KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES
DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

DEVELOPMENT OF A REVERSE-PHASE HPLC METHOD FOR THE QUANTIFICATION OF CRYPTOLEPINE IN THE DRY ROOTS OF CRYPTOLEPIS SANGUINOLENTA .

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN
PHARMACEUTICAL ANALYSIS AND QUALITY CONTROL

BY
EDWARD OFORI
JULY, 2010

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Faculty of Pharmacy and Pharmaceutical Sciences

BY:
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KUMASI
JULY, 2010

DECLARATION

I, Edward Ofori, wish to declare that this thesis report is my own unaided work. I further declare that, to the best of my knowledge, this work has not been previously submitted for any degree anywhere.

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DEDICATION

To my late dad and mum, Mr S.K Mensah and Rose Ameyaa. May their souls rest in peace

ACKNOWLEDGEMENTS

In all things honour and thanksgiving shall be given to the almighty God. I am very indebted to God for His goodness and mercies throughout my work.

I really appreciate the Gift of Life from God.

I am also grateful to KNUST and the Department of Pharmaceutical Chemistry for providing me with the necessary logistics and supportive environment to pursue the Msc. programme.

I wish to express my heart-felt gratitude to the college of pharmacy of the Florida A&M university for their help in the spectral analysis of the isolated cryptolepine.

Thanks go to my family especially my father for their prayers and support throughout the course of the project.

I also wish to appreciate the immense contributions of my supervisor Dr. R. K. Adosraku.

Lastly, to all my friends who made this course bearable, I say thank you all.

ABSTRACT

Cryptolepis sanguinolenta is a slender, thin-stemmed climbing shrub with orange-coloured juice in the cut stem which is distributed throughout the west coast of Africa. The aqueous root extract is a popular antimalarial in West African ethnomedicine. It is commonly known in Ghana as Ghana quinine or "Nibima" (Twi language).

The dry roots of *Cryptolepis sanguinolenta* were obtained from Center for Scientific Research into Plant Medicine (CSRPM), Akropong-Mampong. Roots were authenticated by comparing with herbarium sample at the Department of Pharmacognosy, KNUST.

The major alkaloid in the roots of *Cryptolepis sanguinolenta*, cryptolepine, was isolated by means of gravity column chromatography of the total alkaloidal extract followed by preparative TLC (Mobile phase: ethylacetate:methanol:ammonia (35%) 80:15:5) using a reference cryptolepine(CLP-R) sample. The authenticity of the isolated cryptolepine was confirmed by determining the UV, Infrared, ^{13}C and ^{1}H Nuclear Magnetic Resonance (NMR) and mass spectrometric data and the assignments were in agreement with those reported by by Grellier et al., (1996) and Dwuma-Badu, et al., (1978).

As a step towards efforts to standardize and to ensure quality in herbal preparations containing extracts of the roots of *Cryptolepis sanguinolenta*, a simple reverse-phase HPLC method with UV detection was developed and validated to quantify the levels of cryptolepine in the roots of the Cryptolepis plant. An isocratic elution of extractable cryptolepine in methanolic extract of dry roots of Cryptolepis was performed using **methanol: water (90:9)** modified with trifluoroacetic acid(**TFA**,98%) to a **pH of 2.4** as mobile phase set at flow rate of 1ml/min on a Hichrom Zorbax C_8 Column (5micron 15cm×4.6mm id) and cryptolepine monitored at **366nm**. The average retention time of cryptolepine was found to be **2.723±0.069 min(n=8).**

The method developed was found to be precise since the intra-day and inter-day precision were <2% RSD in the concentration range of **1.02µg/ml-10.2µg/ml**. Since the coefficient of correlation($\mathbf{r^2}$) of **0.9976** for the regression line of peak area(y) against %

concentration of cryptolepine(x) was greater than 0.995, the method was said to be linear in the concentration range. The method was also found to be robust since deliberate alteration of pH, flow rate and wavelength of detection in the range of 2.4±0.2units , 1±0.3ml/min and 366±2nm respectively did not affect the precision of the method. Hence, it could also be inferred that the method was accurate. The Limit of detection(LOD) and Limit of Quantitation(LOQ) of the method were found to be **0.574µg/ml** and **1.740µg/ml** respectively.

The developed method was used to determine the percentage content of extractable cryptolepine in the roots of the Cryptolepis plant and it was found to be **2.763±0.185% w/w (n=9).** This quantity of cryptolepine is the highest reported so far in the roots of the Cryptolepis plant.

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CHAPTER ONE

1 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Plants represent an extraordinary reservoir of novel molecules and there is currently a resurgence of interest in the vegetable kingdom as a provider of new lead compounds for introduction into therapeutic screening programs. Many drugs commonly used today in developing countries are of herbal origin and prescription drugs contain at least one active ingredient derived from plant material either obtained from plant extracts or synthesized to mimic a natural plant compound. Among the commonly used prescription drugs from plants sources include *Digitalis* which is used to treat heart conditions and is derived from the herb Foxglove, *Quinine*, a well-known anti-malaria drug that comes from the bark of the cinchona tree, *Taxol*, which is a potent drug used for cancer therapy and is derived from the yew tree among others.

For the majority of developing countries, the cost of imported drugs on a large scale is almost prohibitive. On the other hand, these countries have an enormous wealth of information on medicinal plants, which are not only cheap and abundant but also culturally acceptable. Furthermore, most developing countries have neither a well-organized pharmaceutical industry nor the manufacturing capacity to isolate large quantities of active principles from plants should they be discovered **(Farnsworth *et al.*, 1985)**

Over the past decade, interest in drugs derived from higher plants, especially the phytotherapeutic ones, has increased expressively. In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care. In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine (e.g. acupuncture). (**WHO Fact Sheet, 2008**). The uses of plants in improving health cannot be overemphasized. For instance Costa Rica has set

aside 25% of its land to preserve the forests, in part to provide plants and other materials for possible pharmaceutical and agricultural applications (**Akerele, 1993**).

Despite the belief that the majority of clinical drugs are synthetic in origin, it is interesting to note that 6 out of the top 20 pharmaceutical prescription drugs dispensed in 1996 were natural products and that over 50% of the top 20 drugs could be linked to natural product research. In recent years the development of sensitive biological testing systems, mainly by industry, has led to the procedure of high through-put screening(**Phillipson, 2001**).

Research into the bioactivity of medicinal plants resides in the fields of phytochemistry, pharmacognosy, and horticulture. In the area of phytochemistry, medicinal plants have been characterized for their possible bioactive compounds, which have been separated and subjected to detailed structural analysis using techniques in H^1NMR/C^{13}NMR, Infrared (IR) spectroscopy, Mass spectroscopy etc.

Recent advancement in drug discovery from plant sources employs high-throughput screening tests used for bioassay-guided fractionation leading to the isolation of active principles that may be developed into clinical agents either as the natural product or a synthetic modification or a synthesised analogue with enhanced clinical action or reduced adverse side effects. Medicinal plants are used to treat and manage a lot of diseases including infectious diseases such as malaria, typhoid fever, Candidiasis, etc and non-communicable diseases such as hypertension, diabetes mellitus, and cancer among others.

Malaria continues to be a major cause of mortality and morbidity especially throughout the developing world. In the last 25 years or so a number of significant advances have been made that have the potential to make a major contribution to the control of this disease. New treatments for malaria are urgently needed due to the increasing problem of drug-resistance in malaria parasite. The long-established use of *quinine* and the more recent introduction of *artemisinin* and its derivatives as highly effective antimalarials demonstrates that plant species are an important resource for the discovery of new antimalarial agents. Furthermore, many plant species continue to be used in traditional medicines for the treatment of malaria and many people depend on such remedies as they cannot afford and/or do not have access to effective antimalarial drugs **(Wright, 2005).**

New antimalarial drugs developed from the discovery and isolation of artemisinin from *Artemisia annua* L., a plant used in China for almost 2000 years and Quinine derivatives from the Cinchona plant have been of immense importance in the treatment of malaria. Other plants with potential antimalarial actions include *Cryptolepis sanguinolenta* (Lindl.) Schlechter (Asclepiadaceae) evidenced by**(Cimanga *et al.*, 1997)** and(**Wright, 2007)**, *Cassia occidentalis* L. (Caesalpiniaceae), Chinese herb *Dichroa febrifuga,* Alstonia species and *Azadirachta indica* A. Juss (Neem). Aslo, Extracts from three of the plants, *Pleiocarpa mutica*, *Cleistopholis patens* and *Uvaria chamae* were found to have significant antiplasmodial activity (**Addae-Kyereme *et al.*, 2001**). The newest antimalarial drug *atovaquone* (Malarone®) is synthetic 2-alkyl-3-hydroxynaphthoquinone compound, an analogue of lapachol (a prenylnaphthoquinone) from the *Tabebuia* species (Bignoniaceae). The discovery of this drug provided a novel lead for antimalarials that resulted in the development of atovaquone.

Most of the biological activities of medicinal plants results from some active secondary metabolites in isolation or acting synergistically *in vitro.* Research trend has been geared towards isolation of such phytochemicals. For instance, cryptolepine is the major alkaloid isolated from *Cryptolepis sanguinolenta* (Lindl.) Schlechter and is principally responsible for its antiplasmodial activity. The isolation and structure elucidation of gedunin, the antimalarial agent of *Azadirachta indica*, was reported by Khalid S.A. et. al (1989). Chinese herb, *Dichroa febrifuga*, contains γ-isomeride of dichroine as the principal antimalarial agent **(Chou *et al.*, 1948).**

Quantification of these secondary active metabolites remains very essential as far as the safety, efficacy and quality of phytopharmaceuticals are concerned. Because herbal medicines are recent cynosure of phytopharmaceuticals industries, effort would have to be made to identify and quantify the major active principles of crude medicinal plant products. This would help curb adulteration and sub-standard products on the market. This would also go a long way to aid in the standardization of phytopharmaceuticals. Safety of medicinal plants is crucial and the general idea that herbal drugs are very safe and free from side effects is false. Plants have hundreds of constituents and some are very

toxic such as the most cytotoxic anti-cancer plant-derived drugs, digitalis, the pyrrolizidine alkaloids, ephedrine, phorbol esters (**Calixto, 2000**). Hence if the quantities of these are known it would help to prevent toxicity in phytopharmaceuticals.

However, the content of these active principles are highly influenced by factor such as temperature, light exposure, water availability, nutrients, period and time of collection, method of collecting, drying, packing, storage and transportation of raw material, age and part of the plant collected.

Cryptolepis sanguinolenta (Lindl.) Schlechter is a potent antimalarial but research has shown that it is also potentially cytotoxic **(Ansah and Gooderham, 2002)** hence to quantify how much of cryptolepine, the major alkaloid, in the plant is very crucial as far as the quality and safety are concerned.

1.2 LITERATURE REVIEW

1.2.1 CRYPTOLEPIS SANGUINOLENTA

- Botanical Name: *Cryptolepis sanguinolenta* (Lindl.)Schlechter synonyms: *Pergularia sanguinolenta* or *Cryptolepis triangularis.*

- Common Names: Nibima (Ashanti language), delboi (Fulani language), gangamau (Hausa language), nombon (Dioule language), ouidoukoi (Bambara language), and kpokpo-yangolei (Mende language), Ghana quinine, and yellow dye root **(Luo *et al.*, 1998)**

- Phytochemical constituents: cryptolepine, quindoline, CSA-3(phenolic derivative of cryptolepine)(Dwuma-Badu *et al.*, 1978), cryptospirolepine (**Tackie *et al.*, 1993**) and cryptolepine isomers; neocryptolepine (cryptotackieine) and Isocryptolepine (**Pousset et al., 1995**), 11-isopropylcryptolepine(Hadden *et al.*, 1999) , hydroxycryptolepine, cryptoheptine, cryptoquindoline (**Paulo et al., 1995**)

- Family**:** *Cryptolepis sanguinolenta* is a member of the Asclepiadaceae family and Periplocoideae subfamily or member of the newly created family Periplocaceae**(Paulo *et al.*, 2000).**

- Habitat and Distribution- *Cryptolepis sanguinolenta* grows in the rainforest and deciduous forest belt. It is distributed throughout the west coast of Africa (**Iwu, 1993**). It is normally found in the forest and thickets but can also be cultivated.

- Description- *Cryptolepis sanguinolenta* is a slender, thin-stemmed climbing shrub with orange-coloured juice in the cut stem(**Paulo and Houghton, 2003)**. The leaves are glabrous, oblong-elliptic or ovalate, shortly acuminate apex, rounded, sometimes acutely cuneate base. The flowers are greenish-yellow, the fruit is a follicle, linear 17–31 cm long, and the seeds are 10–12 mm long with a tuft of silky hairs at the end. (**Simon, Koroch, Acquaye, et al**. **1999**). The roots are rather

tortuous and branched with little or no rootlets, with longitudinal ridges apparent in the dried samples. The root is distinctly yellow in colour and breaks with a short fracture exposing a smooth transverse surface which is yellow in colour (**Iwu 1993**).

Figure 1:1 Roots of *C. sanguinolenta*

Figure 1:2 Cut roots of *C. sanguinolenta*

1.2.2 PHYSIOLOGY, BIOCHEMISTRY OF THE ASCLEPIDEACEAE FAMILY

Not cyanogenic. Alkaloids, Proanthocyanidins(cyaniding) are present. Iridoids are not detected (?). Ellagic acid absent (*Periploca*). Arbutin are absent. Saponins/sapogenins absent. Aluminium accumulation not found. C_3 physiology recorded directly in *Cryptostegia* (**Watson and Dallwitz, 1992)**.

1.2.3 USES OF CRYPTOLEPIS SANGUINOLENTA

The aqueous root extract of Cryptolepis sanguinolenta is a popular antimalarial in West African ethnomedicine**(Ansah and Gooderham, 2002).** Also, the root of *Cryptolepis sanguinolenta* is used in traditional African medicine to treat a variety of diseases including jaundice, hepatitis, urinary tract infections, hypertension, inflammatory conditions and stomach ache. Extracts of the roots are also used as a tonic often taken daily for years without evidence of toxicity (**Appiah, 2009**; **Julian et al., 2009**). *C. sanguinolenta* is used as a decoction by healers in many West African countries to treat a

variety of conditions that could be associated with diabetes, such as vaginal *Candida albicans* infections **(Luo *et al.*, 1998).** Today, the roots of *C. sanguinolenta* are used in most antimalarial herbal preparations in Ghana.

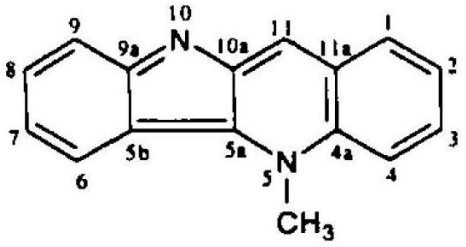
1.2.4 CRYPTOLEPINE

1.2.4.1 HISTORY;

Cryptolepine is a rare example of a natural product whose synthesis was reported prior to its isolation from nature. Cryptolepine was first synthesized in 1906 by Fichter and Boehringer for use as a possible dye while its isolation from *Cryptolepis triangularis* was first reported by Clinquart twenty-three years later in Congo **(Bierer *et al.*, 1998a)**. Clinquart isolated a purple compound (mp 160 °C), assumed to possess a primary NH_2 group, from the roots of *C. triangularis* collected in Kisantu (Belgian Congo). He named this compound cryptolepine and proposed the formula $C_{14}H_{17}N_2O_4$. Delvaux isolated a purple base (mp 193-194 °C) from the same plant material which he named cryptolepine and proposed the formula $C_{17}H_{16}N_2O$. The accepted formula for cryptolepine, $C_{16}H_{12}N_2$, was first isolated as a purple solid (mp 175-178 °C) by Gellert in 1951 from *C. sanguinolenta*. Cryptolepine has also been isolated as the major alkaloid from the Sri Lankan plant *Sida acuta* (family: Malvaceae), although there appears to be no locally reported use of this latter plant in the treatment of malaria (**Kirby, Warhurst et al. 1995)**.

1.2.4.2 CHEMISTRY

Cryptolepine is an indoloquinoline alkaloid. It is 5-methyl quinolo [2': 3': 3: 2] indole with the following molecular structure;



(I)

Figure 1:3 structure of Cryptolepine

Chemical formulae of Cryptolepine; $C_{16}H_{12}N_2$

Molecular weight; 232g/mol

Form salts of hydrochloride, hydrobromide and hydroiodide. The pKa of

Cryptolepine has been found to be 6.34 **(Boakye-Yiadom and Heman-Ackah, 1979)**

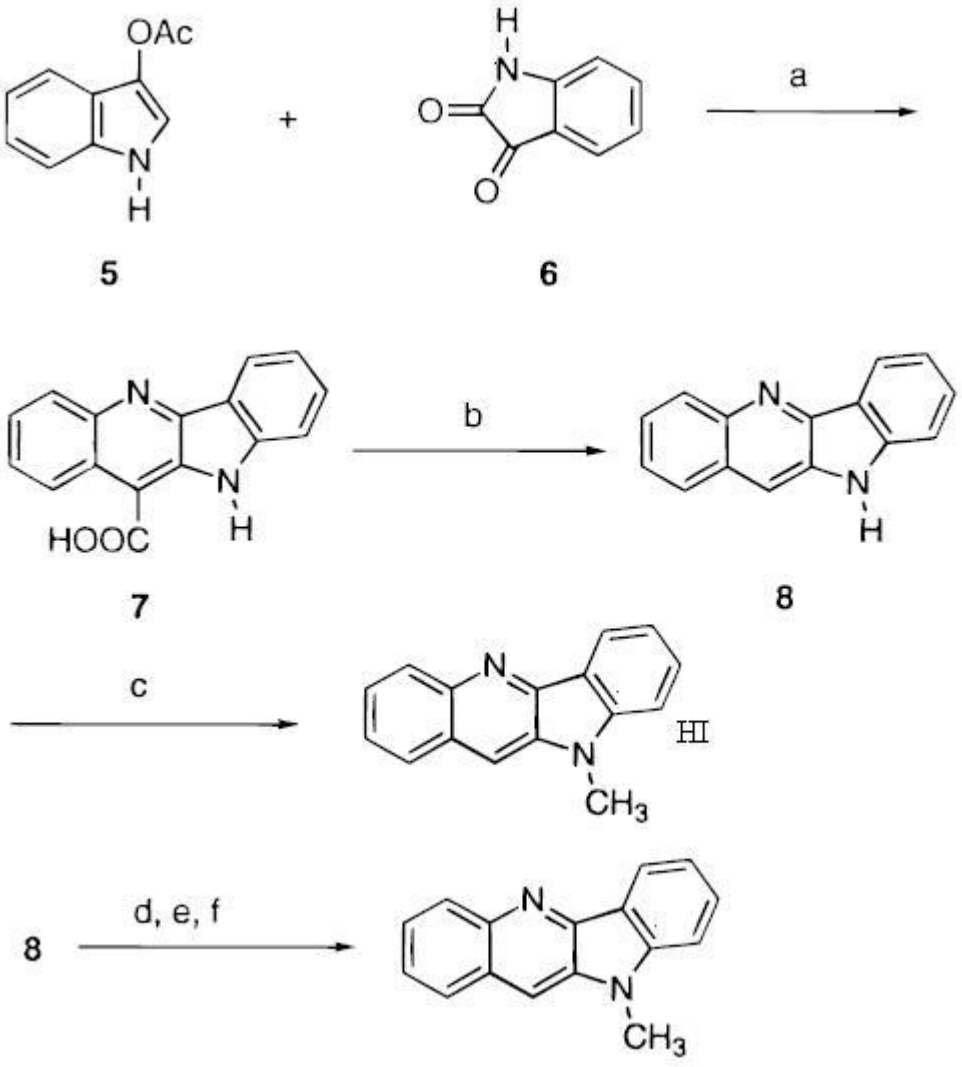
Physicochemical properties; odourless, violet alkaloid (the salts of which are yellow) crystal and has a melting point of 166-169°C. It is soluble in water, methanol, chloroform and ethylacetate.

1.2.4.3 SYNTHESIS OF CRYPTOLEPINE

The following method of synthesis of Cryptolepine was demonstrated by Bierer, Fort et al. This method utilizes the procedures of Holt and Petrow and Deguitis and Ezyarskaite with some modification (**Scheme 1**). 3-indolyl acetate (**5**) was reacted with isatin (**6**) under an inert atmosphere to yield quindoline-11-carboxylicacid (**7**). Decarboxylation in diphenyl ether gave quindoline (**8**). Alkylation of the N-5 nitrogen with methyl iodide was accomplished using the method of Fichter and Boehringer. This method required the use of a bomb but gave good yields of the hydroiodide salt of cryptolepine. A more convenient method amenable to larger scale reactions involved the use of methyl triflate as the alkylating agent, which was accomplished at room temperature and afforded near quantitative yields of the hydrotrifluoromethanesulfonate (hydrotriflate) salt of

cryptolepine. This procedure was the method of choice for preparing multigram quantities of cryptolepine hydrotriflate and, ultimately, cryptolepine. Standard basic extraction methods could be used to convert the hydroiodide and hydrotriflate salts of cryptolepine to its free base.**(Bierer *et al.*, 1998b)**

Scheme 1;



(a) KOH, H_2O, N_2 (b) Ph_2O, 255°C, 6h (c) CH_3I, MeOH, Bomb, 120°C (d) KOH, BaO (e) CH_3I (f) Na_2CO_3.

1.2.5 PHARMACOLOGICAL PROPERTIES OF CRYPTOLEPINE;

Cryptolepine (**I**) and its hydrochloride salt possess a number of reported bioactivities, including antimicrobial (**Boakye-Yiadom K., 1983)**, antibacterial **(Boakye-Yiadom and Heman-Ackah, 1979; Paulo *et al.*, 1994)**, anti-inflammatory, antihypertensive **(Bamgbose, S.O.A.; Noamesi, B. K. 1981; 1983)**,antipyretic, antimuscarinic**(Rauwald, H. W.; Kober, M, et al. 1992)** antithrombotic,**(Oyekan and Okapor, 1989)** noradrenergic receptor antagonistic (**Noamesi, B. K., Bamgbose, S. O. A.1980**) and vasodilative properties**(Oyekan, 1994)**. Cryptolepine also possesses significant antimalarial activity.

1.2.6 RESEARCH UPDATES ON CRYPTOLEPIS SANGUINOLENTA AND CRYPTOLEPINE

1.2.6.1 PROBING THE N-5 REGION OF THE INDOLOQUINOLINE ALKALOID, CRYPTOLEPINE FOR ANTICRYPTOCOCCAL ACTIVITY

N-5 Alkylated analogues of cryptolepine were synthesized and tested for anticryptococcal activity. Evidence provided in this study suggests that the active form of cryptolepine consists of the flat tetracyclic aromatic ring with the methyl group on the N-5 atom. It was also found that changes in the electronic density around the N-5 atom do not appear to affect activity. Steric hindrance of the N-5 substituents seems to decrease activity. Through systematic modification of the N-5 alkyl groups, o-phenylpentyl group was shown to possess the highest potency thus far**(Ablordeppey *et al.*, 1999).**

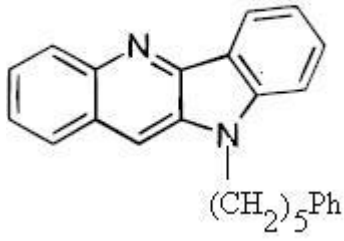


Figure 1:4 Structure of o-phenylpentyl derivative of cryptolepine

1.2.6.2 THE POPULAR HERBAL ANTIMALARIAL, EXTRACT OF CRYPTOLEPIS SANGUINOLENTA, IS POTENTLY CYTOTOXIC.

The aqueous root extract of Cryptolepis sanguinolenta (CSE) is a popular antimalarial in West African ethnomedicine. Cryptolepine (CLP), the major alkaloid of the plant, is a cytotoxic DNA intercalator that has promise as an anticancer agent. To date the aqueous root extract, the traditional antimalarial formulation, has not been evaluated for toxicity. In this study, we have examined the in vitro toxicity of CSE and CLP using V79 cells, a Chinese hamster lung fibroblast frequently used to assess genetic toxicity, and a number of organ-specific human cancer cell lines. CSE and CLP caused a dose- and time-dependent reduction in viability of the V79 cell line. Flow cytometric analysis of CSE- and CLP-treated (24 h) asynchronously growing V79 cells using propidium iodide (PI) staining revealed an accumulation of cells (up to 55%) in the sub-G1 phase of the cell cycle, indicative of cell death. The V79 cells and almost all the organ-specific human cancer cell lines exposed to CSE and CLP were profoundly growth inhibited, as measured in a clonogenicity assay. In a V79 cell mutation assay (hprt gene), CSE (5-50 μg/ml) only induced mutation at the highest dose employed (mutation frequency approximately 4 and 38 mutant clones per 10(6) cells for control and CSE, respectively), but CLP (0.5-5.0 microM) was not mutagenic. These results indicate that CSE and CLP are very cytotoxic and may be weak mammalian mutagens and/or clastogens. The poor genotoxicity of CSE and CLP coupled with their potent cytotoxic action support their anticancer potential.**(Ansah and Gooderham, 2002)**

1.2.6.3 ETHNOBOTANICAL-DIRECTED DISCOVERY OF THE ANTIHYPERGLYCEMIC PROPERTIES OF CRYPTOLEPINE: ITS ISOLATION FROM *CRYPTOLEPIS SANGUINOLENTA*, SYNTHESIS, AND IN VITRO AND IN VIVO ACTIVITIES.

