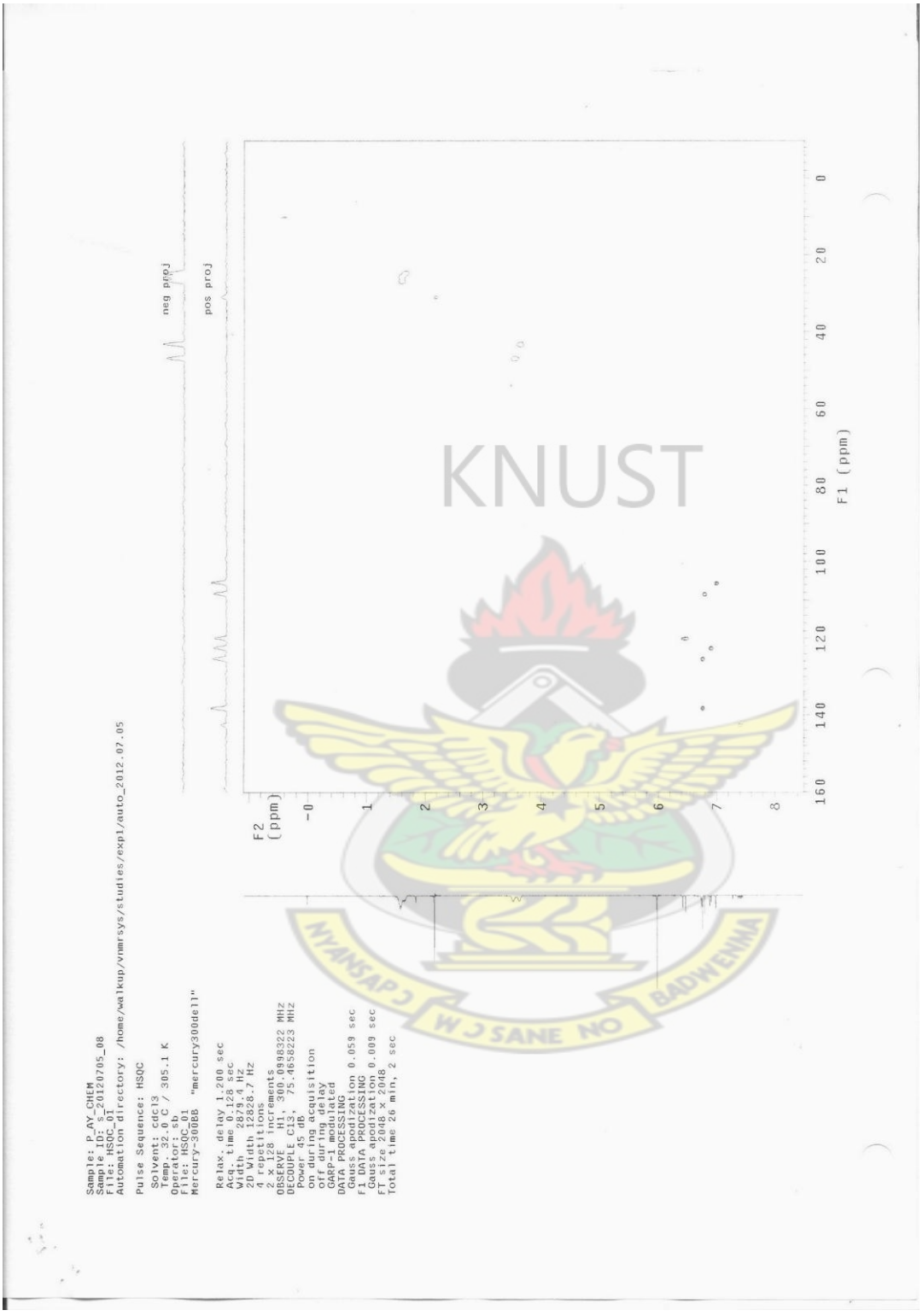


Figure 30: HSQC DEPT of Isolated Piperine

MASS SPECTROSCOPY DATA