Using an ethnobotanical approach in combination with in vivo-guided fractionation as a means for lead discovery, cryptolepine was isolated as an antihyperglycemic component of Cryptolepis sanguinolenta. Two syntheses of cryptolepine, including an unambiguous synthesis are reported. The hydroiodide, hydrochloride, and hydrotrifluoromethanesulfonate (hydrotriflate) salts of cryptolepine were synthesized,

and a comparison of their spectral properties and their in vitro activities in a 3T3-L1 glucose transport assay is made. Cryptolepine and its salt forms lower blood glucose in rodent models of type II diabetes. While a number of bioactivities have been reported for cryptolepine, this is the first report that cryptolepine possesses antihyperglycemic properties. (**Bierer *et al.*, 1998b**)

1.2.6.4 IN VITRO BIOLOGICAL ACTIVITIES OF ALKALOIDS FROM *CRYPTOLEPIS SANGUINOLENTA*

Aqueous and an 80% EtOH extract from the root bark of Cryptolepis sanguinolenta showed potent antibacterial, anticomplementary, and moderate antiviral activities, but no antifungal effect could be detected. Bioassay-guided fractionation of the 80% EtOH extract led to the isolation of three alkaloids: quindoline (1), hydroxycryptolepine (2), cryptolepine.HCl (3), and the corresponding base cryptolepine (4). All compounds strongly inhibited the growth of Gram-positive bacteria (MIC < or = 100 micrograms/ml) and showed a moderate (MIC = 125 or 250 micrograms/ml), a weak (MIC = 500 micrograms/ml), or no activity (MIC > 500 micrograms/ml) against selected Gram-negative bacteria. They also possessed a bactericidal effect depending on the bacterial strain. Compounds 1, 2 and 3 displayed a dose-dependent inhibitory effect on the classical pathway of the complement system while compounds 2 and 3 activated the alternative pathway, except for compound 1. Compound 3 was found to possess an antiherpetic activity. Compounds 1 and 4 showed no antiviral effect, but were quite cytotoxic in the antiviral test system down to a concentration of 1 microgram/ml.**(Cimanga *et al.*, 1996)**

1.2.6.5 CRYPTOLEPINE HYDROCHLORIDE: A POTENT ANTIMYCOBACTERIAL ALKALOID DERIVED FROM *CRYPTOLEPIS SANGUINOLENTA*

The activity of cryptolepine hydrochloride, a salt of the main indoloquinoline alkaloid from the West African medicinal plant *Cryptolepis sanguinolenta*, was assessed against the fast growing mycobacterial species *Mycobacterium fortuitum*, which has recently been shown to be of use in the evaluation of antitubercular drugs. The low minimum

inhibitory concentration (MIC) of this compound (16 microg/mL) prompted further evaluation against other fast growing mycobacteria namely, M. *phlei*, M. *aurum*, M. *smegmatis*, M. *bovis* BCG and M. *abcessus* and the MICs ranged over 2-32 microg/mL for these species. The strong activity of this agent, the need for new antibiotics with activity against Mycobacterium tuberculosis, coupled with the ethnobotanical use of C. *sanguinolenta* extracts to treat infections, highlight the potential of the cryptolepine template for development of antimycobacterial agents.**(Gibbons *et al.*, 2003)**

1.2.6.6 A SIMPLE SELECTIVE AND SENSITIVE HPLC METHOD FOR THE DETERMINATION OF CRYPTOLEPINE IN HUMAN PLASMA.

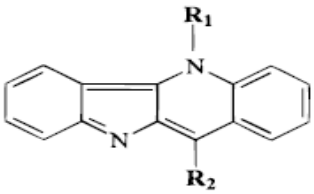
As a first step towards a comprehensive pharmacokinetic study of cryptolepine, a sensitive and robust high performance liquid chromatographic method with photodiode array detection for the identification and determination of the drug in human plasma has been developed.

A non-linear gradient elution of analyte and the internal standard (quindoline hydrochloride) was performed using a reverse-phase Zorbax SB C18 5µm (4.6x250mm) column, Waters 2695 Alliance system with a PDA detector (PDA996) and a column temperature of 35°C. The mobile phase consisted of acetonitrile and 10mM monobasic potassium phosphate buffer at pH 3. Plasma samples were purified by liquid- liquid extraction using ethyl acetate. The eluents were monitored at 366 nm.

Average retention times (Tr) for cryptolepine and the internal standard were 3.1 10.3 minutes respectively. A plot of peak area ratio (of cryptolepine:IS) Versus increasing concentrations of cryptolepine yielded linear calibration curves with correlation coefficients (r2) of > 0.998. Limits of detection (LOD) and quantitation (LOQ) were 3.2 and 9.5ngml-1 respectively. The intra and inter day precision were < 10 % for the concentration range (10-1000 ngml-1). The method proposed in this work for the analysis of cryptolepine in plasma was sensitive, selective and reproducible. (**Kumar, Ononiwu et al., 2004**)

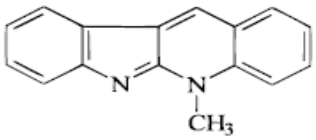
Other alkaloids in C. sanguinolenta

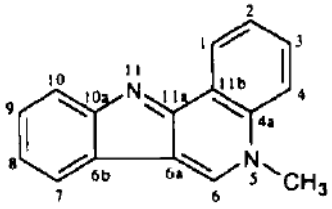
Quindoline (**4**), cryptospirolepine, neocryptolepine (cryptotackieine) (**5**), Isocryptolepine (**6**), hydroxycryptolepine (**3**), cryptoheptine, cryptoquindoline.



	R_1	R_2
1	CH_3	H
2[a]	CH_3	H
3	CH_3	OH
4	H	H

[a]Hydrochloride





(**6**)

Figure 1:5 Structures of other alkaloids isolated from C. *sanguinolenta*

1.3 JUSTIFICATION

C. sanguinolenta forms part of most herbal antimalarial preparation in Ghana. Examples on the Ghanaian market are; Masada herbal mixture, Nibima herbal antimalarial. The major alkaloid, Cryptolepine, is principally responsible for its antimalarial activity and it has potential cytotoxic activity. It has been reported that Cryptolepine occurs at a yield of 0.54%w/w in the roots, 0.48%w/w in the stem and 1.03%w/w in the leaves. **(Iwu, 1993).** However, no attempt has been made to develop a method to quantify the levels in the root which is the main part of the plant used in such preparations. This project tends to develop a RP-HPLC-UV method to quantify the amount of cryptolepine in the roots. This would also go a long way to aid in the standardization of *C. sanguinolenta* decoctions.

Also, according to survey conducted by Centre for Scientific Research into Plant Medicine (CSRPM), Mampong, the Cryptolepis plant now faces extinction in the wild due to increasing demand by manufactures of herbal antimalarials in Ghana and deforestation.(***eyes on malaria online*** **;Ammren Magazine,**). It has necessitated the need to cultivate it on large scale. Because of the increased demand there is the tendency of producers to adulterate the crude material/extracts of the roots. An HPLC method developed for the purpose of quantifying the amount of cryptolepine in the root as a marker of quality would be a step in the right direction for quality assurance of the crude drug.

OBJECTIVES;

1. To isolate and characterize cryptolepine from the dry roots of *C. sanguinolenta* for use as a reference.
2. To develop an HPLC method that separates cryptolepine from other components in the roots of the Cryptolepis plant.
3. To validate the developed HPLC method and use it to determine the percentage by weight of cryptolepine in the dry roots of *C. sanguinolenta.*

1.4 THEORY OF EXPERIMENTAL WORK

1.4.1 ISOLATION AND PURIFICATION TECHNIQUES

The physical state at ambient temperature of the crude mixture highly influences the approach towards purification of the components in the mixture. Thus whether *one-phase* (either *solid* or *liquid)* or a *two-phase (solid/liquid* or *liquid/liquid)* system.

In the case of the *one-phase solid system* 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 respectively. Removal of solvent after drying leads to the recovery of the purified solid for recrystallisation from a suitable solvent.

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 crystallized out from the reaction solvent; the mixture therefore only requires cooling and filtering 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 crystallized 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

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**).

1.4.1.1 SOLVENT EXTRACTION

The term solvent extraction (or liquid-liquid distribution as recommended by IUPAC) refers to the distribution of a solute between two immiscible liquid phases in contact with each other. The solute A, which initially is dissolved in only one of the two liquids, eventually distributes between the two phases. When this distribution reaches equilibrium, the solute is at concentration $[A]_{aq}$ in the aqueous layer and at concentration $[A]_{org}$. Distribution ratio(D) is defined as the ratio of " the total analytical concentration of the substance in the organic phase to its analytical concentration in the aqueous phase, usually measured at equilibrium" irrespective of whether the organic phase is the lighter or heavier. D is also called the distribution coefficient or distribution factor.

$D=[A]_{org}/ [A]_{aq}$

For practical purposes, as in industrial applications, it is often more popular to use the percentage extraction %E (sometimes named the extraction factor), which is given by

$\%E= 100D/ (1+D)$.

Solvent extraction is used in numerous chemical industries to produce pure chemical compounds ranging from pharmaceuticals and biomedicals to heavy organics and metals in analytical chemistry and in environmental waste purification (**Rydberg et al Eds., 2004**).

Batch-extraction processes; extraction is the process of removing a compound of interest from a solution or solid mixture. The separation of a neutral organic compound (or compounds) from a solution or suspension (as either a solid or liquid) in an aqueous medium involves repeated shaking of the solution with an organic solvent in which the compound of interest is soluble and is immiscible (or nearly immiscible) with water. The batch process, involves macerating the tissues with the appropriate solvent in a Waring Blender, soaking for a short time, filtering in a suitable size of Buchner funnel and then returning the residue to fresh solvent for further extraction.

Organic solvent extraction is one process for separating the desired active constituent from plant material. Fresh or dried plants are used for the extraction. The plants are first ground and thoroughly mixed with a solvent such as hexane, benzene, or toluene inside a tank. The solvent choice depends on several factors including the characteristics of the constituents being extracted, cost and environmental issues. Once the solvent dissolves the desired substance of the plant, it is called the "The miscella". The miscella is then separated from the plant material. The major techniques involved in solvent extraction include maceration, percolation, and countercurrent extraction.

Maceration involves soaking and agitating the solvent and plant materials together. The solvent is then drained off. Remaining miscella is removed from the plant material through pressing or centrifuging.

With percolation, the plant material is moistened with the solvent and allowed to swell before being placed in one of a series of percolation chambers. The materials are repeatedly rinsed with solvent until all the active ingredients have been removed. New solvent is used on plant material that is almost completely exhausted, and then re-used on subsequently less exhausted batches. This method is more effective in obtaining active ingredients than the maceration technique. (**Raaman, 2006**)

The solvent selected will depend upon the solubility of the substance to be extracted in that solvent and the ease with which the solvent can be separated from the solute. This is also known as solvent-solvent or liquid-liquid extraction. Solvent-solvent extraction is performed using the separatory funnel.

Ideal solvents for the extraction should meet most of the criteria below;

- High capacity for the species being extracted.
- Selectivity, dissolving to a large extent one of the key components without dissolving other components to any large extent.
- Low mutual solubility with water.
- Easy regeneration.
- Suitable physical properties, such as density, viscosity, and surface tension.
- Relatively inexpensive, nontoxic and noncorrosive.

The solvents commonly used include diethyl ether or di-isopropyl ether, toluene, dichloromethane and light petroleum. **(Rydberg et al Eds., 2004**)

1.4.1.2 FILTRATION TECHNIQUES

Filtration is a technique used for two main purposes. The first is to remove solid impurities from a liquid and the second is to collect a desired solid from the solution from which it was precipitated or crystallized. Two general methods include gravity filtration and vacuum (or suction) filtration.

Gravity filtration; the most familiar filtration technique is probably filtration of a solution through a paper filter held in a funnel, allowing gravity to draw the liquid through the paper. Because even a small piece of filter paper will absorb a significant volume of liquid, this technique is useful only when the volume of mixture to be filtered is greater than 10ml. For many macroscale and microscale procedures, a more suitable technique, which makes use of gravity, is to use Pasteur (or disposable) pipette with a cotton or glass wool plug.

The use of filter cones in gravity filtration technique is the most useful when the solid material being filtered from a mixture is o be collected and used later. The filter cone, because of its smooth sides, can easily be scraped free of collected solids. Because of the many folds, fluted filter paper cannot be scraped easily.

Use of Fluted filters is also useful when filtering a relatively large amount of liquid. Because a fluted filter is used when the desired material is expected to remain in solution, this filter is used to remove undesired material, such as dirt particles, decolorizing charcoal, and undissolved impure crystals. A fluted filter is often used to filter a hot solution saturated with a solute during a crystallization procedure. (**Pavia, 2005**)

Vacuum filtration; when substantial quantities of a solid are to be filtered from suspension in a liquid, a Buchner funnel of convenient size is employed. The use of suction renders rapid filtration possible and also results in a more complete removal of the mother-liquor than filtration under atmospheric pressure. However initial gentle suction often leads to more effective filtration than powerful suction.

Modification of the general technique of isolation by filtration may be necessary in the light of the chemical nature of the reaction mixture, of the particle size of the solid, or of the ratio of the amount of solid to liquid material to be filtered. For example, strongly alkaline or strongly acidic reaction mixtures weaken cellulose filter papers. Acid-hardened grades which are more chemically resistant are commercially available (e.g. Whatman filter papers) but for maximum resistance to chemical attack, glass-fibre paper (e.g. Whatman) or a glass funnel fitted with a fixed sintered glass plate may be used.

The filtration of very finely divided suspended material is often very tedious as a result of the filter paper pores becoming clogged. In such a case the addition of a suitable filter aid (e.g. a high grade diatomaceous earth such as Celite 545, or Whatman filter aids) to the suspension overcomes the problem; alternatively the suspension may be filtered through a bed of filter aid prepared by pouring a slurry of it in a suitable solvent into the filter funnel fitted with the required size of filter paper. The initial application of gentle suction in the filtration is in this case vital. A glass-fibre filter paper, supported on a conventional filter paper in a Buchner or Hirsch funnel, is useful for the rapid removal of finely divided solid impurities from a solution. The selection of a funnel appropriate to the amount of solid rather than the total volume of liquor to be filtered is important. **(Furniss et al., 1989)**.

1.4.1.1 CHROMATOGRAPHIC METHODS FOR SEPARATION OF PHYTOCHEMICALS

The separation and purification of plant constituents is mainly carried out using one or other or a combination of chromatographic techniques; Thin Layer Chromatography(TLC), Column Chromatography(CC), High Performance Thin layer Chromatography(HPTLC), Optimum Performance Laminar Chromatography(OPLC), High Performance Liquid Chromatography(HPLC),Supercritical-fluid Chromatography(Gas Liquid Chromatography(GLC) and Electrophoresis.

Chromatography is a technique used to separate molecules based on their size, shape, or charge. It is employed to analyse and isolate a variety of macromolecules. During chromatography, molecules in some kind of buffer or solvent move through a solid phase that acts as sieving material. As the molecules move through the molecular sieve, they are

separated. Three well-known types of chromatography include paper chromatography, thin-layer chromatography and column chromatography.

Few methods of chemical analysis are truly specific to a particular analyte. It is often found that the analyte of interest must be separated from the myriad of individual compounds that may be present in a sample. Separation on paper (paper chromatography) or thin layers (thin layer chromatography) are the earliest of chromatographic techniques to perform and require simple apparatus. They readily provide qualitative information and with careful attention to detail, it is often possible to obtain qualitative data. Chromatography is one method used to ascertain the amino acid composition of a protein. A sample is digested with proteases. The resulting amino acid mixture is run on a chromatograph. The amino acids travel up the paper to different heights. The distance they travel as compared to the solvent is measured and reported as R_f value. Each amino acid has a characteristic R_f for a given solvent. By determining the R_f values for the sample, we can learn which amino acids are found in a given protein.

Modes of separation

Chromatographic separations are a result of the interactions between the analyte and the two phases. In general, there are five types of interactions: Adsorption, Partition, Ion-Exchange, Affinity and Size-Exclusion.

Adsorption Chromatography

This is also known as displacement, liquid/solid chromatography. It is based on interactions between the solute and fixed active sites on the stationary phases. Stationary phases are normally a solid adsorbent packed in a column, spread on a plate or on a porous paper. The mobile phase is usually a liquid solvent. The active sites of the stationary phase interact with the functional groups of the compounds to be separated by non-covalent bonds, non-polar interactions, Van der Waals forces and hydrophobic interactions. The less tightly bound compounds will be eluted out by the mobile phases at earlier time and

classes of compounds can be separated. Eg: separation of alcohols from hydrocarbons using silica gel. Silanol groups on the gel interact with the polar functional groups on the alcohols.

Partition chromatography

During a partition separation, solute particles interact between two non miscible liquid phases according to their relative solubility. This process is also referred to as liquid/liquid chromatography. The stationary phase is a film of liquid that is strongly adsorbed to an inert support and the mobile phase is a different liquid with different polarity. In general, i.e. normal phase chromatography, the stationary liquid is polar and the mobile phase is non polar. Eg: separation of polar compounds such as amino acids, carbohydrates and water-soluble plant pigments. In reverse-phase chromatography, the stationary liquid is non-polar and the mobile phase is polar. Eg: separation of non-polar compounds such as lipids and fat-soluble pigments. Partition chromatography is a very useful technique because it can resolve minute differences in the solubility of the solutes. It is well suited for separating homologues and isomers.

Ion-Exchange chromatography

This process allows the separation of ions and polar molecules based on the electrical properties of the molecules. The stationary phase is a resin or gel matrix which contains covalently bound positive or negative functional groups. The Cation Exchange column carries negatively charged groups. Anion exchange carries positively charged groups. The mobile phase is a buffered aqueous solution which carries a counter-ion whose charge is opposite and in equilibrium with the total charge of the resin. Once the charged analytes are attached to the exchange groups in the column, they must be eluted out using a buffer with a higher ionic strength or a different pH in order to weaken the electrostatic interactions between the analytes and the exchangers.

Affinity Chromatography

Seperations are based on specific interactions between interacting pairs of substances such as macromolecules and its substrate, cofactor, allosteric effector or inhibitor. The stationary phase is a gel matrix to which a specific ligand is attached. The mobile phase is a buffered solution. During an affinity chromatography, a mixture of substances is applied to the column. Substances that have no affinity for the ligand are washed through with the buffer used and the desired compound is bound to the ligand. A buffer with a different pH or an increased ionic strength is used to elute the desired compound out. The choice of ligand is important in affinity chromatography. The ligand must interact specifically and reversibly with the molecule of interest. In addition, it must be suitable for coupling to a matrix.

Size exclusion chromatography

This is also known as gel filtration, gel permeation chromatography and molecular sieve chromatography. In this process, no chemical attraction or interaction occurs between the solutes and the stationary phase. The molecules are separated according to their size. Higher molecular weight molecules ranging between 2000 to 25,000,000daltons can be separated. The stationary phase made up inert material such as gel or a porous glass or porous silica beads. The mobile phase is water or an aqueous solution that solely serves as a carrier for the analyte. The degree of retention is dependent on the size of the solvated solute molecule relative to the size of the pore. Smaller molecules will permeate the smaller pores, intermediate sized molecules will permeate some pores and larger molecules are eluted at earlier time. By knowing the relative elution time, molecular weights of unknown compounds can be estimated.(**Raaman, 2006**).

1.4.1.1.1 Thin-layer chromatography

Thin-layer chromatography (TLC) was developed because of a specific need for a rapid method which would separate small amounts of compounds. Thin-layer chromatography is one of the most popular and widely used separation techniques. Thin-layer chromatography is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents. TLC is a quick, inexpensive microscale technique that can be used to determine the number of components in a mixture, verify a substance's identity, monitor the progress of a reaction, determine appropriate conditions for column chromatography, and analyze the fractions obtained from column chromatography.

Although alumina and silica are the most common stationary phases used for TLC, there are many different types including the following (arranged in increasing order of polarity); Reverse Phase (hydrocarbon-coated silica e.g. C-18), Paper, Cellulose, Starch, Calcium sulfate, Silica (silica gel), Florisil (Magnesium silicate) Magnesium oxide, Alumina (aluminum oxide; acidic, basic or neutral),Activated carbon (charcoal; Norit pellets). **(Thin-layer chromatography)**

Thin layer chromatography is a separation method in which uniform thin layer of sorbent or selected media are used as carrier medium. The sorbent is applied to a backing as a coating to obtain a stable layer of suitable size. The most common support is a glass plate, but other supports such as plastic sheets and aluminium foil are also used. The four sorbents mostly commonly used are silica gel, alumina, kieselguhr (diatomaceous earth), and cellulose. Silica gel (silica acid) is the most popular layer material. It is slightly acidic in nature. In order to hold the silica gel firmly on the support, a binding agent such as plaster of Paris (calcium sulphate hemihydrates) is commonly used.

Two ultraviolet (UV) indicators, which aid in the location of separated substances, are also incorporated, either singly or together, in silica gel or other layer materials. Zinc silicate fluoresces when exposed to ultraviolet light of 254nm wavelength, so that substances adsorbing this wavelength will contrast sharply by appearing dark through quenching of the greenish-yellow fluorescing background. The sodium salts of hydroxypurene-sulfonic

acids fluoresce at 366nm and provide a contrasting background for substances that absorb at this frequency.

Alumina (aluminium oxide) is chemically basic and for a given layer of thickness it will not separate quantities of material as large as can be separated on silica gel. Alumina is more chemically reactive than silica, and care must be exercised with some compounds and compound classes to avoid decomposition or rearrangement of these substances during sample application, storage before development or development. Diatomaceous earth (kieslghur) is a chemically neutral sorbent that does not separate or resolve as well as either alumina or silica gel. Precoated TLC plates are commercially available.

Compared to paper chromatography, the special advantages of thin layer chromatography are the versatility, speed of separation and sensitivity. TLC is used for the separation of substances in a wide variety of fields. Amino acids from protein, hallucinogenic alkaloids from plant, steroids from the urine of a newborn infant, morphine in the blood of an overdose victim and pesticides from soil may be separated by TLC, with sensitivities of 1μg or less(**Touchstone, 1992**).

Diagnostic/ Qualitative TLC

The objective of diagnostic or qualitative TLC is to determine the number of components in a system and if possible to learn what they are without isolating them. This technique can be used to monitor the components of eluents in column chromatography and subsequently combining fractions with similar components.

Quantitative or Analytical TLC

This deals with how much of each component present in the sample mixture. The thickness of the adsorbent layer is typically around 0.1 – 0.25 mm for analytical purposes. (**Raaman, 2006**). In order to use TLC as a quantitative method of analysis, it is essential to quantify the spots along with definition for all of the usual parameters (specificity, range of the domain of linearity, precision ect.). This is done by placing the plate under the

lens of a densitometer (or scanner) that can measure either adsorption or fluorescence at one or several wavelengths. This instrument produces a pseudo-chromatogram that contains peaks whose areas can be measured. In fact it is an isochronic image of the separation at the final instant. In TLC a spot is usually detectable if it corresponds at least to a few ng of a compound UV absorbent.(**Rouessac, F. and Rouessac, A., 2007).**

Preparative TLC*;*

TLC can be used on a microscale to monitor a reaction and determine if the product or products were successfully produced using only microgram quantities of materials. It is difficult to separate gram quantities using TLC and therefore column chromatography is used at this scale. However, larger TLC plates, called a Preparative Plates, can be used for separations of milligram quantities of materials because they are coated with thick layers (1-3mm) of stationary phase. Sample is applied to the plate as a thin even layer horizontally to and just above the solvent level. When developed with solvent the compounds separate in horizontal bands rather than horizontally separated spots. Each band (or a desired band) is scraped off the backing material. The adsorbent material is then extracted with a suitable solvent and filtered to give the isolated material upon removal of the solvent.

The "Chromatotron" is a novel and highly convenient piece of equipment for preparative thin-layer separations. Basically it consists of a slanted circular glass plate which is spun about a central shaft by means of an electric motor. The glass plate carries the adsorbent layer of 1.2 or 4mm thickness. The plate is spun during loading and solvent development and the sample and separated components are as radial bands. **(Furniss et al., 1989)**.

Visualization of spots on TLC plates

Any compounds have to be visualized after TLC separation in order for the compounds to be seen. There are a number of visualization reagents which can be used to detect any number of compounds either by reacting with a specific chemical structure (a ketone, aldehyde or carboxylic acid) or by forming a complex with a double bond, or finally by oxidizing to visible intermediates or if carried to the extreme, to carbon itself.

Among the most spraying reagents employed include;
Aluminium chloride for Flavonoids, Anisaldehyde and Sulfuric acid for steroids, terpenes, sugars. Bromothymol blue for lipids. 4-Dimethylaminobenzaldehyde: HCl for indole derivatives. Diphenylamine for glycolipids. Potassium hydroxide for coumarins. Dragendorff's solution I and II for alkaloids.(**Raaman, 2006)**

1.4.1.1.2 Column chromatography

Chromatography is based on differential migration rates of components of a liquid or gas as it moves past adsorptive materials. Some combination of adsorbent, conditions, and carrier fluid allows application of a mixture of materials to a column of adsorbent and to flush so that differential migration rates separate the materials before they exit. In column Chromatography the stationary phase (which can be solid or liquid) and a mobile phase (usually liquid or gas) are both placed in a column container

The conventional technique of liquid-solid column chromatography employs the continuous passage of a single eluting solvent through the column under gravity or under pressure applied to the top of the solvent reservoir (Flash column chromatography).
If the desired compound is coloured or strongly fluorescent under ultraviolet light, their location in selected eluent fractions presents no problems. Hence suitable fractions are combined and concentrated to recover the purified material. Also, each of the individual fractions collected could be examined directly by TLC (employing one of the non-selective detecting agents e.g. iodine vapour) **(Furniss et al., 1989)**.

The most common adsorbents used in column chromatography include; aluminium oxide (alumina), silica gel, cellulose, Charcoal, Dextran, Agarose, Polyacrylamide, Polystyrene.

The Solvent system (Eluent) must be significantly less polar than the components of the mixture. If the solvent is more polar and strongly adsorbed, then the components of the extract will remain in the mobile phase and little separation will take place. It is also essential that the mixture is soluble in the solvent; otherwise it will remain permanently adsorbed on the adsorbent. Polar solvent are used to elute strongly adsorbed components

while non-polar solvents are used for weakly adsorbed components of a mixture. The eluotropic series serves as a guide to selection of solvent or mixture of solvents based on their polarity.

ELUOTROPIC SERIES

Solvent

Pentane
Hexane
Iso-octane
Cyclohexane
Carbon tetrachloride
l-Chlorobutane
Xylene
Toluene
Chlorobenzene
Benzene
Ethyl ether
Dichloromethane
Chloroform
1,2-Dichloroethane
Methyl ethyl ketone
Acetone
Dioxane
1-Pentanol
Tetrahydrofuran
Methyl t-butyl ether
Ethyl acetate
Dimethyl sulfoxide
Diethylamine
Acetonitrile 1-Butanol
Pyridine
2-Methoxyethanol
n-Propyl alcohol
Isopropyl alcohol
Ethanol
Methanol
Ethylene glycol
Dimethyl formamide
Water
Acetic acid.

1.4.1.1.3 High Performance Liquid Chromatography (HPLC);

HPLC represents the modern culmination of the development of liquid chromatography. The user begins by placing samples on a tray for manual/ automatic injection into the column. Solvent is continually pumped through the column, and the separated compounds are continuously sensed by a detector as they leave the column. The resulting detector signal plotted against time is the *chromatogram.*

The basic information attained from the Chromatogram include;

- The number of peaks, which appear, indicates the level of complexity of the sample.
- Qualitative information about the sample is obtained by comparing peak positions with those of standards.
- Quantitative assessment of the relative concentration of components is obtained from peak area comparisons.(**Raaman, 2006**)

This technique is the prime analytical method for compounds which are involatile or thermally unstable. They include the natural products (carbohydrates, steroids, alkaloids, peptides and amino acids, antibiotics, nucleosides, etc.), and the synthetic and naturally occurring compounds arising from research in the pharmaceutical, agricultural and food industries. Analytically useful information obtainable from the chromatogram can show whether a given sample is pure or not. Compared to other separation procedures, HPLC is exceptional in terms of the following characteristics:

• Almost universal applicability; few samples are excluded from the possibility of HPLC separation.

• Remarkable assay precision (±0.5% or better in many cases).

• A wide range of equipment, columns, and other materials is commercially available, allowing the use of HPLC for almost every application. **(Snyder et al., 2010).**

Reversed-Phase Chromatography (RPC)

Reversed-phase chromatography is the term used to describe the state in which the stationary phase is less polar than the mobile phase. Chemically bonded octadecylsilane (ODS), an n-alkane with 18 carbon atoms, is the most frequently used stationary phase.

C8 and shorter alkyl chains and also cyclohexyl and phenyl groups provide other alternatives. Phenyl groups are more polar than alkyl groups.

Water is often described as the strongest elution medium for chromatography, but in fact this is only true for adsorption processes. Water may interact strongly with the active centres in silica and alumina, so that adsorption of sample molecules becomes highly restricted and they are rapidly eluted as a result. Exactly the opposite applies in reversed-phase systems: water cannot wet the non-polar (hydrophobic¼water-repellent) alkyl groups and does not interact with them in any way. Hence it is the weakest mobile phase of all and gives the slowest sample elution rate. The greater the amount of water in the eluent, the longer is the retention time.

The better Sample compounds are retained by the reversed-phase surface the less water soluble (i.e. the more non-polar) they are. The retention decreases in the following order: Aliphatics > induced dipoles (e.g. CCl4) > permanent dipoles (e.g. CHCl3) >weak Lewis bases1 (ethers, aldehydes, ketones) > strong Lewis bases (amines)> Weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids). **(Meyer, 2004).**

HPLC Instrumentation

The main components of an HPLC system are a high-pressure pump, a column and an injector system as well as a detector (Figure 1:6.1**).** The system works as follows: eluent is filtered and pumped through a chromatographic column, the sample is loaded and injected onto the column and the effluent is monitored using a detector and recorded as peaks.

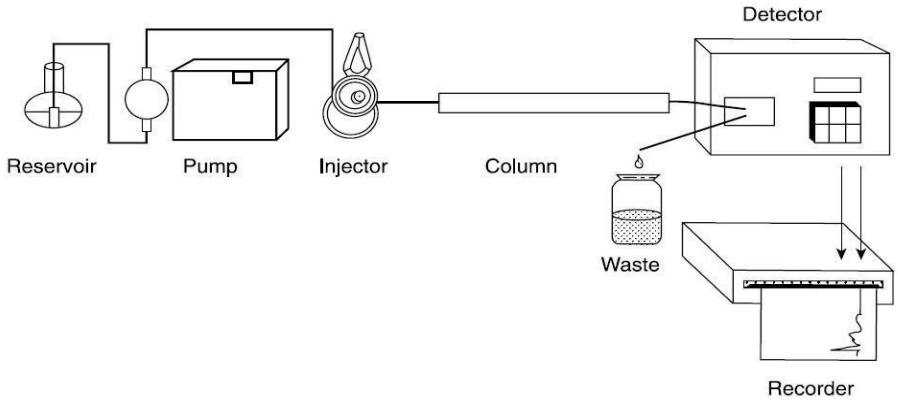


Figure 1:6 Diagram showing a typical HPLC chromatograph

The Chromatogram And Its Purport

The eluted compounds are transported by the mobile phase to the detector and recorded as Gaussian (bell-shaped) curves. The signals are known as peaks (Figure 1:8) and the whole entity is the chromatogram.

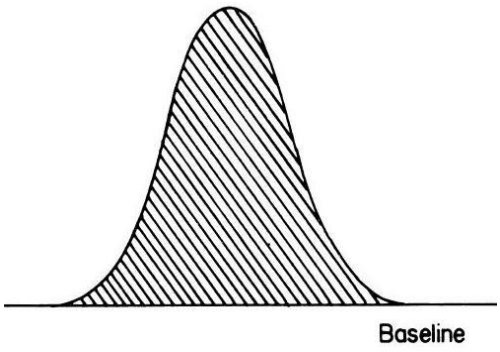


Figure 1:7 Diagram showing a typical HPLC peak (follows Gaussian distribution).

The peaks give qualitative and quantitative information on the mixture in question:

(a) ***Qualitative***: the retention time of a component is always constant under identical chromatographic conditions. The retention time is the period that elapses between sample injection and the recording of the signal maximum. The column dimensions, type of stationary phase, mobile phase composition and flow velocity, sample size and temperature provide the chromatographic conditions. Hence, a peak can be identified by injecting the relevant substance and then comparing retention times.

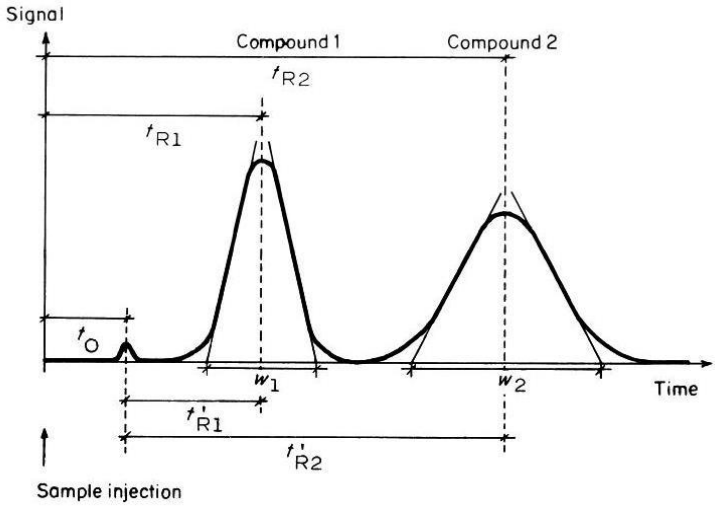


Figure 1:8 Diagram showing a typical HPLC chromatogram

(b) ***Quantitative***: both the area and height of a peak are proportional to the amount of a compound injected. A calibration graph can be derived from peak areas or heights obtained for various solutions of precisely known concentration and a peak-size comparison can then be used to determine the concentration of an unknown sample.

The chromatogram can be used to provide information on separation efficiency

Here:

w = peak width at the baseline, t_o = dead time or retention time of an unretained solute, i.e. the time required by the mobile phase to pass through the column (also called the breakthrough time).

Hence the linear flow velocity, **U**, can be calculated as;

$$\mathbf{U=L/t_o}$$

Where **L**= length of the column.

$\mathbf{t_R}$ = the retention time; this is the period between sample injection and recording of the peak maximum.

Two compounds can be separated if they have different retention times.

$\mathbf{t'_R}$ = Net retention time or adjusted retention time.

Figure **1:7.1** shows that $\mathbf{t_R = t_o + t'_R}$.

$\mathbf{t_o}$ is identical for all eluted substances and represents the mobile phase residence time. $\mathbf{t'_R}$ is the stationary phase residence time and is different for each separated compound. The longer a compound remains in the stationary phase, the later it becomes eluted.

Retention time is a function of mobile phase flow velocity and column length. If the mobile phase is flowing slowly or if the column is long, then $\mathbf{t_o}$ is large and hence so is $\mathbf{t_R}$; $\mathbf{t_R}$ is therefore not suitable for characterizing a compound. Therefore the retention factor or **k** value (formerly known as the capacity factor, k_o) is preferred:

$$k = \frac{t'_R}{t_0} = \frac{t_R - t_0}{t_0}$$

k is independent of the column length and mobile phase flow-rate and represents the molar ratio of the compound in the stationary and the mobile phase **(Meyer, 2004).**

Resolution;

The aim of chromatography is to separate components in a mixture into bands or peaks as they migrate through the column. Resolution, R, provides a quantitative measure of the ability of a column to separate two analytes.

The resolution of two neighbouring peaks is defined by the ratio of the distance between the two peak maxima, i.e. the distance between the two retention times, $\mathbf{t_R}$, and the arithmetic mean of the two peak widths, **w.**

$$R = 2\frac{t_{R2} - t_{R1}}{w_1 + w_2} = 1.18\frac{t_{R2} - t_{R1}}{w_{1/2_1} + w_{1/2_2}}$$

Where $w_{1/2}$ is the peak width at half-height.

COLUMN EFFIECIENCY

A chromatographic column is divided into **N** theoretical plates. A thermodynamic equilibrium of the analytes between the mobile phase and stationary phase occurs within each plate. The efficiency of the column is expressed as the number of theoretical plates, **N**.

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

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$$

Poor column efficiency results in band/peak broadening.

The height of a theoretical plate, **H,** is readily calculated provided the length of the column (**L)** is known;

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

HPLC-HYPHENATED TECHNIQUES

Coupled and complementary methods of analysis in HPLC are employed to bring about separation of constituents of a mixture and other analytical methods of analysis for detection and identification of species. This has developed into what is referred to as hyphenated methods of analysis**.**

Hyphenation of liquid chromatographic (LC-hyphenation) methods primarily began with the advent of diode array detector (DAD) or Photodiode array detector (PAD). Mass spectrometry (MS) has been coupled to liquid chromatography, as have Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR), and inductively coupled plasma (ICP) spectroscopies. Also, different types of chromatographies have been coupled such as LC-GC.

In LC-MS hyphenated system, the chromatographic technique separates the components of a mixture, while MS provides structural information with regards to each of the eluting molecular species. **(Ewing, 2005)**

Quantitation of analyte using HPLC technique.

Peak height or peak area can be measured, either manually or with electronic devices. Peak height measurements have the advantage of simplicity but are sensitive to changes in peak shape; hence it is advised that peak areas should be used where peaks are broad and tailing.

For a given system, a calibration graph must be constructed for each compound to be analysed because the detector response to each will be different. This graph of peak height (or area) against drug concentration can then be used to quantify the unknown sample by extrapolation. Such external calibration requires careful control of the injection volumes and valve injection should be used.

However, external calibration is susceptible to errors arising from fluctuations in column performance and the internal standard technique gives better precision. This involves the addition of a fixed amount of a substance (internal standard) to the sample before injection. Quantification is carried out using peak height (or area) ratios of drug to internal standard.

The general assumption used in quantitation of analyte using HPLC technique is that the peak area/ratio, **A,** is proportional to the concentration, **c,** of the analyte under the same chromatographic conditions.

$\mathbf{A} \alpha \mathbf{c}$

$\mathbf{A} = k\,\mathbf{c}$

So to quantify a sample X, a known concentration of a reference sample, c_s is prepared and run under the same chromatographic conditions as sample X and their peak areas compared as shown below;

$\mathbf{A_s/A_r = c_s/c_r}$. Hence, concentration of sample X, $\mathbf{c_s}$, can be calculated.

Where**;**

$\mathbf{A_s}$= peak area (or ratio) of sample $\mathbf{A_r}$= peak area (or ratio) of the reference sample.

1.4.2 SPECTROPHOTOMETRIC ANALYSIS OF PHYTOCHEMICALS.

Photometric techniques remain the fulcrum of phytochemical identification and possible structural elucidation of isolated phytochemicals. It ranges from simple flame photometers to expensive spectrometers such as ultraviolet-visible, Infra-red, Nuclear magnetic resonance spectrometers which are used in structural and quantitative analysis of phytochemicals.

1.4.2.1 VISIBLE/ ULTRAVIOLET (UV) SPECTROSCOPY

Analytical absorption spectroscopy in the ultraviolet and visible regions of the electromagnetic spectrum has been widely used in pharmaceutical and biomedical analysis for quantitative purposes and with certain limitations, for the characterisation of drugs, impurities, metabolites, and related substances.

Absorption of light in the visible and ultraviolet regions of the spectrum is due to the presence of a Chromophore in the absorbing molecule. Conjugated double bonds give pronounced effects on the absorption of visible and ultraviolet radiation (**Beckett and Stenlake, 1988)**.

Electronic absorption spectroscopy alone can only rarely provide a complete solution to a structural problem, although it is an essential adjunct to other forms of physical measurement and to chemical studies.

Certain generalisation can be made concerning the shape of absorption curves; intense absorption in the range 200-300nm (ε 10000-20000) usually signifies the conjugation of at least two chromophores which may or may not be the same.

Sometimes, the shape of the absorption curve is so characteristic that electronic spectral studies can be used in diagnostic role for small amount of material.

For instance the spectra of quinoline (Fig1:10) gives the following data; λ_{max} (ε) 226nm (35500), 270nm (3500), 300nm (2000), 313nm (2500) in cyclohexane. Also, that of indole (Fig 1:11) exhibits a band at λ_{max} (ε) 220nm (26000), 262nm (6310), 280-290nm (~5000) in cyclohexane (**William and Fleming, 1995**).

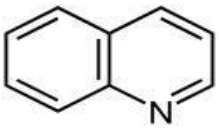


Figure 1:9 Structure of quinoline.

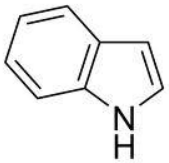


Figure 1:10 Structure of indole

Some compounds show variation in structure in different solvents, and those with ionisable groups are affected by change in pH. Frequently such changes are manifested by alteration in the shape of the absorption spectral spectrum. Such environmental dependence may sometimes provide diagnostic evidence leading to the recognition of particular functional group as, for example phenolic groups or aromatic amino substituent.

1.4.2.2 INFRA-RED (IR) SPECTROSCOPY

The infra-red region of the electromagnetic spectrum extends from 0.8μm (800nm) to 1000μm and is subdivided into near infrared (0.8 to 2μm), middle infrared (2 to 15μm), and far infrared (15 to 1000μm) (**Beckett and Stenlake, 1988)**.

The two major application of IR spectrometry in the characterization of various molecules are;

- Determination of the identity of a compound by means of spectral comparison with that of an authentic sample, and
- Verification of the presence of functional groups in an unknown molecule.

The latter aspect is quite important in the structural elucidation of synthetic organic compounds or substances isolated from natural sources.

When a molecule is subjected to infra-red radiation, transitions take place between rotational and vibrational energy in the ground electronic state. In order for IR radiation to be absorbed by a molecule, two criteria must be met ; the molecule should posses a vibrational or rotational frequency identical to that of the impinging electromagnetic(EM) radiation, and a net change in magnitude or direction of the dipole moment should occur as a result of radiation-molecule interaction.
When IR radiation impinges upon a molecule at the proper frequency, the vibration and or rotation of the molecule is altered. If the frequency of the impinging EM radiation matches a natural frequency of the molecule, a net transfer of energy occurs that creates greater amplitude of vibration and as result absorption of energy (**Abdou, 1990**).

The position of the absorption bands due to stretching and in-plane bending vibrations of the functional groups such as C=O, C—H, N—H, O—H are somewhat independent of the influence of the neighbouring groups. These bands usually occur at 4000-1300cm^{-1}. The position of the band below 1300cm^{-1} is influenced markedly by the neighbouring groups. The portion of the spectrum from 1300 to 400cm^{-1} is referred to as "fingerprint". Many of these bands overlap and it is often very difficult to identify specific absorptions. This region is used for matching the spectra of unknown compounds with the spectra of known compounds, as one matches fingerprints; hence the name fingerprint region.

Some Commonly Encountered IR Vibration Bands

C—H stretching and bending vibrations occur at 3300-2800cm^{-1}.

Saturated acyclic and cyclic hydrocarbons have stretching υ at 2960-2850cm^{-1} and in-plane bending is at 1470-1360cm^{-1}.

Aromatic C—H stretching υ occurs at 3100-3000 cm^{-1} and out of plane bending is at 900-650 cm^{-1}.

The most characteristics band for aromatic compounds is at 1610-1590(due to aromatic skeletal vibration).

N—H stretching vibration occurs at 3500-3300 cm^{-1}.

C—N of aliphatic compounds occurs at 1210 cm^{-1} and for aromatic at 1250-1350 cm^{-1}.

C=N stretching vibration υ occurs at 1680-1640 cm^{-1}(**Abdou, 1990**).

Sample Preparation for IR spectrometry

Sample for IR can be prepared in the form of a gas, liquid or solid. Liquid samples are prepared "neat" (pure form) or in solution using a liquid cell. Carbon tetrachloride and carbon disulphide are two commonly used solvents.

Solid samples are prepared either as a KBr disc or in the form of dispersion in mineral oil(example is Liquid paraffin, Nujol) to give a mull(Nujol mull). A KBr disk of a sample is prepared by grinding the sample with KBr powder, placing the mixture between a punch and die and applying a pressure of about 50,000 psi.

Fourier Transform Infra-Red spectrometry (FT-IR)

The wide availability of high powered microcomputers at reasonable cost has helped popularise the application of Transform spectroscopy in general and FT in particular to several branches of spectrometry. These include IR, NMR and MS.

FT-IR has rapidly grown due to superior sensitivity and resolution of the technique, absolute wavelength accuracy and higher precision of measurements.

Light covering the whole frequency range typically 5000-400 cm^{-1} , is split into two beams. Either one beam is passed through the sample or both are passed , but one is made to transverse a longer path than the other. Recombination of the two beams produces signal patterns known as *interferogram* and looks nothing like a spectrum. However Fourier Transformation of the interferogram, using computer built into the spectrometer converts it into a plot of absorption against wavenumber which resemble the usual spectrum obtained by the traditional method (**William and Fleming, 1995**).

1.4.2.3 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

NMR spectroscopy is arguably the most important analytical method available today. It gives information about the number, type, and connectivity of hydrogen and carbon atoms. With the advent of spectrometers operating at very high magnetic fields (up to 21.1 T, i.e. 900 MHz proton resonance frequency) it has become an extremely sensitive technique, so that it is now standard practice to couple NMR with high pressure liquid chromatography (HPLC).

1.4.2.3.1 Origin of NMR spectra

Under appropriate conditions, a sample can absorb electromagnetic radiation in the radio-frequency region at frequencies governed by the characteristics of the sample. Absorption is a function of certain nuclei in the molecule. A plot of the frequencies of absorption peaks versus peak intensities constitutes the NMR spectrum.

The technique is only applicable to those nuclei which possess a spin quantum number (I) greater than zero. The most important of such nuclei are ^{1}H and ^{13}C, both of which have a spin quantum number of ½. Other nuclei with non-zero spin quantum number are ^{19}F and ^{31}P, with I=1/2; ^{14}N and ^{2}D, with I= 1; and ^{11}B and ^{35}Cl, with I = 3/2. Spin quantum number determines the number of orientations a nucleus may assume in an external uniform magnetic field in accordance with the formula $2I+1$.

Since atomic nuclei are associated with charge, a spinning nucleus gives rise to an electric current and this has a magnetic field associated with it. The magnitude of the magnetic dipole μ has a characteristic value for a given nucleus.

When a spinning nucleus is placed in a uniform magnetic field H_o, the nuclear magnet experiences a torque which tends to align it with the field and because of the interaction of the nucleus with the main magnetic field, the spinning nucleus precesses about the magnetic direction. For a proton, two energy levels are possible; alignment with the field (low energy state), and against the field. The energy separation between the two energy states is given by;

$$E= \mu H_o/I.$$

When the precessing nucleus rotates at the same frequency as the incidented radiowave, nuclear magnetic resonance occurs and this results in absorption of energy which is recorded as the NMR spectrum. At this condition, the tipping field suffers a drop in amplitude. The energy absorbed by the nucleus is used to promote it to a higher energy state.

1.4.2.3.2 Apparatus and sample handling

The schematic diagram of a basic NMR spectrometer is shown in Fig 1:12 below. The instrument can be described in the following components;

1. A strong magnet whose homogeneous field can be varied continuously and precisely over a relatively narrow range using a sweep generator.
2. A radio-frequency oscillator.
3. A recorder, calibrator, and integrator.
4. A sample holder

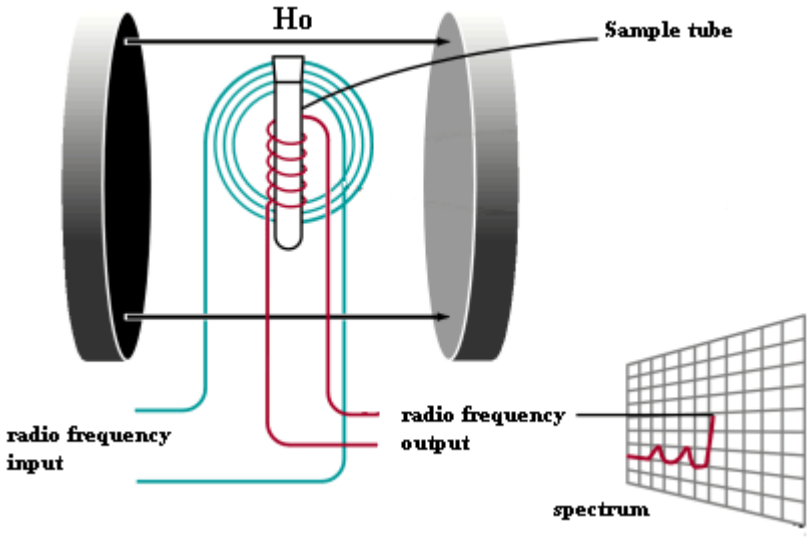


Figure 1:11 Typical NMR spectrophotometer

The sample, a liquid or a solution in a suitable solvent, is contained in a 5-mm O.D. glass tube. Ordinarily about 0.4ml of a neat liquid or somewhere between 10-50mg of a liquid or solid dissolved in a 0.4ml of a solvent is used.