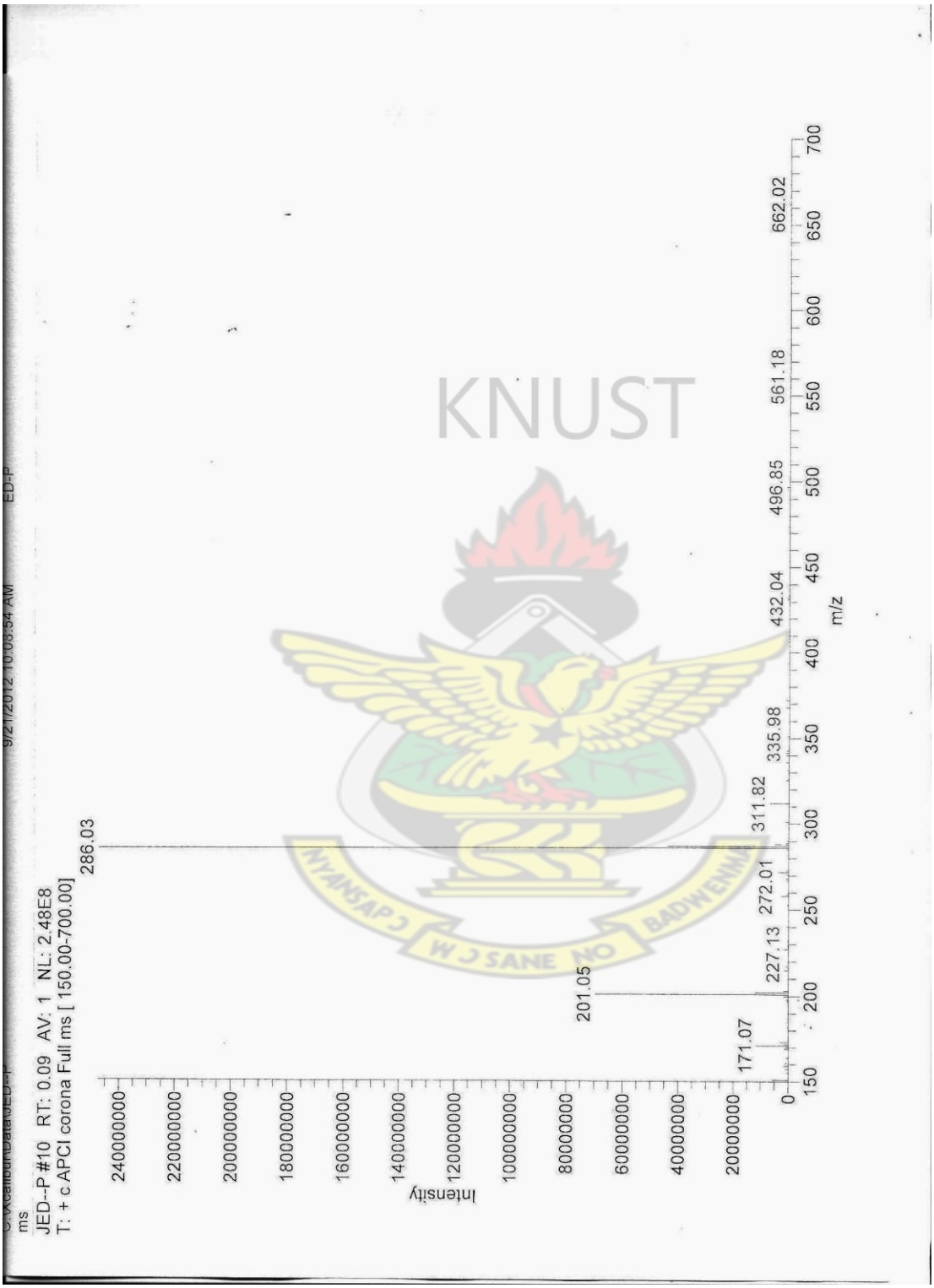


Figure 31: Mass Spectrum of isolated Piperine

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