The ideal solvent should contain no protons, be inexpensive, low-boiling, non-polar, and inert. The most widely used solvent is deuterated chloroform ($CDCl_3$). Commercial grade

solvents are available in deuterated form with an isotopic purity (atom %D) of 98-99.8%. For instance, Dimethy-d_6 Sulfoxide (DMSO-d_6) (Merck Sharp and Dohme of Canada, ltd) has isotopic purity (atom %D) of 99.5% and the methyl residual protons occur at chemical shift δ 2.5.

1.4.2.3.3 Chemical Shift

The position of an absorption peak relative to that of the reference compound is known as the chemical shift. The most generally useful reference compound is tetramethylsilane (TMS). Each nucleus in a different environment experiences a slightly different local magnetic field due to the circulation of electrons in neighbouring bonds and to through-space effects. A slightly different applied magnetic field is therefore required for resonance and absorption occurs in different regions of the spectrum. Effects which cause shifts to lower fields (downfield) are termed deshielding; the opposite effect (upfield shift) is termed shielding (**Furniss et al., 1989**).

1.4.2.3.4 Spin-spin coupling

The concept of spin spin coupling can be visualised by considering the effect of one proton on a neighbouring proton connected by not more than three bonds. The splitting of the peaks occurs because of the tendency of the electron to pair its spin with that of the nearest proton or nucleus . The multiplicity caused by the effect of one group on the neighbouring group is given by the formula 2nI+1 where n= number of equivalent nuclei of spin I. The relative intensity is determined by the Pascal's triangle. The distance between multiplets is referred to as the coupling constant, J, expressed in Hz.

Qualitative measurement of NMR spectra

The following can be determined from the NMR spectra

- Chemical shift establishes the general nature of a proton
- The multiplicity of proton resonance indicates the nature of the proton environment and the interaction of the protons

The integration of the spectrum areas enables assignment of relative values to the areas and determines the number of protons that a particular multiplet represents (**Abdou, 1990**).

1.4.2.3.5 ^{13}C Nuclear Magnetic Resonance

This is the most exceedingly interesting nuclei for which an increasing amount of work has been reported recently. ^{13}C has nuclei spin of ½ but due to the low natural abundance of this isotope (1.1%, compared to ^{12}C), its resonance has only 1.6% the sensitivity of ^{1}HNMR. It has longer relaxation time.

This recent studies showed that the complicated ^{13}C spectra could be simplified by completely decoupling the ^{13}C nuclei from all the ^{1}H nuclei using spin-spin decoupling technique (double resonance). The development of superconducting magnets, together with Fourier transform techniques and advanced computer technology has helped ^{13}C NMR to develop into one of the most important techniques in structural elucidation (**Abdou, 1990**).

Unlike proton NMR spectroscopy, the relative strength of carbon NMR signals is not normally proportional to the number of atoms generating each one. Because of this, the number of discrete signals and their chemical shifts are the most important pieces of evidence delivered by a carbon spectrum

Distortionless Enhancement By Polarization Transfer (DEPT)

Modern NMR spectrometers come equipped with computer controlled radio frequency transmitters that can be used to manipulate nuclei in ways that create new types of spectra containing additional structural information. One of these techniques is referred to as DEPT. The DEPT technique produces different effects depending on the number of hydrogen atoms bonded to a carbon atom. By doing a series of spectra it is possible to identify the number of hydrogen atoms attached to the carbon atom producing each of the signals in a 13C NMR spectrum. **(NMR Spectroscopy: ^{13}C and DEPT-135)**

1.4.2.4 MASS SPECTROMETRY

Mass spectrometry is an indispensible analytical tool in chemistry, biochemistry, pharmacy, and medicine. Mass spectrometry is used to analyze combinatorial libraries, sequence biomolecules, and help explore single cells. Structure elucidation of unknowns, environmental and forensic analytics, quality control of drugs, flavors and polymers; they all rely to a great extent on mass spectrometry.

The basic principle of mass spectrometry (MS) is to generate ions from either inorganic or organic compounds by any suitable method, to separate these ions by their mass-to-charge ratio (m/z) and to detect them quantitatively by their respective m/z and abundance. The analyte may be ionized thermally, by electric fields or by impacting energetic electrons, ions, or photons. The ions can be single ionized atoms, clusters, molecules or their fragments or associates. Ion separation is effected by static or dynamic electric or magnetic fields. Although this definition of mass spectrometry dates back to 1968 when organic mass spectrometry was in its infancy, it is still valid. However, two additions should be made. First, besides electrons, (atomic) ions or photos, energetic neutral atoms and heavy cluster ions can also be used to effect ionization of the analyte. Second, as demonstrated with great success by the time-of-flight analyzer, ion separation by m/z can be effected in field free regions, too, provided the ions possess a well defined kinetic energy at the entrance of the flight path.(**Gross, 2004**)

Instrumentation

A mass spectrometer consists of an ion source, a mass analyzer and a detector which are operated under high vacuum conditions. A closer look at the front end of such a device might separate the steps of sample introduction, evaporation and successive ionization or desorption/ionization, respectively, but it is not always trivial to identity each of these steps clearly separated from the others.

- *Ion source*- this forms the part of mass spectrometer that ionizes the analyte and transported by a magnetic or electric field to the mass analyzer. Electron ionization and chemical ionization are used for gases and vapours. Chemical ionization is a lower energy process which yields less fragmentation and usually

a simple spectrum. Two techniques often used with liquid and solid biological samples include electronspray ionization(**ESI**) and matrix-assisted laser desorption/ionization(**MALDI**). Inductively coupled plasma(ICP) sources are used primarily for cation analysis of a wide array of sample types. Chemical ionization in an atmospheric pressure electric discharge is called atmospheric pressure chemical ionization (**APCI)** and the analyte in this case is a gas or liquid spray and ionization is accomplished by corona discharge.

- *Mass analyzer*; mass analyzers separate the ions according to their mass-to-charge (*m/z)* ratio.
- *Mass spectrum*; it is the two-dimensional representation of signal intensity (ordinate) versus m/z (abscissa). The intensity of the peak directly reflects the abundance of ionic species of the respective m/z ratio which have been created from the analyte within the ion source.

Terminologies for the interpretation of mass spectrum of compounds

1. **Base peak;** it is the largest peak in a mass spectrum- the one with a relative intensity of 100%. It corresponds to the mass of an ion that is long lived and reaches the detector in greater quantity than any other (**Sorell, 2006**).
2. **Molecular ion (M^+);** it is an ion formed by the removal from (positive ions) or addition to (negative ions) a molecule of one or more electrons without fragmentation of the molecular structure. The mass of this ion corresponds to the sum of the masses of the most abundant naturally occurring isotopes of the various atoms that make up the molecule (with a correction for the masses of the electron(s) lost or gained). **(IUPAC compendium of Chemical Terminology).** A molecular ion may be formed by protonation of a molecule[M+H] and it is also referred to as a molecular ion or *quasi* or *pseudo-molecular ion*. To determine the molecular formula of a compound from a Chemical ionization or Fast Atom Bombardment(FAB) mass spectrum, 1 amu would have to be subtracted from the *m/z value* of the peak considered to be M^+. This is because that peak actually corresponds to the species MH^+. (**Sorell, 2006**)

1.4.2.5 HPLC METHOD VALIDATION

Method validation establishes, by means of laboratory studies, that the performance characteristics of the test method meet the requirements of the intended analytical application. Method validation provides an assurance of reliability during normal use, and this process is sometimes referred to as providing documented evidence that the method does what it is intended to do.

More recently, new information has been published that updates previous guidelines and provides more detail and harmonization with International Conference on Harmonization (ICH) guidelines **(Snyder et al., 2010).**

TERMS AND DEFINATIONS

Several analytical performance characteristics may be investigated during any method validation protocol including:

- Accuracy
- Precision
- Specificity
- Limit of detection
- Limit of quantitation
- Linearity
- Range
- Robustness

1.4.2.5.1 Accuracy

It is the measure of exactness of an analytical method, or the closeness of agreement between an accepted reference value and the value found in a sample. Established across the range of the method, accuracy is measured as the percentage of analyte recovered by the assay.

According to ICH Harmonised Tripartite Guidelines on Validation of analytical Procedures: methodology, Accuracy may be inferred once precision, linearity and specificity have been established (**ICH topic Q2B**).

1.4.2.5.2 Precision

The precision of an analytical method is defined as the closeness of agreement among individual test results from repeated analyses of a homogeneous sample. Precision is commonly performed as three different measurements: repeatability, intermediate precision, and reproducibility.

Repeatability (intra-day precision) is the ability of the test method to generate the same results over a short time interval under identical conditions (intra-assay precision) should be determined from a minimum of nine determinations. Their repeatability should cover the specified range of the procedure (i.e., three concentrations, three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Intermediate (Inter-day) precision refers to the agreement between the results from within laboratory variations due to random events that might normally occur during the use of a test method, such as different days, analysts, or equipment It is not considered necessary to study these effects individually.

The acceptance criterion is relative standard deviation of ≤2% of the peak areas in HPLC method validation **(Snyder et al., 2010).**

1.4.2.5.3 Limit of Detection (LOD)

It is the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from the background noise (2000 Washington conference).

The IUPAC defines LOD as the concentration that gives rise to a signal 3times the standard deviation of the blank. LOD of the method may be calculated using the formula provided by the ICH Topic Q2B 1996 Guidelines.

1.4.2.5.4 Limit of Quantitation (LOQ)

It is the lowest amount of an analyte in a sample that can be determined quantitatively with suitable precision and accuracy (2000 Washington conference).

The IUPAC defines LOD as the concentration that gives rise to a signal 10 times the standard deviation of the blank. LOQ of the method may be calculated using the formula provided by the ICH Topic Q2B 1996 Guidelines.

1.4.2.5.5 Linearity

A linear relationship should be evaluated across the range of the analytical procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by calculation of a regression line by the method of least squares which Microsoft Excel has in built operation for it. Linearity can be inferred if $r^2 \geq 0.995$

1.4.2.5.6 Range

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

CHAPTER TWO

2 EXPERIMENTAL

2.1 INSTRUMENTS and MATERIALS

- Integrator-shimadzu CR 501 chromatopac.
- Cecil CE 2041 single beam UV-VIS spectrophotometer.
- Buchi rotary evaporator.
- Buchi recirculator chiller.
- Buchi water bath.
- Stuart Scientific Flask shaker
- HPLC Apparatus- LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied biosystems).
- Analytical Balance (Adams Instrument).
- Melting point apparatus(Stuart).
- Ovens.
- Desaga spreader
- Column- Hichrom Zobrax C_8 5micron 15cm×4.6mm id
- UV viewing system (CHROMATO-VUE® C-70G)
- Precoated plates-silica gel Gf 254, 0.25mm Merck W. (Germany)
- column chromatographic Silica gel 70/230(Auro,India)
- Silica gel for preparative layer chromatography 60 PF_{254}
- pH meter (Eutech).
- Ceramic mortar and pestle
- Reference cryptolepine and *C. sanguinolenta* roots were obtained from CSRPM, Akropong-Mampong. Roots were authenticated by comparing with herbarium sample(Voucher Number=FP/09/57) by Kofi Annan, PhD, at Department of Pharmacognosy, KNUST.

2.2 REAGENTS;

- Methanol (BDH)
- Ethylacetate (FISONS)
- Petroleum ether(40-60) BDH
- Ammonia (35%) (BDH)
- Chloroform(FISONS)
- Dragendorff's solution A and B
- 1% Acetic acid in water
- Trifluoroacetic acid (98%) (BDH chemicals ltd. UK)
- Diethylamine (FISONS

2.3 METHOD

2.3.1 PHYTOCHEMICAL TEST ANALYSIS OF ROOTS OF CRYPTOLEPIS SANGUINOLENTA

The methanolic: water (1:1) extract of the roots of *Cryptolepis sanguinolenta* was screened for the presence of Alkaloids, Tannins, Glycosides and Steroids.

2.3.1.1 ALKALOIDS

1.5ml of 10% HCl was added to 5ml of extracts of the roots in a test tube. The mixture was heated for 20 minutes. It was cooled and filtered 1ml of the filtrate was tested 5 drops of equal mixture of Dragendorff reagents A and B. A reddish-orange precipitate was observed in the test tube indicating the presence of alkaloids.

2.3.1.2 TANNINS

3 drops of 5% ferric chloride was added to 1ml of the extract. No greenish black precipitate observed in the extract indicating the absence of tannins in the extract.

2.3.1.3 GLYCOSIDES

10ml of 50% HCl was added to 2ml of the extracts in a test tube. The mixture was heated in boiling water for 30minutes. 5ml of Fehling's solution was added and the mixture was boiled for 5 minutes. No brick-red precipitate observed.

2.3.1.4 STEROIDS

Liebermann's Burchard test: 1ml of the extract was dissolved in 0.5ml of acetic anhydride and cooled well in ice. This was mixed with 0.5ml of chloroform and 1ml of concentrated H_2S0_4 was then carefully added by means of a pipette. No reddish-brown ring was formed at the separating interface of the two liquids indicating the absence of steroids.

2.3.2 EXTRACTION OF CRUDE ALKALOID

500g of dry powdered roots of *Cryptolepis sanguinolenta* (Lindl.) Schlt were percolated in 1%v/v acetic acid in water (4.5litres) at room temperature for 48hours. The filtered aqueous extract was extracted with chloroform (3×2litres) and the chloroform layer was separated and then discarded. The remaining aqueous extract was basified using ammonium hydroxide till a pH of 9 and extracted three times with Chloroform(3×2litres). The combined chloroform extracts were washed with water, dried over anhydrous sodium sulphate and concentrated under reduced pressure to dryness to yield 3.0865g of a purple crude total alkaloid extract (**0.6173%w/w**).

2.3.3 ISOLATION OF CRYPTOLEPINE

2.3.3.1 *GRAVITY COLUMN CHROMATOGRAPHY*

The column for the column chromatography was cleaned and dried. A wad of cotton was placed on top of the glass wool. The adsorbent, column chromatographic Silica gel 70/230(Auro,India), was measured into the column to about 10cm. The side of the column was tapped to ensure even packing. The solvent for the isocratic elution was carefully poured into the column. To eliminate air packets the column was gently tapped again. The solvent was drained until the level of the solvent was to the top of the adsorbent. 3.0g of the crude alkaloid extract were dissolved in 5ml of chloroform and mixed with 8.0g of activated column chromatographic Silica gel 70/230(Auro,India).The mixture was heated on a water bath with stirring until dry and homogeneous. This mixture was packed on top of the absorbent. A layer of cotton was placed on top of the coloured mixture to avoid disturbing the surface of the mixture when the mobile phase is poured into the column.

Isocratic elution was carried out using the following mobile phases sequentially; 100% Petroleum ether(40-60), 10% ethylacetate in Petroleum ether, 30% ethylacetate in Petroleum ether, 10% Chloroform in ether, 20% Chloroform in ether and 40% Chloroform in ether and 20×20ml colourless fractions of each mobile phase were collected. The eluents collected were tested for the presence of alkaloids using the Dragendorff's reagent and each fraction tested

negative. Thin layer chromatography (TLC) using precoated plates (250μm) and solvent system I; Ethylacetate: Methanol: Ammonia (35%) 80:15: 5 on the eluents showed no spots. The eluents were bulked separately for each mobile phase.

The elution was continued using 100% ethylacetate and 20×10ml colourless fractions of each mobile phase were collected. The eluents collected were tested for the presence of alkaloids using the Dragendorff's reagent and each fraction tested negative. TLC performed on the eluents showed no spots. The eluents were bulked.

20% of methanol in ethylacetate was used next for the elution and 20ml×40 fractions of mobile phase were collected. The eluents were colourless for 1-4th fractions but yellowish-brown for 5-34th fractions and then colourless for 35-40th fractions. The coloured fractions tested positive with Dragendorff's reagent. TLC on each fraction was conducted using precoated plates and solvent system I. Fractions with similar components were bulked.

The mobile phase was changed to 40% of methanol in ethylacetate and 20ml×20 fractions of colourless mobile phase were collected and the eluents collected were tested for the presence of alkaloids using the Dragendorff's reagent and each fraction tested negative. TLC on the eluents showed no spots. The eluents were bulked.

The mobile phase was changed to 100% methanol and 20ml×150 fractions were collected. Yellow fractions were collected for 4-32nd fractions and colourless for 33-45th fractions. The elution was continued with methanol and the yellow eluents were collected. The yellow coloured fractions tested positive with Dragendorff's reagent. TLC on each fraction was conducted and fractions with similar components were bulked.

The bulked fractions were concentrated under reduced pressure using the Rotavapor at 50°C. The bulked fractions which gave positive test for presence of alkaloids and their TLC had components with same R_f -values as the reference cryptolepine. They were further purified using preparative TLC.

2.3.3.2 *PREPARATIVE TLC ON THE CONCENTRATED ELUENTS*

Preparation of the TLC plates

Absorbent; silica gel for preparative layer chromatography 60 PF_{254},

Supports; Glass plates of size 20×20cm

Composition of slurry: Silica gel for preparative layer chromatography 60 PF_{254} in water in a ratio of 1:2. The appropriate weight of silica gel was weighed and mixed with the corresponding volume of water stirred well enough adding extra silica gel or water till a homogeneous slurry was formed. The plates were coated with the slurry to a thickness of 0.75mm using a Desaga spreader. Coated plates were air dried at a room temperature for 15 minutes and then oven heated at 110°C for 1 hour. The dried plates were stored in desiccators. Used plates were re-activated at 100°C for 30 minutes before use for subsequent works. Spots were applied using disposable capillary tubes. The solvent of application was allowed to dry off before subjecting the plates to development.

Chromatographic tanks: 22cm×22cm×10cm, filled with 100ml of mobile phase-ethylacetate: Methanol: Ammonia (35%) 80:15: 5 . Saturation of the tank with the mobile phase was achieved by spreading a piece of filter paper in the bottle to help create an atmosphere saturated with solvent. Plates were developed in well saturated tanks allowing the solvent front to travel a distance of 15cm before removing the plates from the chamber. The mobile phase was dried off from the plates before detection.

Solvent system I; Ethylacetate: Methanol: Ammonia (35%) 80 : 15 : 5

Detection of Spots/Bands

Cryptolepine fluoresces under Ultraviolet light at 354nm and hence detection of cryptolepine was monitored at this wavelength using the UV viewing system (CHROMATO-VUE® C-70G)

Isolation of the band of Cryptolepine

Under the UV viewing system, the yellow bands corresponding to cryptolepine were marked out using a pencil and scraped into flasks. The scraped silica gel was extracted with methanol till filtrate of the extraction tested negative with the Dragendorff's reagent. To the yellow filtrate

obtained, anhydrous magnesium sulphate was added as a drying agent and the supernatant concentrated using the rotavapor in *vacuo* and then allowed to dry in a grease free desiccator over blue silica gel. Crystals obtained was scraped into sample tube as violet crystals and labelled as isolated cryptolepine and kept in desiccator. The crystals obtained were run with reference cryptolepine (CLP-R) using precoated analytical TLC plates with mobile phases; Ethylacetate: Methanol: Ammonia (35%) 80: 15: 5 and ethylacetate: chloroform: diethylamine 80:15:5(solvent system II).

Isolated cryptolepine showed a single spot under UV light at 254nm and 354nm with the same R_f-value as reference cryptolepine in both solvent systems (Fig 5.1 and 5.2). The melting point of isolated cryptolepine was determined to be166-168℃ (lit. 166-169° C) and the structure of isolated cryptolepine was further authenticated using Ultraviolet-Visible, Infra-Red, Nuclear Magnetic Resonance and Mass spectrophotometry.

2.3.4 SPECTROPHOTOMETRIC ANALYSIS OF ISOLATED CRYPTOLEPINE

2.3.4.1 ULTRAVIOLET/VISIBLE ABSORPTION SPECTRUM OF ISOLATED CRYPTOLEPINE

Calibration of Absorbance Scale of the UV/Vis Spectrophotometer

Potassium dichromate was dried to a constant mass at 130 ° C. 60mg of the potassium dichromate was accurately weighed, dissolved in 0.005M sulphuric acid and diluted further to 1000ml with the same acid. The UV/Vis absorption spectrum of the solution was determined over the wavelength range 225-450nm using 1cm cell and the acid as reference. The A (1%,1cm) at λ_{max} and λ_{min} were compared with the British Pharmacopoeia stipulations (**BP 2005**). The A (1%, 1cm) at λ_{max} and λ_{min} were within maximum tolerable values.

Determination of Ultraviolet/visible absorption spectrum of isolated Cryptolepine.

Several dilutions of methanolic solution of the isolated cryptolepine were made and scanned over wavelength range of 200-550nm using the calibrated Cecil CE2041 (2000series) UV/VIS spectrophotometer in a 1cm cell using methanol as reference. The absorptivities, ε, were

recorded at the following wavelengths: λ_{max} 223nm (log ε=3.98), 246 (3.72), 275 (4.17), 281(4.25) 355sh (3.92), 370(4.13) and compared with that of pure cryptolepine as described by Dwuma-Badu, Ayim et al. 1978.

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

The isolated cryptolepine was sent to the College of Pharmacy, Florida A&M university, Tallassee, in the USA. The IR, NMR and MS analysis of the isolated cryptolepine was done under the supervision of Prof. Seth Ablordeppey and the data was sent to us in Ghana. The Proton and Carbon-13 nuclear magnetic resonance spectra of the isolated cryptolepine were obtained on Mercury-300BB Varian Spectrometer, the sample was dissolved in deuterated Dimethyl Sulfoxide ($DMSO_d$). The infrared spectrum of isolated cryptolepine was obtained on the PerkinElmer Express Version 1.02.00. Mass spectra data were obtained on LCQ Advantage MAX mass spectrometer (Thermo Electronic Inc) with ACPI probe. The sample was dissolved in methanol and introduced through an infusion pump. 30 - 35% normalized collision energy was applied on the parent mass to get the MS/MS spectra.

2.3.5 EXTRACTION OF THE ROOTS OF CRYPTOLEPIS SANGUINOLENTA PRIOR TO ISOCRATIC REVERSE PHASE HPLC ANALYSIS.

0.1000g of the powdered dry roots of *Cryptolepis sanguinolenta* (Lindl.) was accurately weighed using an electronic balance into a clean dry beaker and transferred into a conical flask. It was shaken with 4×50ml methanol for 20minutes each using a flask shaker. The extraction was complete when the methanolic extract tested negative for alkaloids with Dragendorff's reagent. The methanolic extract was filtered under gravity into a 250ml volumetric flask using a Whatman No.1 filter paper. The filtrate was topped up to the 250ml mark using methanol. Accurately, 6.7ml, 5ml, and 2ml of the stock solution was pipette into a 10ml volumetric flask and diluted to the mark with methanol to produce 1.5times, 2times and 5times dilution of the stock solution. Each of the three concentrations was subjected to HPLC analysis.

2.3.6 INVESTIGATION OF MOBILE PHASE FOR THE HPLC ANALYSIS.

Candidate mobile phase compositions used included the following; Methanol: water 90:9 and pH modified to 2.4 using glacial acetic acid, Methanol: Monobasic Phosphate buffer (0.02M, pH adjusted to 2.4 using phosphoric acid) 90:10, Acetonitrile: TFA, (0.5%v/v) 90:10(pH 2.22), methanol: TFA, (0.05%v/v) 90:10 and methanol: water (90:9) modified with trifluoroacetic (TFA,(98%)) acid to a pH of 2.4. The complete chromatograph system used is described in 2.3.7 below. The effect of the different solvents on the chromatogram was noted. The mobile phase composition chosen was methanol: water (90:9) modified with trifluoroacetic acid (TFA,(98%)) to a pH of 2.4(see **table 3.2**)

2.3.7 HPLC ANALSIS OF CRYPTOLEPINE IN THE ROOTS OF CRYPTOLEPIS SANGUINOLENTA

The complete chromatograph consisted of Shimadzu LC-6A pump with programmable absorbance detector (783A Applied Biosystems) and Shimadzu CR 501 chromatopac Integrator. Column used was Hichrom Zobrax C_8 15cm×4.6mm id. The mobile phase consisted of methanol: water (90:9) modified with trifluoroacetic acid(TFA,(98%) to a pH of 2.4 eluted isocratically at 1.0ml/min. 20ul portions of a suitable concentrations of isolated cryptolepine as well as methanolic root extract were loaded and injected in turn unto the column and run for a maximum of 10min. The eluent was monitored at 366 nm and AUFS of 0.500(attenuation=0). The column temperature was ambient. The peak areas were estimated from the chromatogram and used as a measure of concentration. The retention time of isolated cryptolepine was noted from the chromatogram. A calibration curve was drawn for isolated cryptolepine and the content of isolated cryptolepine in the methanolic root extract extrapolated from the curve. This was repeated for different samples of the root at different times and days.

2.3.8 HPLC METHOD DEVELOPMENT AND VALIDATION;

2.3.8.1 PRECISION:

Repeatability (intra-day precision)

0.100g of the powdered roots was subjected to extraction as described in **2.3.5** above and the stock solution was diluted 1.5 times 2 times and 5 times to produce three concentrations within the range of the method using methanol prior to HPLC analysis. Solution of each concentration was injected six times and the peak areas recorded in one day. The relative standard deviation (RSD) of the percentage w/w of isolated cryptolepine in the powdered roots for each concentration was calculated

Intermediate precision (intra-day precision)

0.1000g of powdered roots was accurately weighed and subjected to extraction as described above and the stock solution was diluted 2 times, 5 times and 1.5 times to produce three concentrations within the range of the method using methanol prior to HPLC analysis. This was repeated for three consecutive days and the relative standard deviation (RSD) of the percentage w/w of isolated cryptolepine in the powdered roots for each concentration was calculated.

2.3.8.2 ROBUSTNESS:

The following parameters of the HPLC method for the quantification of isolated cryptolepine in the roots were sequentially varied but keeping all other chromatographic parameters of the system constant; mobile phase composition, pH, flow rate and wavelength of the UV detector.

The effect on the chromatogram and the precision of the method (RSD of %w/w of Cryptolepine) was studied.

CHAPTER THREE

3 RESULTS AND CALCULATIONS

3.1 Phytochemical test

Table 3.1 below shows the results of the phytochemical tests performed on the water/methanolic (1:1) extract of the roots of *Cryptolepis sanguinolenta*;

Table 3.1 Phytochemical test on C. *sanguinolenta* roots

TEST FOR→	ALKALOID	TANNINS	GLYCOSIDES	STEROIDS
RESULT→	Positive	Negative	Negative	Negative

3.2 Method Development

Investigation into ideal mobile phase for the HPLC analysis of cryptolepine

Table 3.2 Investigation into ideal mobile phase for the HPLC analysis.

Mobile phase	**Nature of Peak**	**Retention time**(R_t) (min)
Methanol :water 80:20	Peak tails significantly (T>2) at the base	2.068
Methanol: water 90:9 and pH modified to 2.4 using glacial acetic acid	Peak tails significantly (T>2) at the base and broadens slightly at the peak maximum.	3.616
Methanol:Monobasic Phosphate buffer (0.02M, pH adjusted to 2.4 using H_3PO_4) 90:10	Peak tails slightly at the base (T≈2.0).	3.087
Acetonitrile:0.5%v/v TFA,(pH 2.22) 90:10	Peak tails slightly at the base (T≈2.0).	3.956
Methanol:TFA,(0.05%v/v) 90:10	Peak tails slightly at the base (T≈2.1).	2.975
Methanol:water(90:9) modified with trifluoroacetic (TFA,(98%)) acid to a pH of 2.4	No tailing peak(T<<2)	2.678

3.3 HPLC Method Development and Validation Data;

Table 3.3 Peak areas of various concentrations of isolated cryptolepine

Concentration of isolated cryptolepine	Peak area
0.001020	104.0
0.000816	86.33
0.000510	50.67
0.000408	40.33
0.000102	4.17

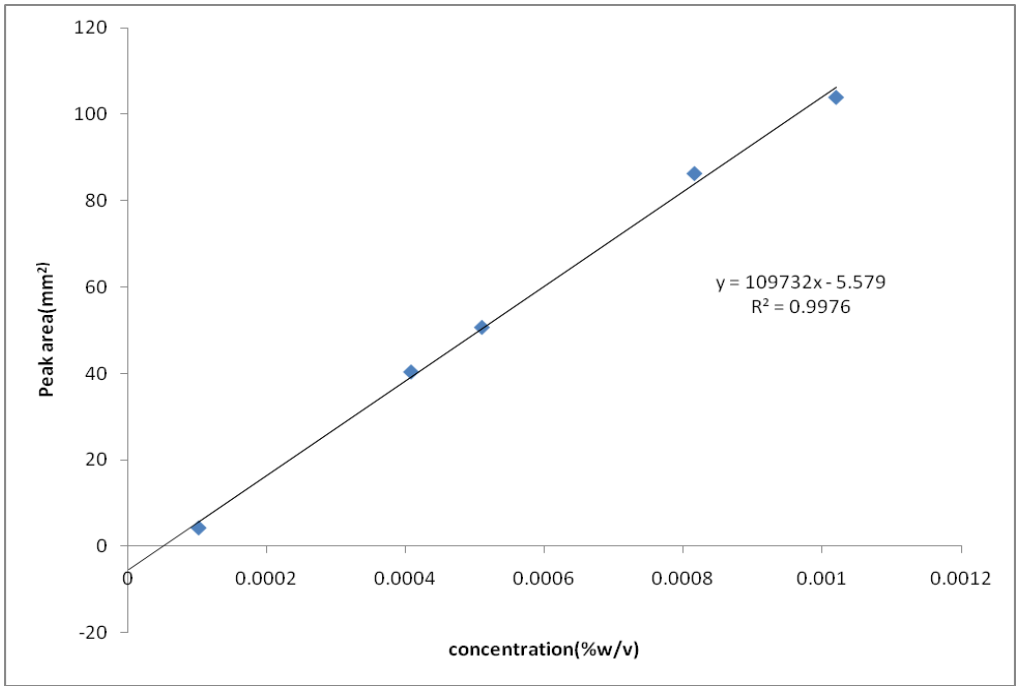


Figure 3:1 A calibration curve plot of conc. of isolated cryptolepine vrs. peak area.

Range =1.02µg/ml-10.2µg/ml Linearity; Coefficient of correlation, $R^2 = 0.9976$

3.3.1 REPEATABILITY /INTRA-DAY PRECISION

Table 3.4 shows the results for 1.5times dilution of the stock methanolic extract of the roots;

Table 3.4 results for 1.5times dilution of the stock.

Peak area(mm^2)	Weight of cryptolepine in root sample(g)	% w/w of Cryptolepine in root
71.94	0.002649	2.649
72.25	0.002660	2.660
72.40	0.002665	2.665
73.10	0.002689	2.689
72.75	0.002677	2.677
74.10	0.002723	2.723
		Average=2.677%w/w
		Std Dev=0.02637
		RSD=0.99%

Table 3.5 results for 2times dilution of the stock.

Peak area(mm^2)	Weight of cryptolepine in root sample(g)	% w/w of Cryptolepine in root
54.83	0.002753	2.753
54.70	0.002747	2.747
53.10	0.002674	2.674
55.01	0.002761	2.761
55.30	0.002774	2.774
56.24	0.002817	2.817
		Average content=2.754%w/w
		Std Dev=0.04662
		RSD=1.69%

Table 3.6 results for 5times dilution of the stock

Peak area(mm^2)	Weight of cryptolepine in root sample(g)	% w/w of Cryptolepine in root
21.54	0.003089	3.089
20.89	0.003015	3.015
21.93	0.003134	3.134
21.72	0.003110	3.110
21.43	0.003077	3.077
20.98	0.003025	3.025
		Average content = 3.075%w/w
		Std Dev=0.046917
		RSD=1.53%

3.3.2 INTERMEDIATE/INTER-DAY PRECISION

Day	Concentrations(dilutions of stock)	Average % w/w of Cryptolepine (n=6)	RSD
1	1.5 times 2 times 5 times	2.677 2.754 3.075	0.99% 1.67% 1.53%
2	1.5 times 2 times 5 times	2.579 3.021 2.654	1.76% 1.09% 1.87%
3	1.5 times 2 times 5 times	2.929 2.625 2.552	0.98% 1.98% 1.47%

3.3.2.1 CALCULATION OF THE PERCENTAGE CONTENT OF CRYPTOLEPINE IN THE ROOTS

Sample calculation;

From the calibration curve above (**Fig3:1)**

The equation of the regression line for the calibration curve is given by;

y = 109732x - 5.579 ---------- (Equation1)

where y is the peak area and x is the concentration(%w/v) of the methanolic solution of isolated cryptolepine.

Hence for peak area= **71.94mm^2** from the 1.5times dilution of the stock (see **table 3.4**), substituting into equation 1 above, the corresponding concentration of isolated cryptolepine in the diluted extract, x, is given by;

x= (y+5.579)/109732

x= (71.94+5.579)/109732

x= 0.0007064%w/v

Hence concentration of cryptolepine in the stock solution= 1.5×0.0007064%

= 0.0010596%w/v

This implies that;

Each 100ml of the methanolic extract contains 0.0010596g of cryptolepine

Hence 250ml contains;

(250ml/100ml)× 0.0010596g = **0.002649g**

For 0.1g of powdered root extracted,

Percentage by weight of cryptolepine in the roots analysed = (0.002649g/0.1000g)×100%

= **2.649**%w/w.

The percentage content of cryptolepine for all the data were calculated as above and the average percentage content of Cryptolepine in the roots for the three different days = **2.763±0.185% w/w(n=9)**

3.3.3 ROBUSTNESS

Table 3.7 Influence of changing mobile phase composition on the chromatogram of Cryptolepine and method precision.

EFFECT→ MOBILE PHASE ↓	PEAK OF ISOLATED CRYPTOLEPINE	RETENTION TIME (min)	PRECISION OF METHOD
Methanol: water 85:14 modified to pH 2.4 with TFA,(98%).	Tails slightly (tailing factor, T≈1.3).	3.234	RSD<2% Precise.
Methanol: water 98:2 modified to pH 2.4 with TFA,(98%).	Significant tailing (T>>2) of the peak at the base.	3.640	RSD>2% Not precise.
Methanol: TFA,(98%) 99:0.1 (pH=2.20).	Broadens at the peak maximum and tails significantly.	None	None

Methanol: water 40:59 modified to pH 2.4 with TFA,(98%).	Significant tailing of the peak.	3.871	Not precise
Methanol: 0.05% v/v TFA,(98%) 60:40 (pH=2.68)	Broadens at the peak maximum and tails.	Broad, ≈2.668	Not precise

Table 3.8 Influence of changing mobile phase pH on the chromatogram of Cryptolepine and method precision.

EFFECT→ pH OF MOBILE PHASE ↓	PEAK OF ISOLATED CRYPTOLEPINE	RETENTION TIME(MIN)	PRECISION OF METHOD
2.21	No tailing	2.676	Precise (RSD<2%)
2.33	No tailing	2.582	Precise (RSD<2%)
2.56	No tailing	2.634	Precise (RSD<2%)
2.60	Slight tailing at the base (T>2%)	2.724	Precise (RSD<2%)

Table 3.9 influence of flow rate variation on the chromatogram of Cryptolepine and method precision.

EFFECT→ FLOW RATE(ML/MIN) ↓	PEAK OF ISOLATED CRYPTOLEPINE	RETENTION TIME (min)	PRECISION OF METHOD
1.3	No tailing peak	2.030	Precise ; RSD<2%
1.1	No tailing peak	2.406	Precise; RSD<2%
0.9	No tailing peak	3.567	Precise; RSD<2%
0.7	Peak tails slightly(T≈2.0)	4.730	Precise; RSD<2%

Table 3.10 influence of variation of wavelength of UV detection on the chromatogram of Cryptolepine and method precision.

Wavelength(nm)	Peak of ISOLATED CRYPTOLEPINE	Retention time(min)	Precision of Method
364	Single peak No tailing	2.678	Precise; RSD<2%
365	Single peak No tailing	2.706	Precise; RSD<2%
367	Single peak No tailing	2.667	Precise; RSD<2%
368	Single peak No tailing	2.634	Precise; RSD<2%

3.3.4 LIMIT OF DETECTION (LOD)

ICH Q2B 1996 Guidelines gives the following formula for calculation of LOD;

LOD= (3.3δ)/s

δ=residual standard deviation of the regression line(RSDR)

s= slope of the calibration curve.

x(%w/v)	$y_{est}(mm^2)$	Y	$y-y_{est}$
0.000408	39.19166	40.33	1.138344
0.000816	83.96231	86.33	2.367688
0.00102	106.3476	104.0	-2.34764
0.00051	50.38432	50.67	0.28568
0.000102	5.613664	4.17	-1.44366
			Residual std=1.909121

LOD of method= (3.3×1.909121)/109732=**0.574µg/ml**

3.3.5 LIMIT OF QUANTITATION (LOQ).

LOQ= (10 δ)/s

= (10×1.909121)/109732= **1.740µg/ml**

CHAPTER FOUR

4 DISCUSSION AND CONCLUSION

4.1 CHARACTERIZATION OF ISOLATED CRYPTOLEPINE

4.1.1 MELTING POINT

The melting point of isolated cryptolepine was determined, as a measure of purity, to be 166-168^{o}C and it was within the range of 166-169^{o}C reported by Grellier,. et al.(1996) and Dwuma-Badu et al.,(1978) as the melting point of cryptolepine.

4.1.2 RETARDATION FACTOR(R_F)

The TLC of isolated cryptolepine(**fig 5.1**) showed only one spot with the same retardation factor as reference cryptolepine (CLP-R) in two different solvent systems. The average R_f –value for isolated cryptolepine in solvent system I: Ethylacetate: Methanol: Ammonia (35%) 80: 15:5, was found to be **0.536±0.043 (n=3)** and that of isolated cryptolepine in solvent system II: ethylacetate: chloroform: diethylamine 80:15:5, was found to be **0.252±0.01 (n=3).**

4.1.3 HPLC

The HPLC chromatogram of isolated cryptolepine gave a single peak**(Fig. 5.5**) with average retention time of **2.723±0.069 min(n=8)**. This implies that with the chromatographic conditions described in section **2.4.7**, the average retention time of isolated cryptolepine was found to be **2.723±0.069 (n=8)** min. The acidic condition (pH=2.4) was chosen for the HPLC analysis to ensure that cryptolepine was fully ionised in solution since partial ionisation could lead to double peaks and tailing.

4.1.4 SPECTROPHOTOMETRIC ANALYSIS OF ISOLATED CRYPTOLEPINE;

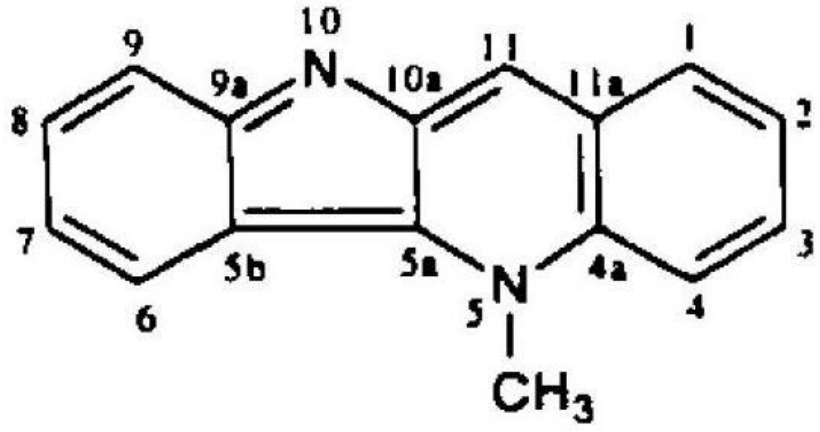


Figure 1:2.1 Chemical structure of Cryptolepine

4.1.4.1 ^{13}C NMR SPECTROPHOTOMETRIC ANALYSIS OF ISOLATED CRYPTOLEPINE

Heteroaromatic carbons give signals within the range δ 100-165ppm(**Carbon NMR Spectroscopy)** and **Fig. 5:14** shows nine peaks within this range of chemical shift under CH ^{13}C DEPT analysis of isolated cryptolepine. This confirms the nine protonated heteroaromatic carbons in cryptolepine: C-1,2,3,4,6,7,8,9, and 11.

There are no CH_2 (sp^2 hybridised) carbons in isolated cryptolepine as there was no peak observed for the CH_2 ^{13}C DEPT analysis of isolated cryptolepine and this is in congruence with the chemical structure of cryptolepine.

sp^3 hybridised carbons absorb at lowest field (δ 0-50ppm) as compared to sp^2(δ100-160ppm aromatic) and sp(δ 65-90ppm) hybridised carbons(**C^{13}-NMR Interpretation**). **Figure 5:14-15** reveals under the CH_3 carbons that the^{13}C DEPT analysis of isolated cryptolepine showed a singlet at δ 40.3ppm which confirms the presence of the only methyl carbon on the quinoline nitrogen at position 5 of cryptolepine (>N-CH_3).

Dimethyl Sulfoxide (DMSO) usually gives a singlet signal at δ 39.51ppm from TMS (**NMR Solvent Data Chart**) hence with reference to **Figure 5:14**, DEPT signal at about 40ppm from TMS under the CH carbons (sp hybridised) for the isolated cryptolepine might be residual solvent peak for the two symmetrical methyl carbons of DMSO used for the ^{13}C NMR analysis of the isolated cryptolepine.

Quaternary carbons are not detected by DEPT analysis (**Mitchell and Costisella, 2007)** and hence **Figure 5:14** shows there was no peaks for carbon number 9a, 10a, 5b, 5a, 4a, and 11a.

4.1.4.2 ^{1}H NMR SPECTROPHOTOMETRIC ANALYSIS OF ISOLATED CRYPTOLEPINE

The ^{1}H NMR of isolated cryptolepine with reference to **Fig. 5:10** was consistent with the data reported by Grellier et al., (1996).

The ^{1}H NMR of isolated cryptolepine showed an intense singlet signal at δ 5.023ppm with integral of 36mm corresponding to 3H of the methyl group of cryptolepine at position 5**.** Since the methyl group at position 5 of cryptolepine has no neighbouring protons to couple it, it gave a singlet peak at δ 5.023ppm typical of signal produced by methyl group attached to a tertiary nitrogen.

The proton NMR of isolated cryptolepine**(Fig. 5:10)** showed aromatic multiplets from δ 7.467 to 8.808ppm and an aromatic singlet at δ 9.304ppm corresponding to eight aromatic protons and one isolated aromatic hydrogen of cryptolepine respectively. The aromatic singlet at δ 9.304ppm from TMS may be due to the hydrogen at position 11 of cryptolepine (H-11) which is the only H without neighbouring hydrogen atoms to split its signal.

With reference to **Fig 5:11**, the multiplet produced from δ 8.808 to 8.740ppm from TMS was due to two hydrogens(2H) which may be due to protons at position 4 and 6 (H-4, δ 8.808ppm, doublet(d), J=8.4 and H-6, δ 8.771ppm,d, 9.3).

The chemical shift position from δ 7.952 to 7.848ppm showed multiplet peaks which were fused together and corresponds to three protons. The fused double doublets peaks produced could be due to H-2, H-9 and H-8 of cryptolepine.

Residual protons of $DMSO_d$ occur at δ 2.5 (**NMR Solvent Data Chart**) and hence the peak at this chemical shift could be due to the methyl protons of the residual DMSO in the solvent used to determine the ^1H NMR of isolated cryptolepine.

4.1.4.3 INFRARED SPECTROPHOTOMETRIC ANALYSIS OF ISOLATED CRYPTOLEPINE

With reference to **Fig 5:18**, the IR spectrum of isolated cryptolepine shows absorption bands between 650cm^{-1} and about 1640 cm^{-1} followed by apparently no significant absorption bands from about 1800 to 2600cm^{-1} and finally broad absorption bands at 2600-3500cm^{-1}.

The IR spectrum of isolated cryptolepine shows a broad absorption band at 3077.57 cm^{-1} which may be due to heteroaromatic C—H stretching bands of cryptolepine at which usually occur at 3077-3003cm^{-1} region (**Silverstein and Bassler, 1967**). There was a weak absorption band at about 2922 cm^{-1} which may be due to the aliphatic symmetric stretch of the methyl goup of cryptolepine at position 5 which occurs at about 2820-2760$cm^{-1}$for methyl group attached to nitrogen atom(**Silverstein and Bassler, 1967)**.

In-plane skeletal vibrations of aromatics and heteroaromatics rings resulting from expansion and contraction of the carbon-carbon bonds usually occurs near 1600, 1580, 1500 and 1450 cm^{-1} (**Furniss et al., 1989**). Hence IR absorption bands at 1615.47, 1580.16 and 1507.88 cm^{-1} with respect to **Fig. 5:18** can be attributed to the skeletal vibrations of the heteroaromatic ring of cryptolepine.

Besides, similar band pattern of 1611, 1585 and 1505 cm^{-1} was observed by Dwuma-Badu, et al., (1978) when the IR spectrum of isolated cryptolepine was determined. The band at 1450 cm^{-1} is usually interfered by C—H bending vibrations.

The band at 1458.77 and 1430.34 cm^{-1} with respect to **Fig 5:18** may principally comprise of absorption due to asymmetric bending vibration of CH_3(**Silverstein and Bassler, 1967)** at position 5 of cryptolepine and in-plane skelatal vibration of the heteroaromatic ring.

Symmetrical CH_3 bending vibrations usually occurs near 1375 cm^{-1} and hence the band at 1369.86 cm^{-1} may be due to the bending deformation of the CH_3 at position 5 of cryptolepine.

Heteroaromatic C—H in-plane bending bands occur in the 1300-900 cm^{-1} region and hence IR bands for isolated cryptolepine occuring at 1290.42, 1259, 1219.73, 1139.58, 1040.60, 1075.11, 924.69 and 989.39 cm^{-1} in the fingerprint region may be due to heteroaromatic C—H in-plane deformations.

Aromatic and heteroaromatic compounds display strong out-of plane C—H bending and ring bending absorption bands in the 909-650cm^{-1} region(**Silverstein and Bassler, 1967).** Hence with reference to **Fig 5:18**, the fingerprint bands at 883.35, 846.02, 793.32, 772.02. 753.04 and 664.33cm^{-1} may be due to out-of plane heteroaromatic C—H bending and ring bending vibrations of cryptolepine. There was a strong and sharp absorption at 753.04cm^{-1} (**fig 5:19)** which is characteristic of 1,2 aromatic di-substituted compounds with four adjacent H atoms which are present at both the indole and the quinoline moieties(i.e. H-6,7,8,9 and H-1,2,3,4).

Tertiary amines have C—N stretching vibrations occuring at 1360-1310cm^{-1} (**Silverstein and Bassler, 1967)** and hence the band at 1352.81cm^{-1} might be due to the C—N stretching vibrations in cryptolepine. C=N in conjugation with —C=C— usually have stretching vibrations occuring at the 1660-1630cm^{-1} region and hence with reference to **Fig. 5:19**, the band for isolated cryptolepine at 1638.31cm^{-1} may be due to the C=N stretch vibrations in cryptolepine.

The IR spectrum of isolated cryptolepine showed similar absorption bands consistent with that of cryptolepine as reported by Dwuma-Badu, et al., (1978).

4.1.4.4 MASS SPECTROMETRY OF ISOLATED CRYPTOLEPINE

The base peak of mass spectrometric analysis of isolated cryptolepine, with reference to **Fig 5:22,** was found to be at *m/z* value of 233.27 corresponding to 100% relative intensity. However, the mass spectrometer used for the analysis employed atmospheric pressure chemical ionisation(APCI) at in which most of the ionic species are protonated and hence to determine the molecular formula of a compound from a Chemical ionization or Fast Atom Bombardment(FAB) mass spectrum, 1 amu would have to be subtracted from the *m/z value* of the peak considered to be M^+: the species corresponds to MH^+ (**Sorell, 2006**). Hence, the base peak of the mass spectrum of the isolated cryptolepine which also corresponds to the molecular ion actually occured at *m/z* **232.27**. This value is in agreement with the literature value of the molecular weight of cryptolepine which was 232g.

The peak occuring at *m/z* **219.33**(29.36%) with reference to **Fig 5:22** might correspond to N-demethylated fragment of cryptolepine.

4.1.5 METHOD DEVELOPMENT AND VALIDATION

4.1.5.1 IDEAL MOBILE PHASE

Several mobile phase systems for the isocratic elution of cryptolepine were investigated. Initial trial with methanol: water 80:20 yielded significantly tailing peaks (tailing factor, T >2). This implied that the mobile phase would have to be modified to an acidic medium to ensure complete ionization of cryptolepine.

Hence different acid modifiers including glacial acetic acid, monobasic phosphate buffer and Trifluoroacetic acid (TFA,(98%)) were used (**Table 3.2**) in different proportions but it was evident that the ideal mobile phase was methanol:water 90:10 modified with TFA,(98%) to pH of 2.4. This low pH was used because it ensured complete ionization of cryptolepine and gave well resolved peaks of cryptolepine.

4.1.5.2 LINEARITY AND RANGE

The method as described at section **2.3.7** was found to be linear with equation of line of y=102815x-0.7092 and coefficient of correlation(r^2) of 0.9995 for the regression line of peak area(y) against % concentration of cryptolepine(x) which was greater than 0.998 as stipulated by

the ICH guidelines. The HPLC analytical method for the quantitation of cryptolepine was found to be linear within cryptolepine concentration range of **1.06 µg/ml-10.6 µg/ml.** This range corresponds to concentrations of cryptolepine (inclusive) that could be determined with acceptable precision, accuracy, and linearity using the method.

4.1.5.3 PRECISION

Repeatability (Intra-day precision).

According to the ICH guidelines the accepted criteria for repeatability of an analytical method should be ≤2% RSD of six replicate determinations each for three different analyte concentrations. With reference to the results obtained from the repeatability studies carried out on the analytical method in section **3.4.1**, the RSD for 1.5times, 2times and 5times dilutions of the stock for the intra-day were 0.99%,1.69% and1.53% respectively. Hence, it can be inferred that the HPLC method developed to quantate the levels of cryptolepine in the roots of Cryptolepis was repeatable since all the RSD were less than 2%.

Intermediate(Inter day) precision

According to the ICH guidelines the accepted criterion for repeatability of an analytical method was ≤2% RSD of six replicate determinations each for three different analyte concentrations on three consecutive days. With reference to the results obtained from the intermediate precision studies carried out on the analytical method in section **3.3.2**, the RSD for 1.5times, 2times and 5times dilutions of the stock for each of the three days were less than 2%. The method can be said to be precise under random events such as different days.

4.1.5.4 ROBUSTNESS

The method was found to be robust when the mobile phase composition, pH , flow rate and wavelength of detection were delibrately altered. The precision of the method was not affected when pH, flow rate and wavelength of detection was varied in the range of 2.4±0.2units , 1±0.3ml/min and 366±2nm respectively.

4.1.5.5 LIMIT OF DETECTION(LOD)

The LOD of the HPLC method developed for the quatification of cryptolepine levels in the roots of *C. sanguinolenta* was found to be **0.574µg/ml** and this was the lowest concentration of

cryptolepine which could be reliably differentiated from the background noise by the HPLC analytical method.

4.1.5.6 LIMIT OF QUANTITATION(LOQ)

The LOQ of the method was found to be **1.740µg/ml** and this represented the lowest concentration of cryptolepine which could be quantified with suitable precision, accuracy and give 10 times the background signal when the HPLC analytical method was used to determine the percentage content of cryptolepine in the dry roots of Cryptolepis.

4.1.5.7 ACCURACY

Accuracy may be inferred once precision, linearity and specificity have been established and hence the analytical method developed was also accurate.

4.1.6 PERCENTAGE CONTENT OF CRYPTOLEPINE IN THE DRY ROOTS OF CRYPTOLEPIS SANGUINOLENTA USING ISOLATED CRYPTOLEPINE AS A SECONDARY REFERENCE STANDARD.

The HPLC method developed was used to determine the average percentage content of cryptolepine in the dry roots of *C. sanguinolenta* and it was found to be **2.763±0.185% w/w% (n=18).** This implies that each 100g of dry roots of *C. sanguinolenta* contained 2.763±0.185g of cryptolepine. It was reported by Iwu, M.M.(1993) that cryptolepine occurred at a yield of **0.54%w/w** in the roots but the method employed was not HPLC. The HPLC method developed in this project was more sensitive to detect and quantitate as highest levels as **2.763±0.185% w/w** of cryptolepine in the dry roots of *C. sanguinolenta*. This value is however subject to variations in the environment where the plant grows.

4.2 CONCLUSION

Cryptolepine was successfully isolated from the dry roots of *C. sanguinolenta*. The melting point of the isolated cryptolepine was found to be **166-168^{o}C** and its authenticity was confirmed by determination of UV, IR, NMR and Mass spectral data assignments which were in agreement with those reported by Grellier et al., (1996) and Dwuma-Badu, et al., (1978).

The reverse phase HPLC-UV method developed for separation and quantification of cryptolepine in the roots of *Cryptolepis sanguinolenta* had the folowing chromatographic parameters; **Column**; Hichrom Zorbax C_8 5micron 15cm×4.6mm id, **Mobile phase**; methanol: water (90:9) modified with trifluoroacetic acid(TFA,(98%)) to a pH of 2.4, **Flow rate**; 1ml/min, **Injection volume**; 20µl, **Wavelength of detection**; 366nm, **AUFS**; 0.5 and **Column temp**; ambient.

The HPLC method developed was validated and found to be precise, accurate, robust, and linear within cryptolepine concentration range of **1.02µg/ml-10.2µg/ml**. Hence, the method can be used to standardise herbal preparations containing extracts of *C. sanguinolenta*. The Limit of detection(LOD) and Limit of Quantitation(LOQ) of the method were found to be **0.574µg/ml** and **1.740µg/ml** respectively.

The percentage content of cryptolepine in the dry roots of *C. sanguinolenta* was found to be **2.763±0.185% w/w (n=9)** using the reverse phase-HPLC method developed.

4.3 RECOMMENDATIONS

- The percentage content of cryptolepine in the dry roots of *C. sanguinolenta* must be investigated seasonally to establish the appropriate period to harvest the roots with highest levels of the major alkaloid, cryptolepine.

- The stability of cryptolepine in herbal aqueous extracts must be investigated to aid in the standardisation of herbal preparations containing extracts of the roots of *C. sanguinolenta*.

- Suitable methods should be developed to quantify the possible degradation products of cryptolepine and establish permissible limits for toxic degradation products.

- Finally, photodegradation of cryptolepine would have to be investigated to establish its stability to light.

APPENDIX

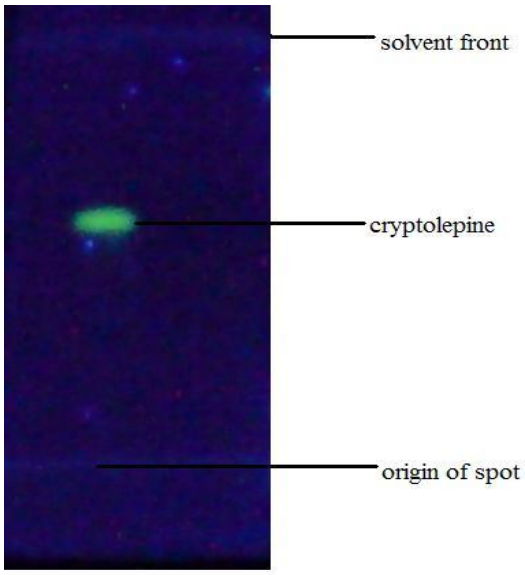


Figure 0:1 TLC chromatogram of isolated cryptolepine (MP=Solvent system I)

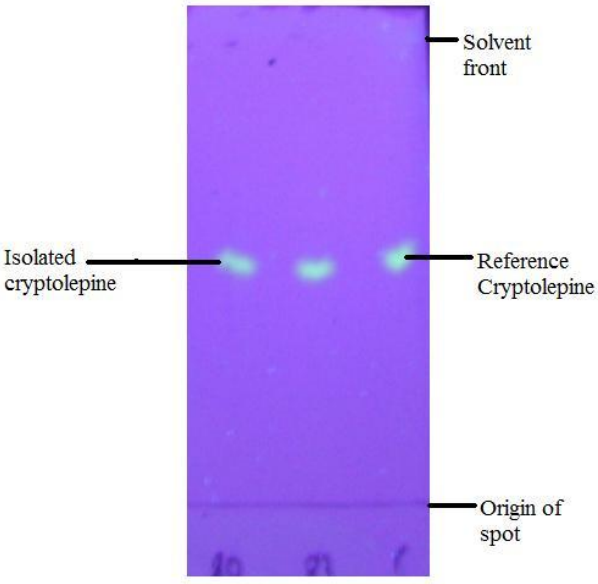


Figure 0:2 TLC chromatogram of isolated cryptolepine along with reference cryptolepine (CLP-R) (MP* solvent system I)

* MP=mobile phase

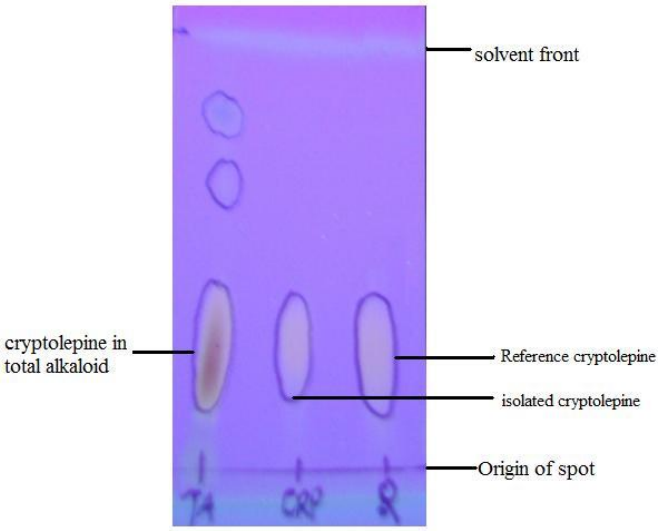


Figure 0:3 TLC chromatogram of total alkaloid, isolated cryptolepine and CLP-R (MP=Solvent system II)

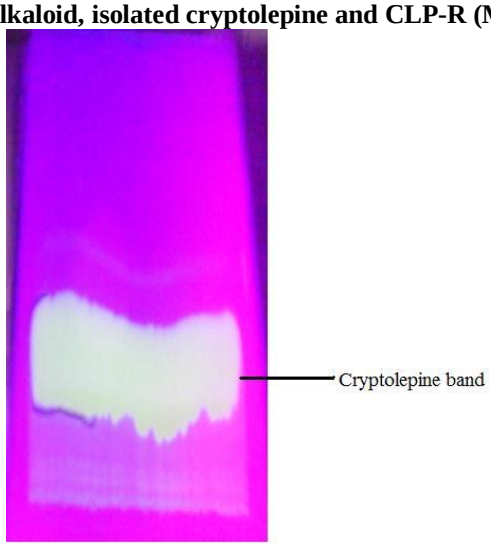


Figure 0:4 Preparative TLC chromatogram of concentrated eluents containing isolated cryptolepine

NB; Developed TLC plate observed under 365nm UV light.

RETARDATION FACTOR (R_f – VALUE) OF ISOLATED CRYPTOLEPINE.

Solvent system	Distance moved by isolated cryptolepine spot(x)/mm			Distance moved by solvent front(y)/mm			R_f value= x/y		
I	23.00	21.50	23.00	45.00	43.00	38.50	0.511	0.500	0.597
II	9.00	10.00	10.50	40.00	42.00	40.00	0.225	0.238	0.263

Hence R_f –value for solvent system I = 0.536± 0.043 and solvent system II= 0.252± 0.01

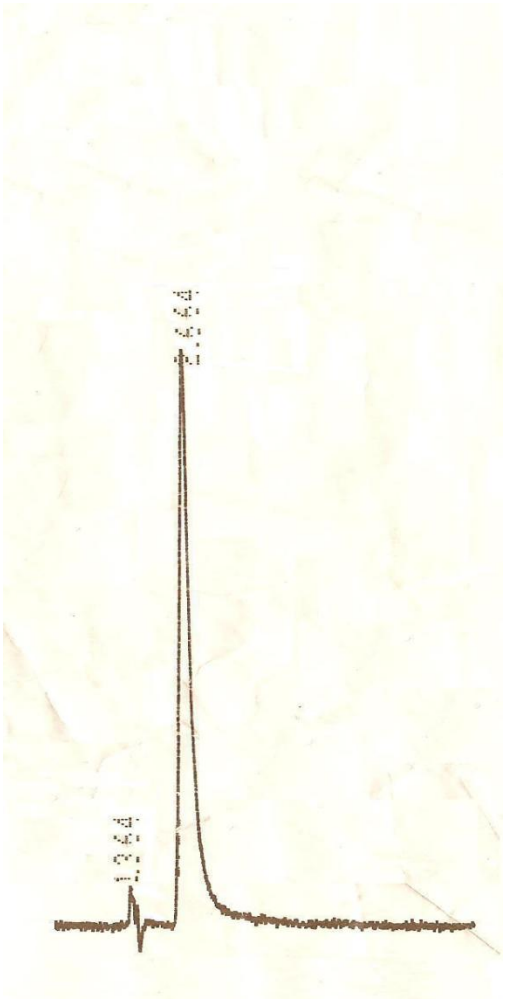


Figure 0:5 HPLC chromatogram of isolated cryptolepine

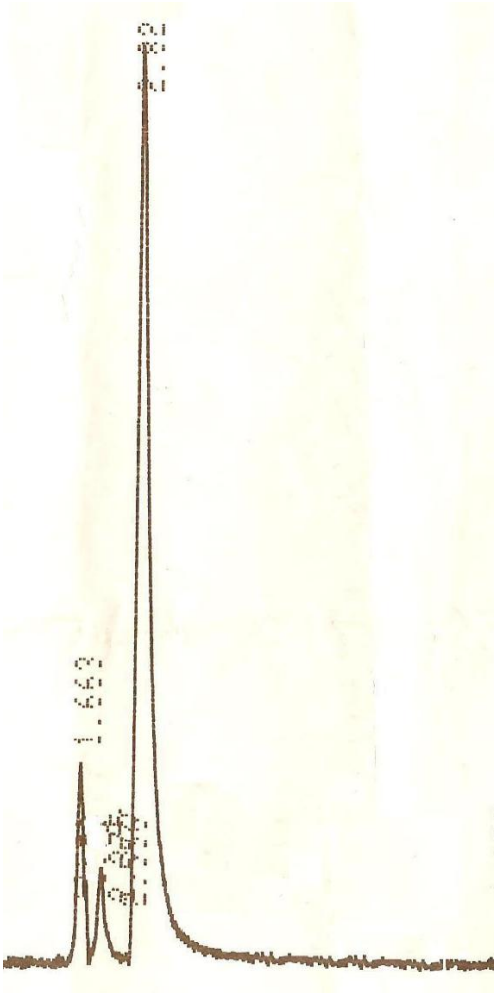


Figure 0:6 HPLC chromatogram of methanolic extract of roots of *C. sanguinolenta*

Average retention time for isolated cryptolepine = (2.664 +2.674+2.663+2.659+2.721+2.741+2.812+2.850)/8

= 2.723±0.069 min.

SOME OF THE HPLC CHROMATOGRAM OF THE DILUTED STOCK SOLUTION OF THE ROOT EXTRACT.

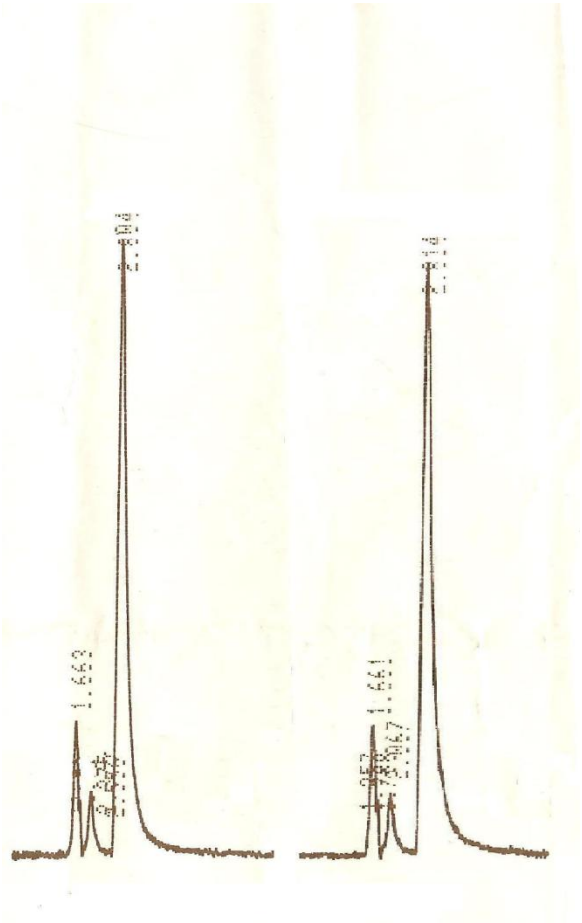


Figure 0:7 Chromatograms for1.5times dilution of stock

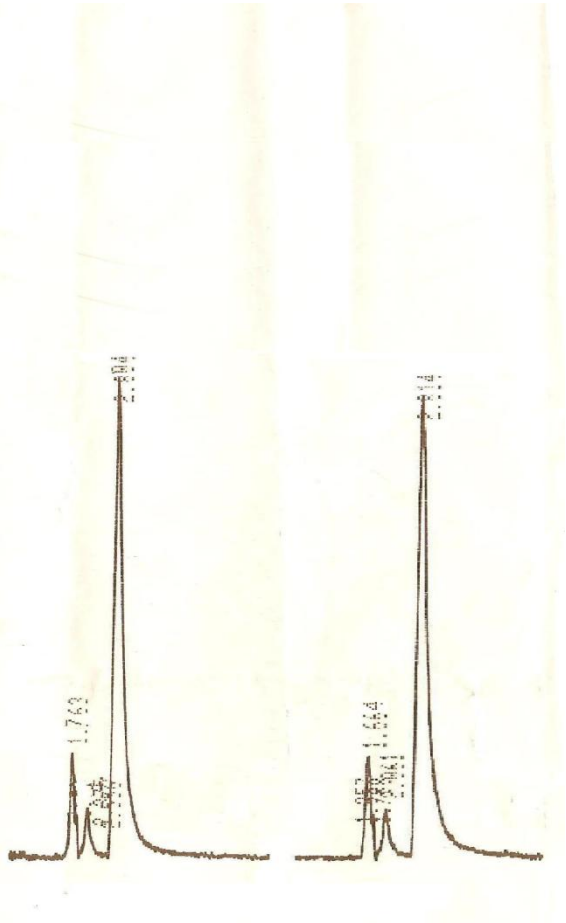


Figure 0:8 Chromatograms for 2 times dilution of stock

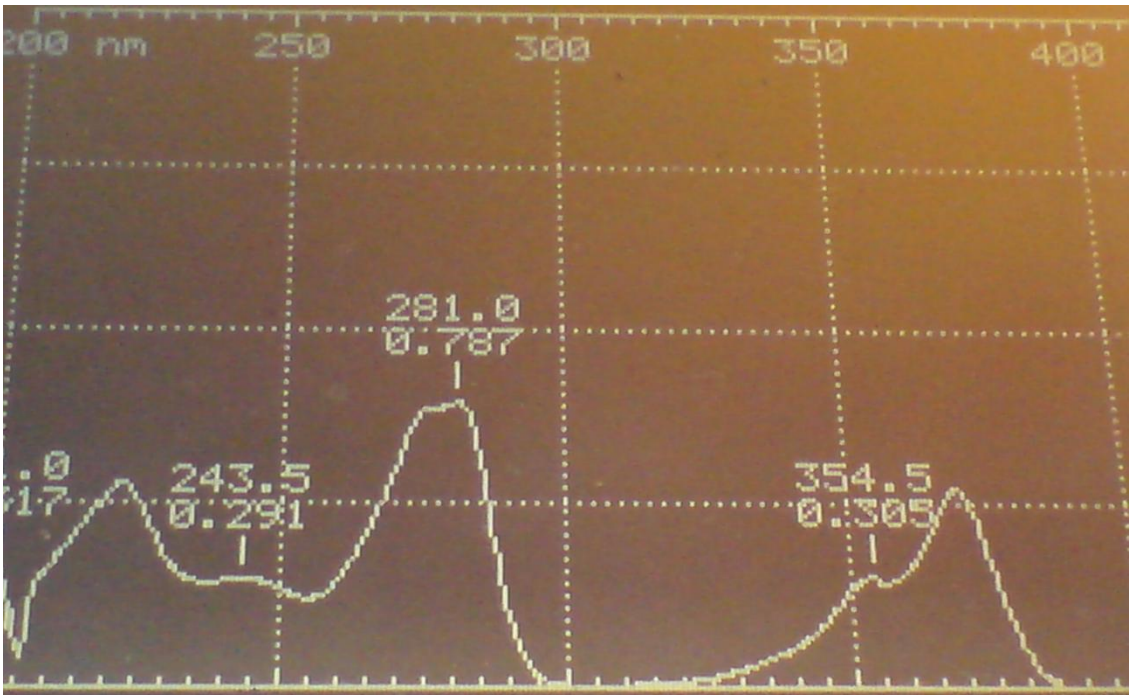


Figure 0:9 UV/Vis spectrum of methanolic solution of isolated cryptolepine.

The methanolic solution of isolated cryptolepine yielded the following data for UV spectrum; λ_{max} 223nm (log ε=3.98), 246 (3.72), 275 (4.17), 281(4.25) 355sh (3.92), 370(4.13)(see Section **2.3.4.1**)

[1]HNMR SPECTRUM OF ISOLATED CRYPTOLEPINE

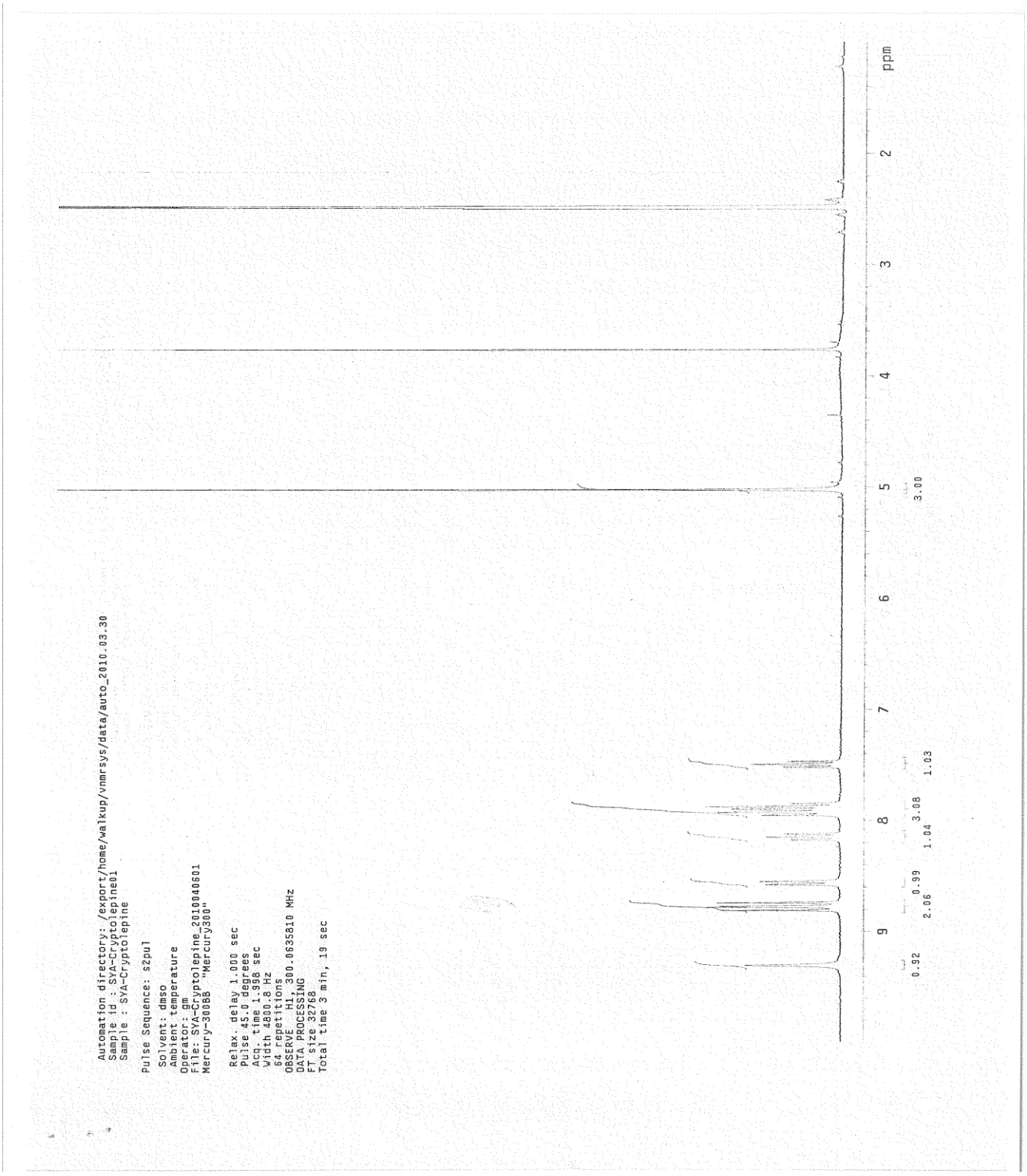


Figure 0:10 [1]HNMR of Isolated Cryptolepine.

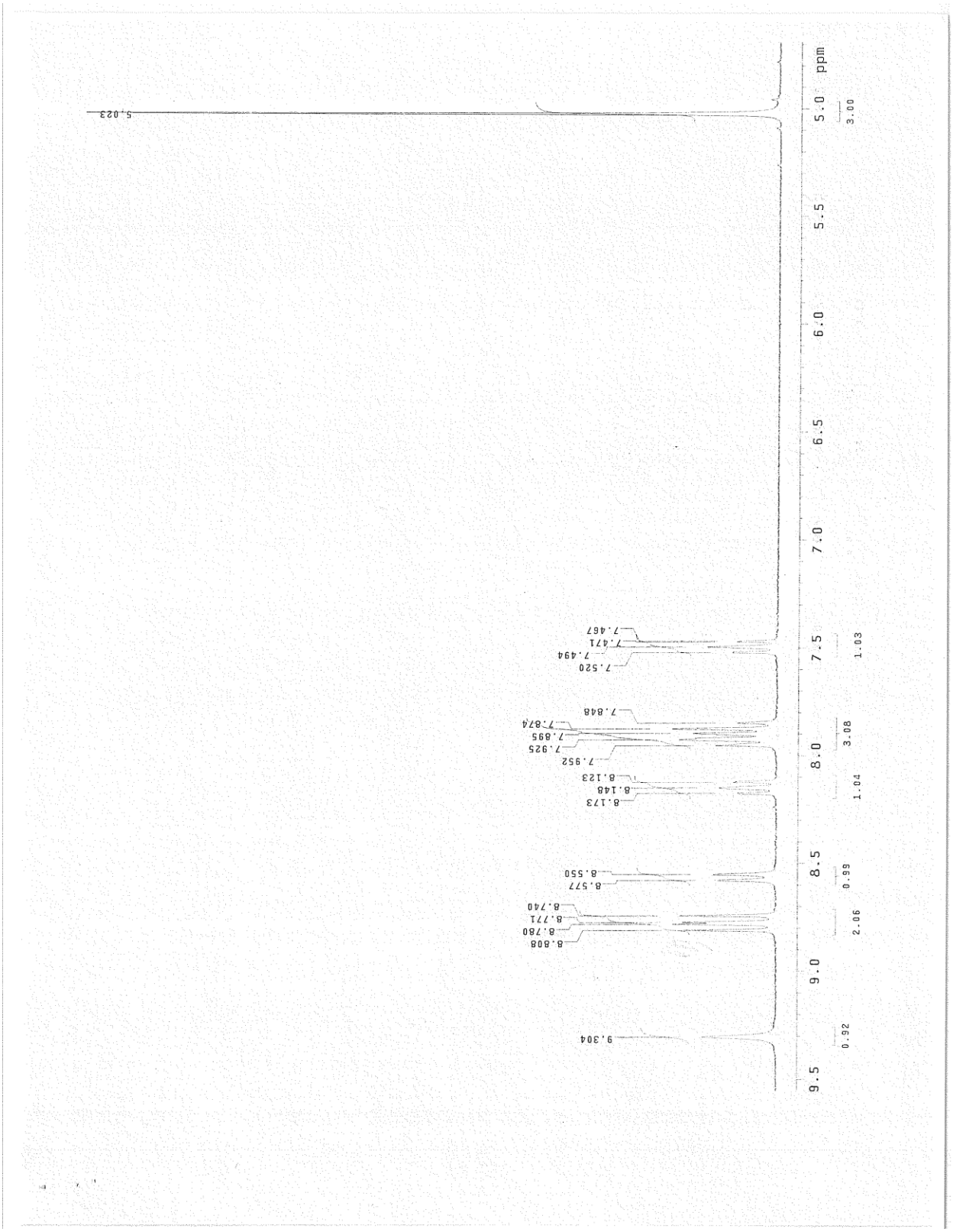


Figure 0:11- Expanded ^{1}HNMR of isolated cryptolepine

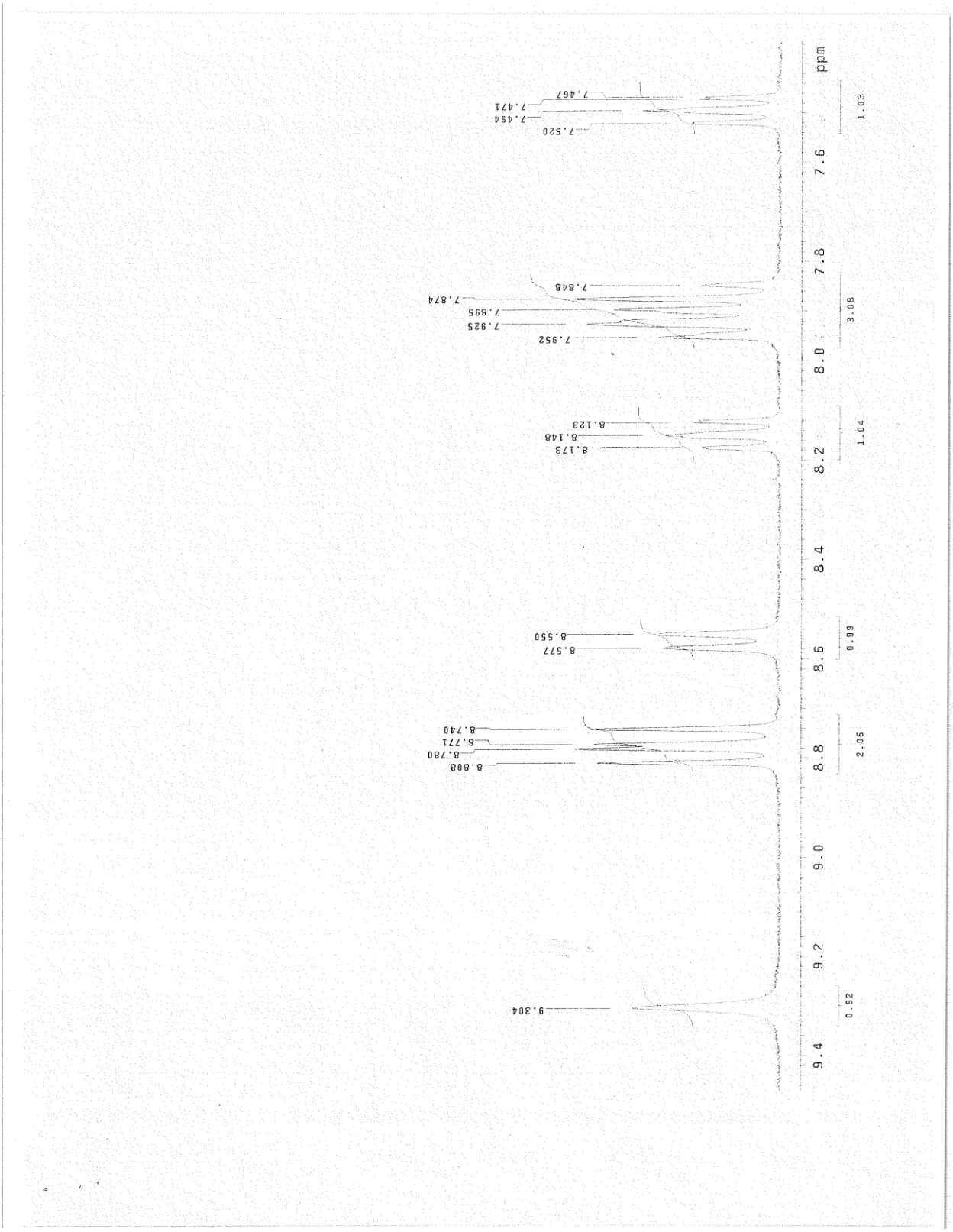


Figure 0:12- Expanded ^{1}HNMR of isolated cryptolepine.

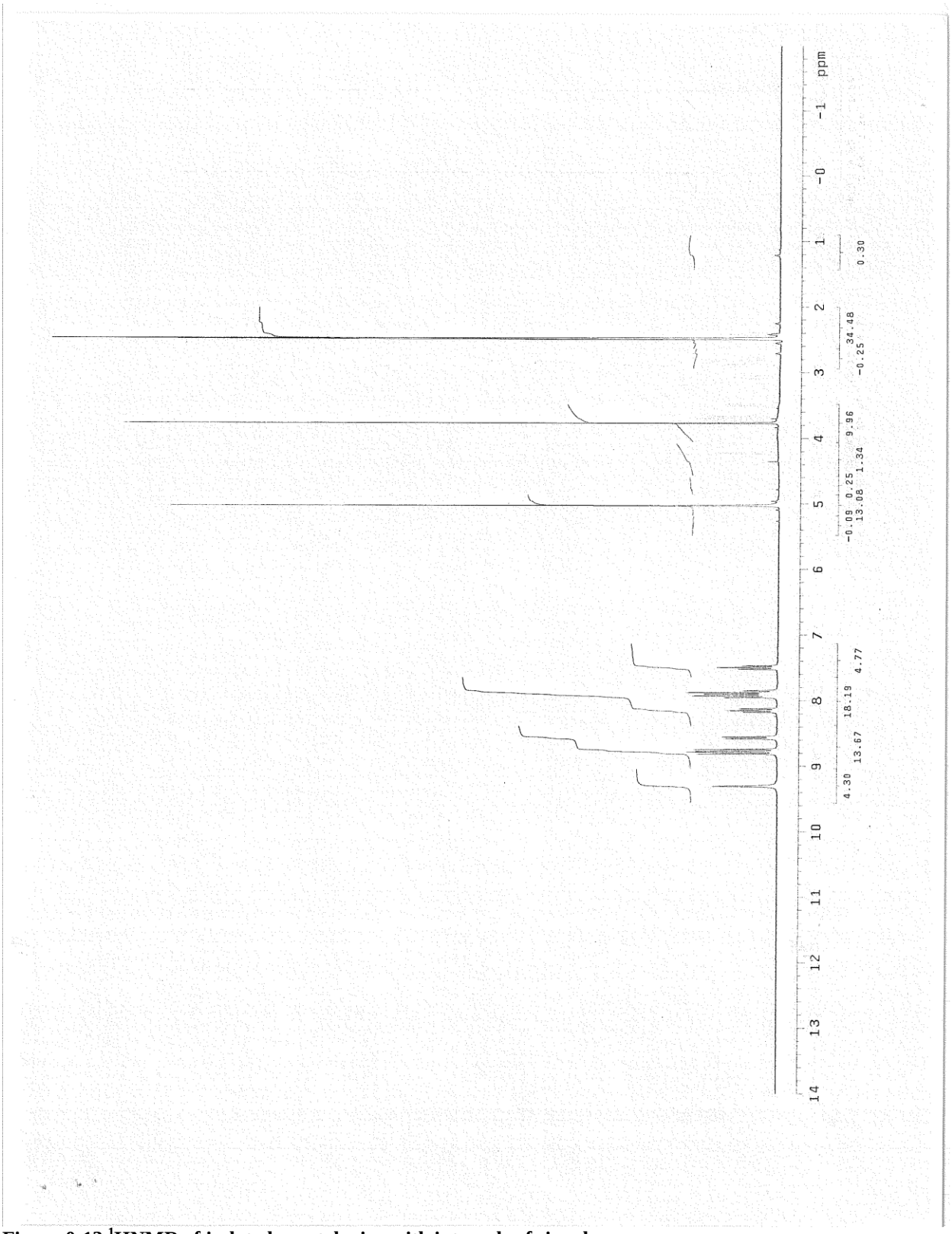


Figure 0:13 [1]HNMR of isolated cryptolepine with integrals of signals.

^{13}C NMR FOR ISOLATED CRYPTOLEPINE

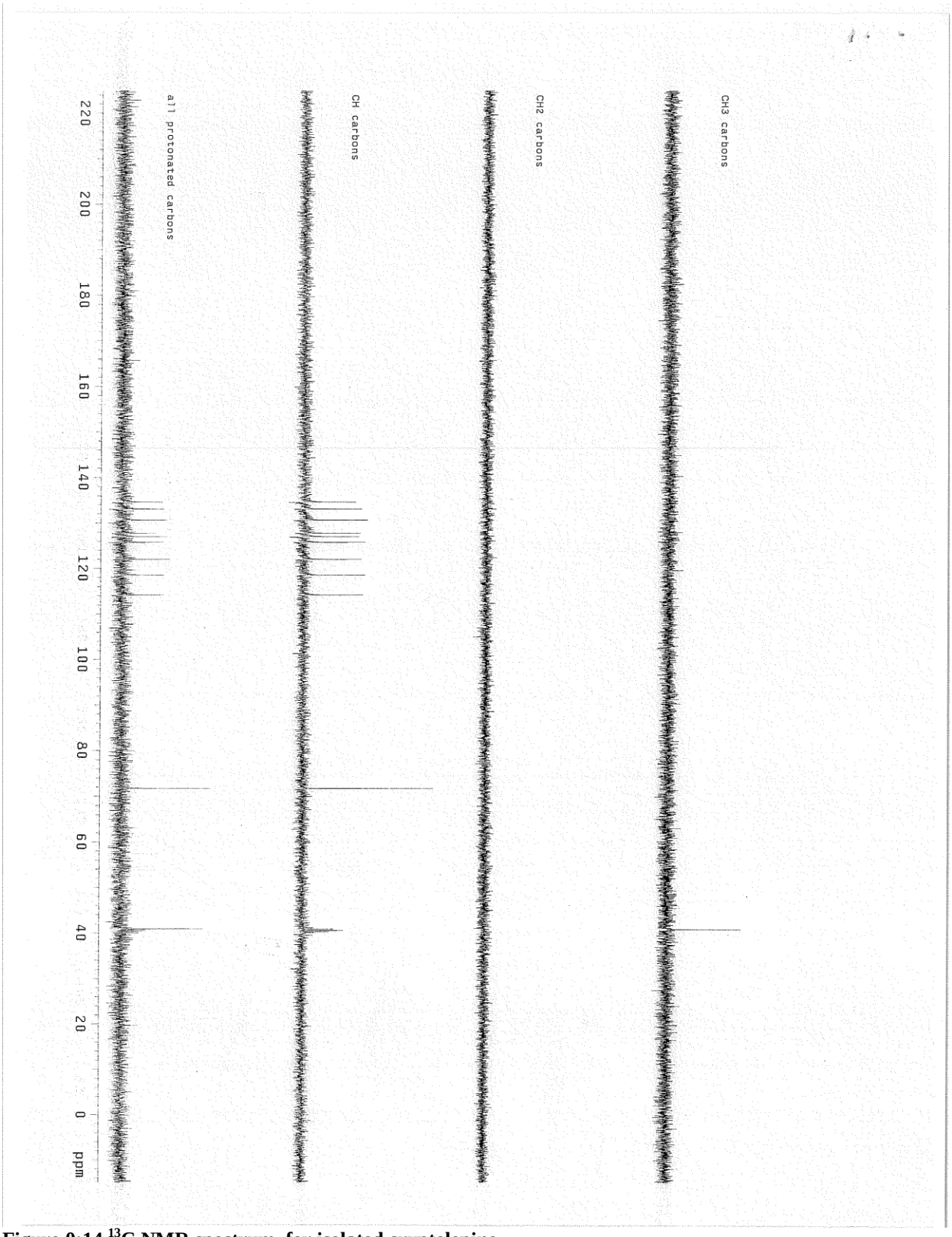


Figure 0:14 ^{13}C NMR spectrum for isolated cryptolepine

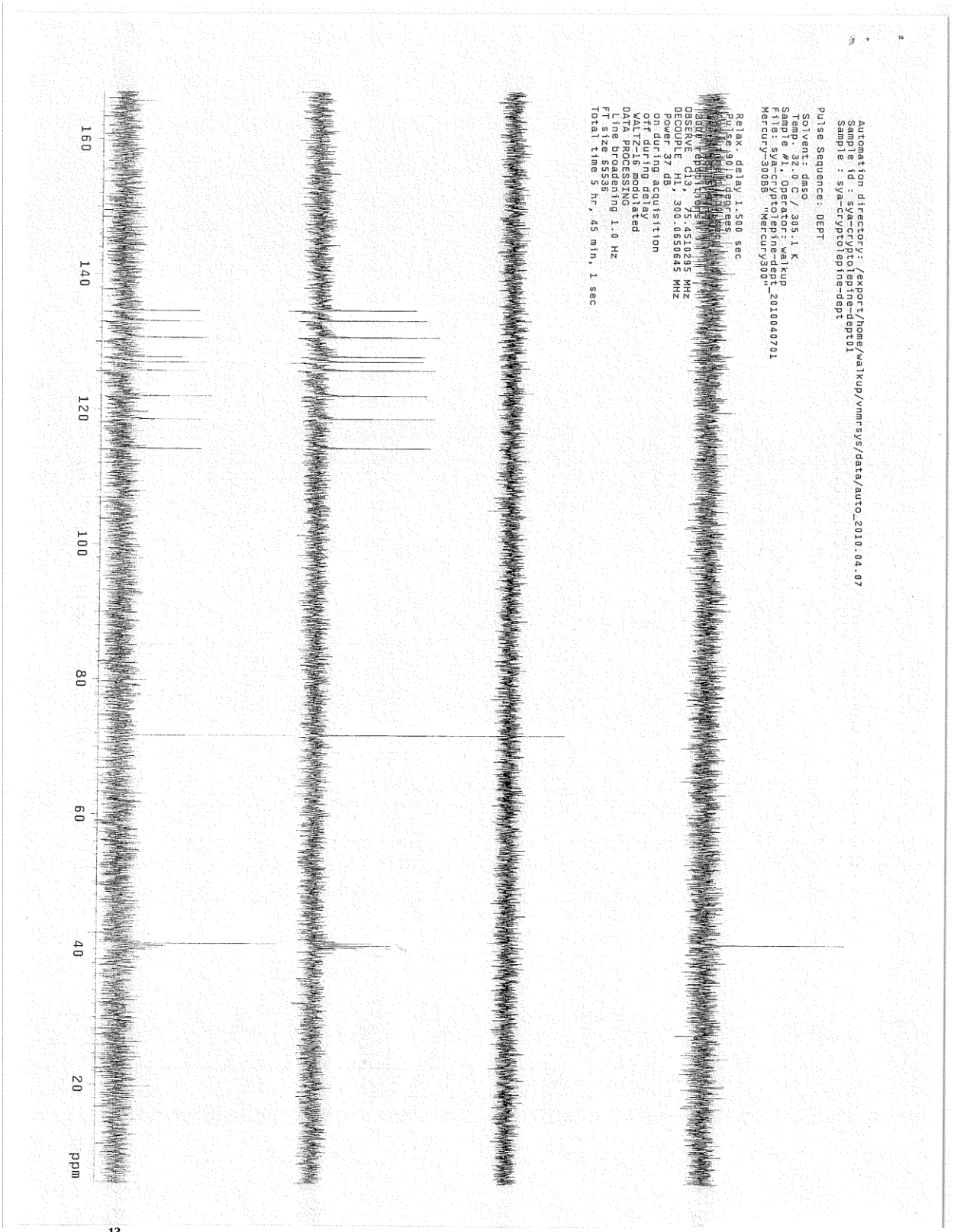


Figure 0:15 ^{13}C NMR spectrum for isolated cryptolepine (enlargement of Fig. 5:14)

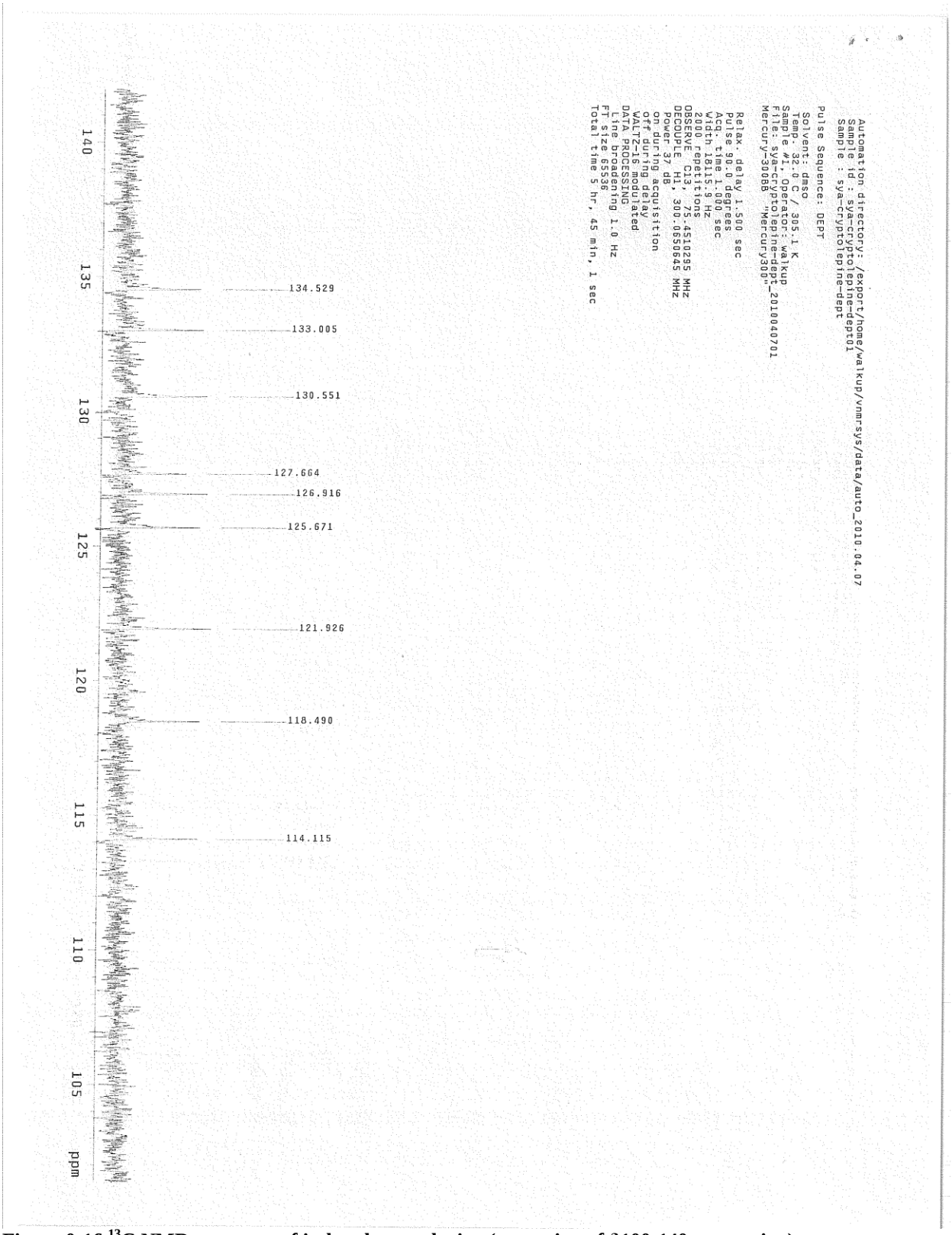


Figure 0:16 ^{13}C NMR spectrum of isolated cryptolepine (expansion of δ100-140ppm region)

IR SPECTRA FOR ISOLATED CRYPTOLEPINE

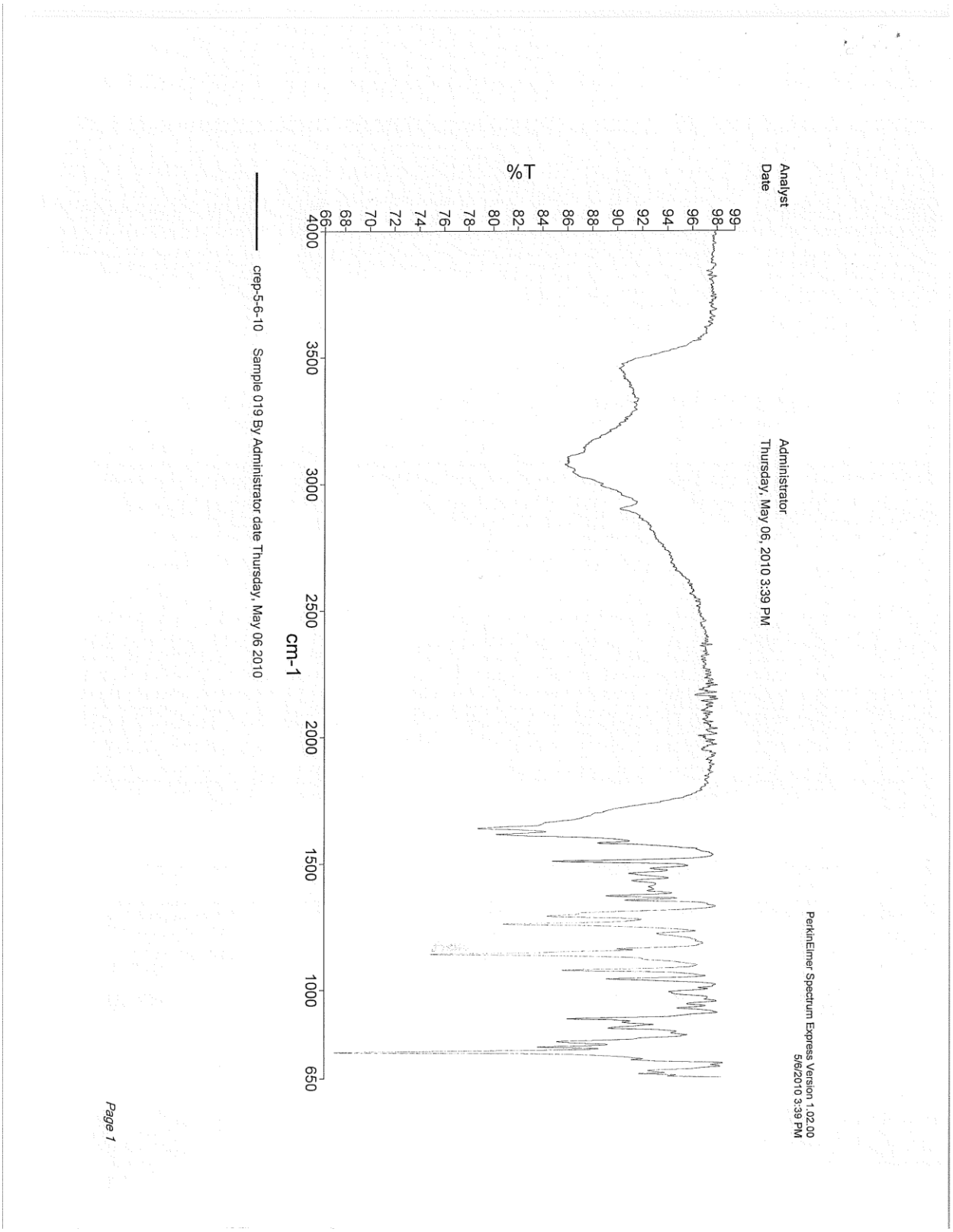


Figure 0:17 Infrared spectrum of isolated cryptolepine

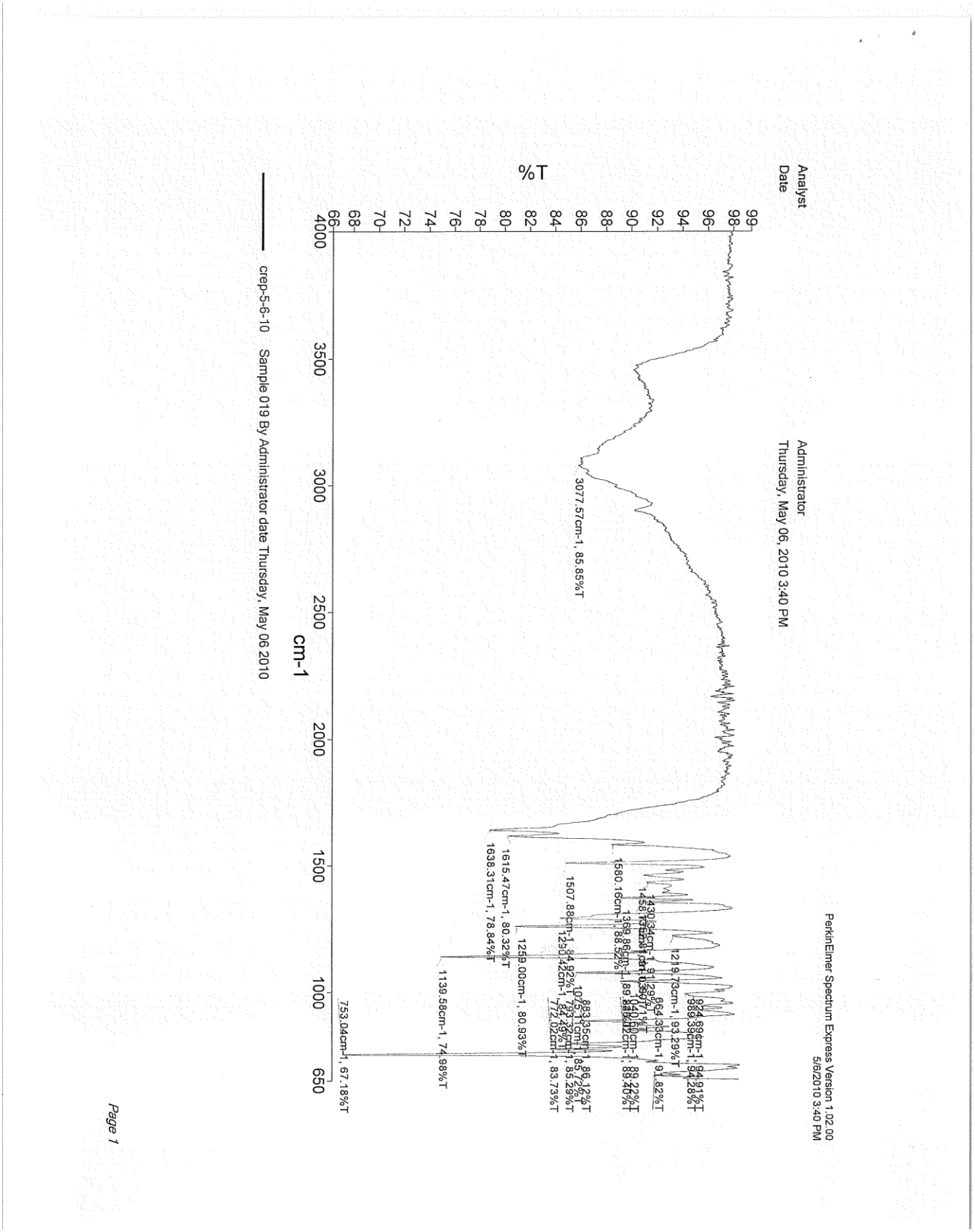


Figure 0:18 Infrared spectrum of isolated cryptolepine (with respective band wavenumbers and percentage transmittances)

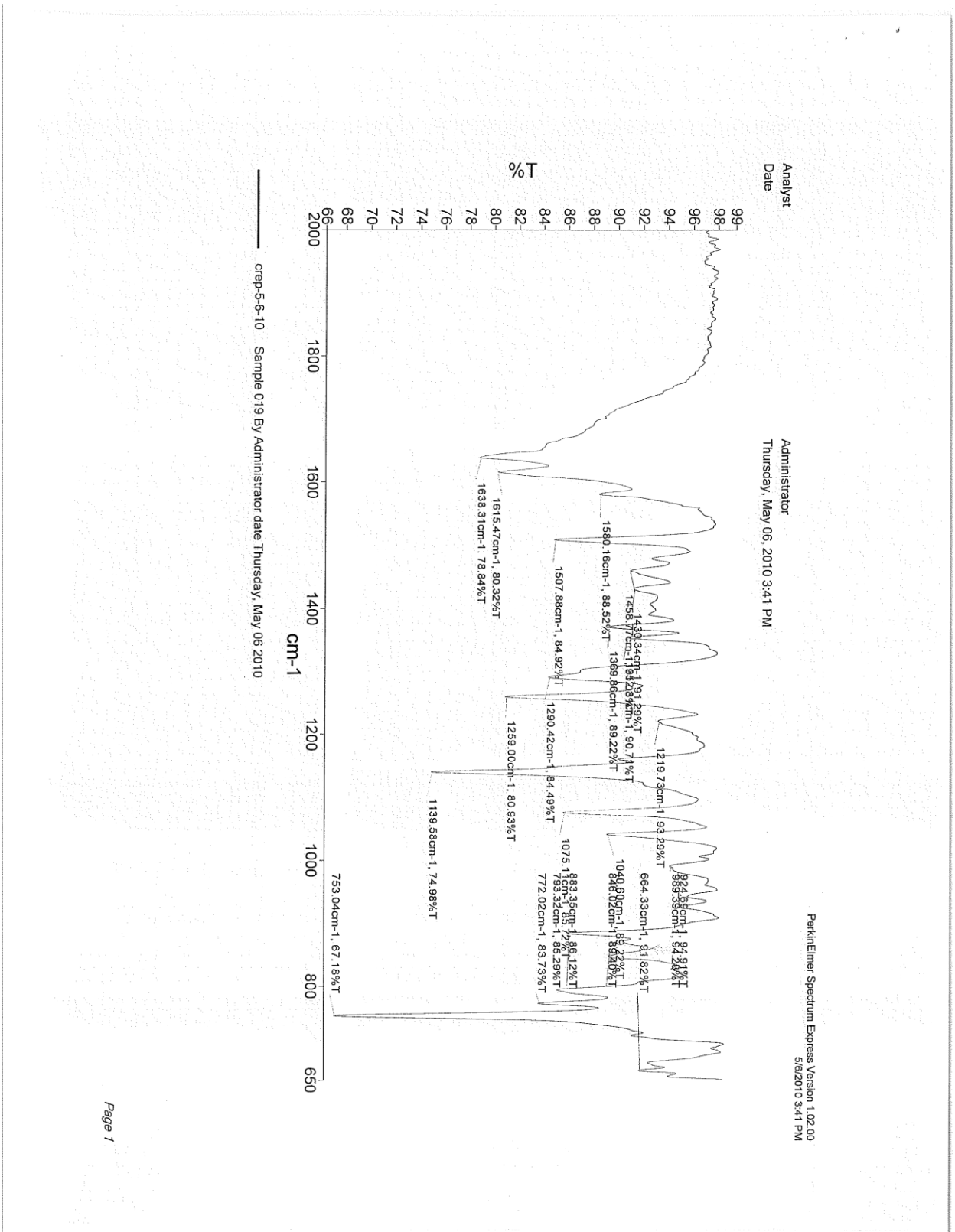


Figure 0:19 Infrared spectrum of isolated cryptolepine (expansion of 650-2000 cm^{-1} region of Fig. 5:18).

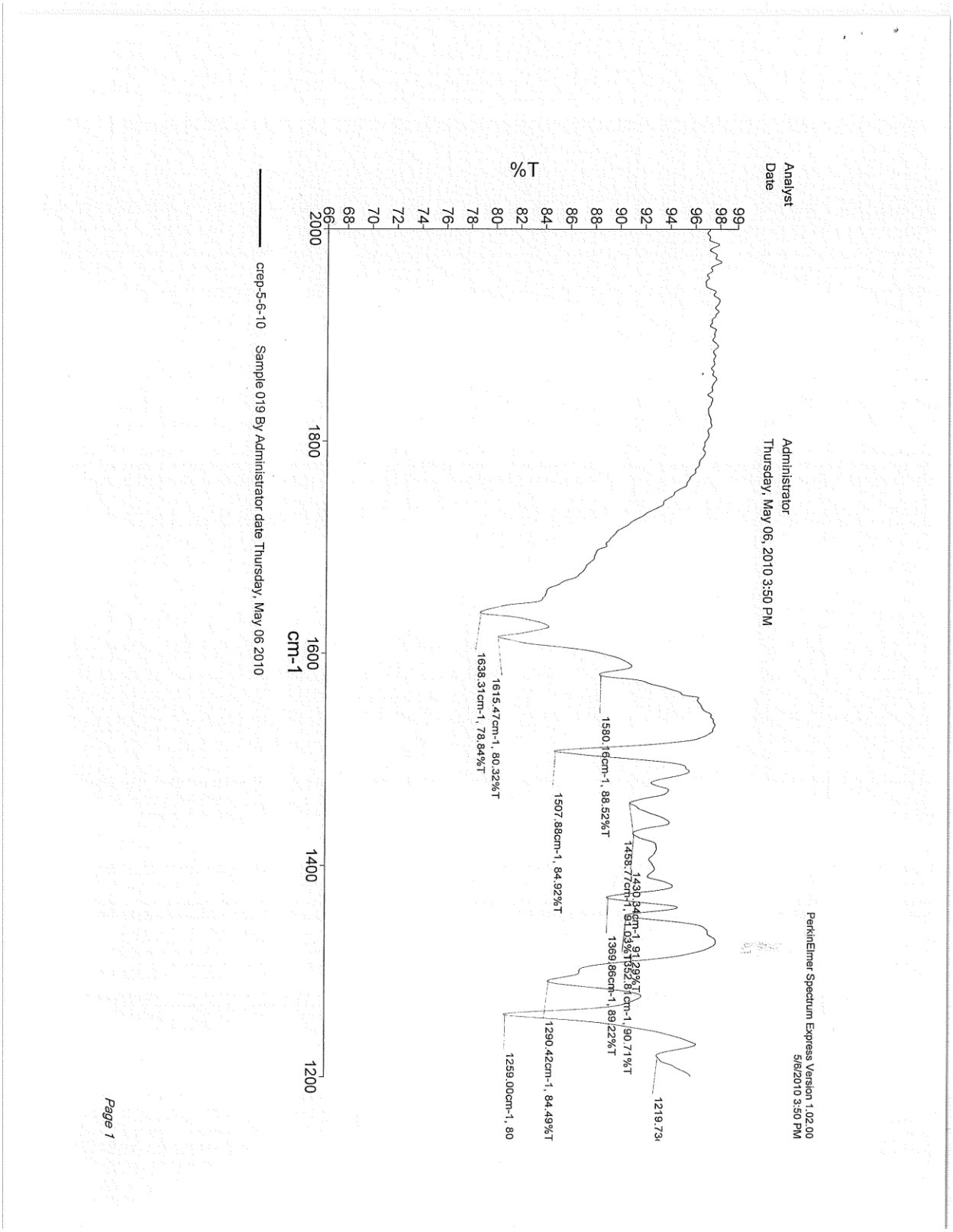


Figure 0:20 Infrared spectrum of isolated cryptolepine (expansion of 1200-2000 cm-1 region of Fig. 5:19).

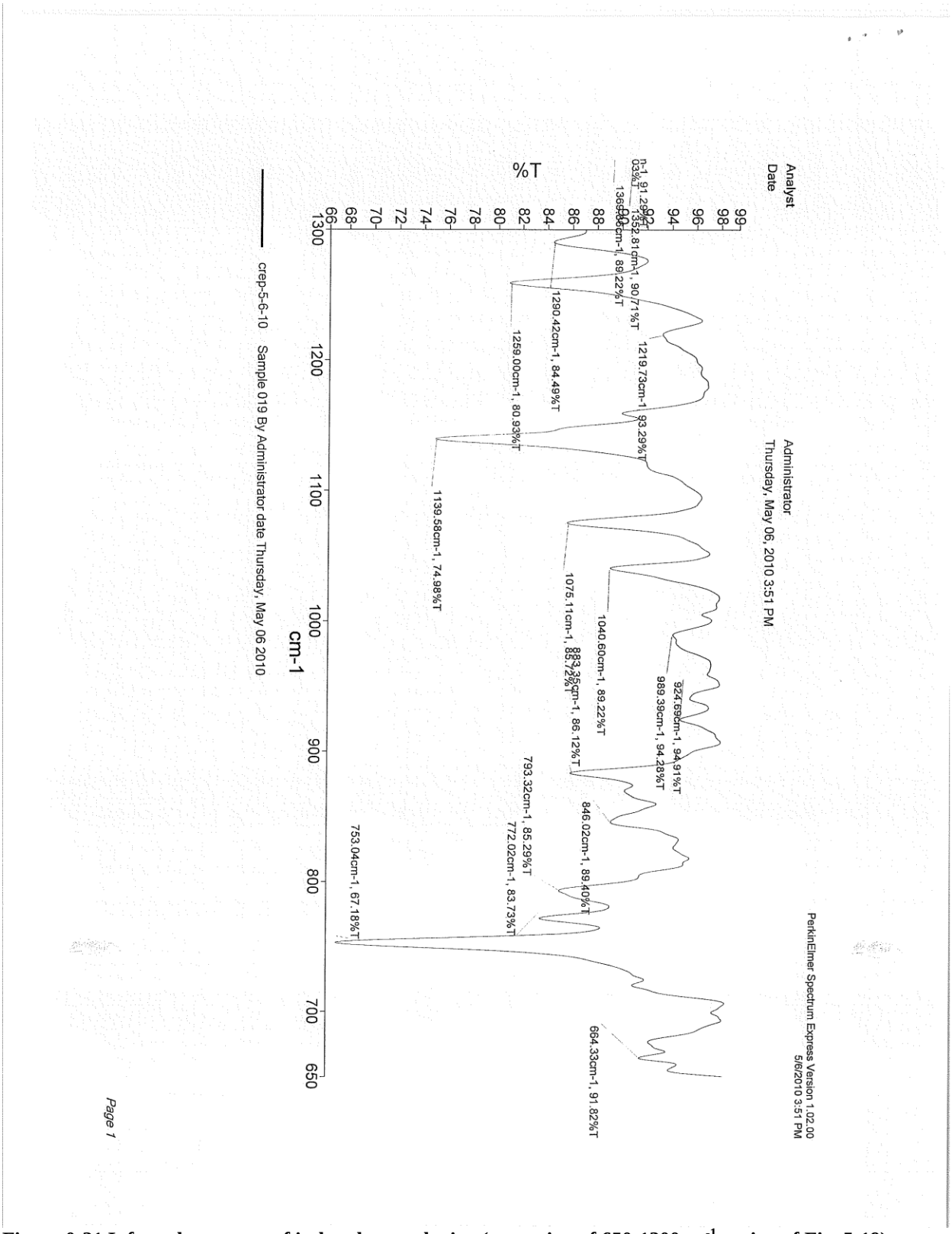


Figure 0:21 Infrared spectrum of isolated cryptolepine (expansion of 650-1300cm^{-1} region of Fig. 5:19).

MASS SPECTRUM OF ISOLATED CRYPTOLEPINE

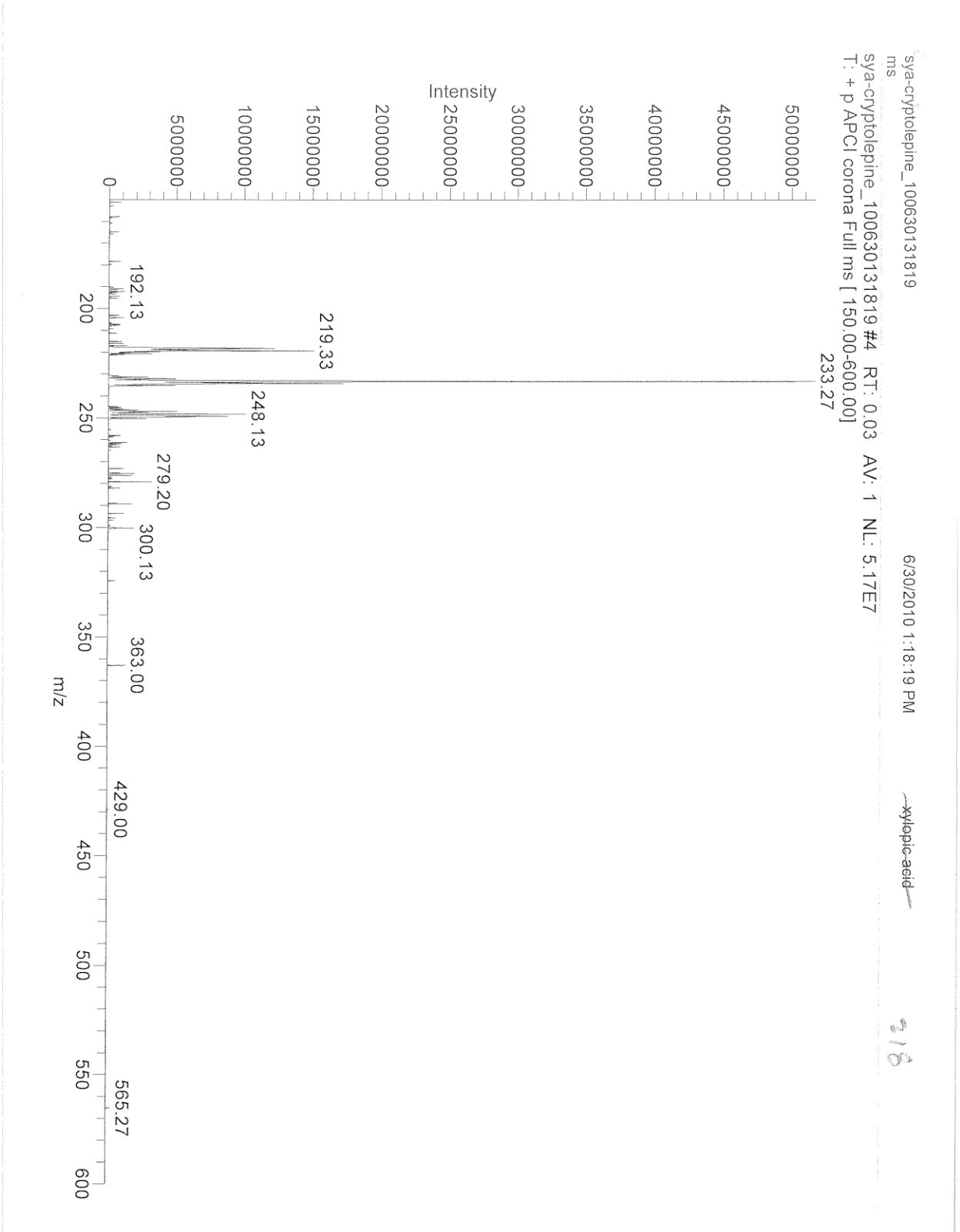


Figure 0:22 Mass spectrum of isolated cryptolepine (m/z 150-600)

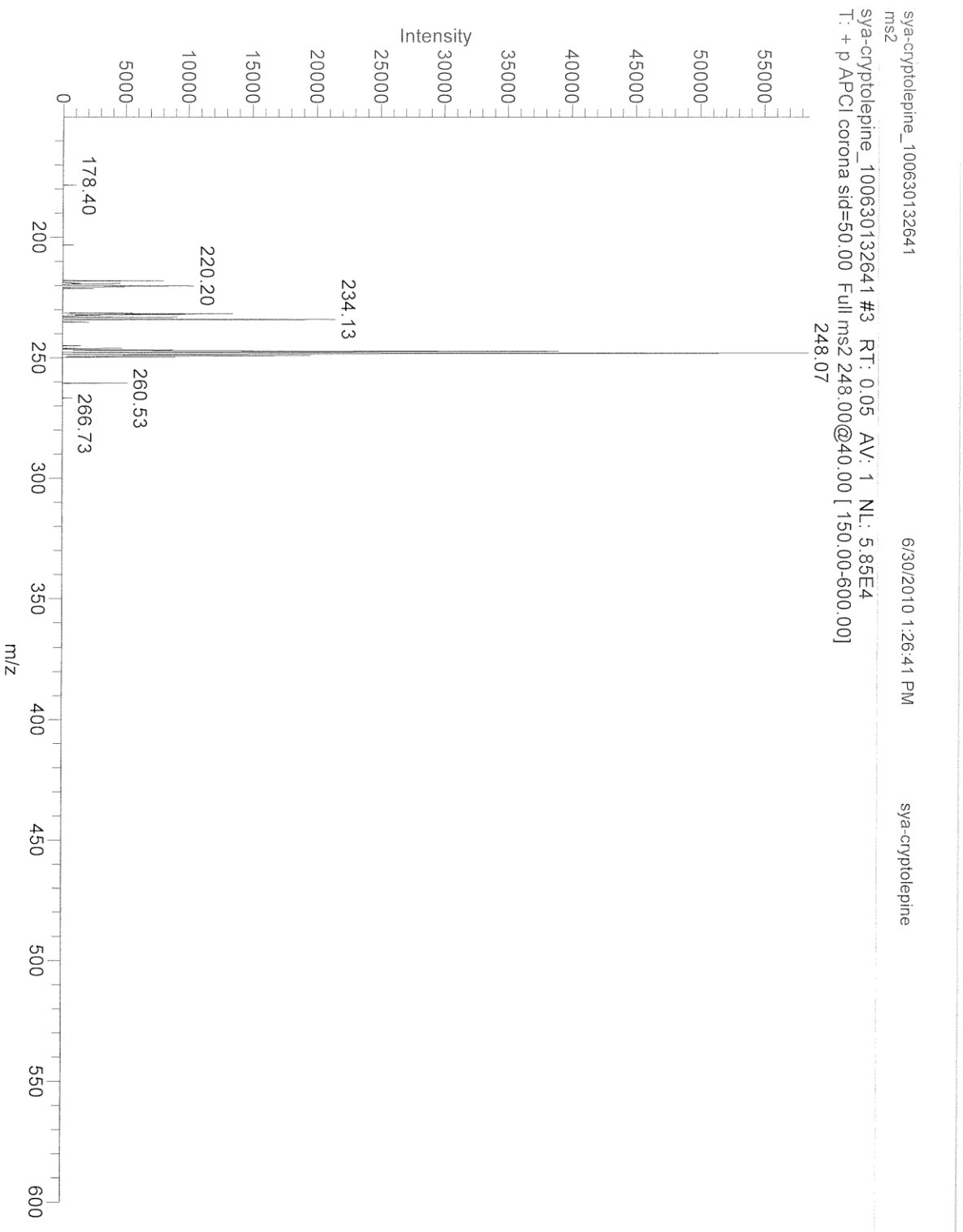


Figure 0:23 Mass spectrum of isolated cryptolepine

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