KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

LOCAL PRODUCTION OF 5-HTP FROM THE SEEDS OF GRIFFONIA SIMPLICIFOLIA

SUBMITTED BY

JOHN NII ADOTEY ADDOTEY

JULY, 2009

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A DEGREE OF

MASTER OF PHILOSOPHY
PHARMACEUTICAL CHEMISTRY

THE DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

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KUMASI, GHANA

JULY, 2009

DECLARATION

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

……..
JOHN NII ADOTEY ADDOTEY
(STUDENT)

Submitted on …..... day of July, 2009

……
DR. REIMMEL ADOSRAKU
(SUPERVISOR)

DEDICATION

To my mum, Nana Difie Bawua

ACKNOWLEDGEMENTS

What shall I render to my God for all His mercies store? I will take the gifts He has bestowed and humbly ask for more.

I am grateful to the Almighty for the gift of life.

I am also grateful to KNUST and the Department of Pharmaceutical Chemistry for providing me with the scholarship to pursue the MPhil programme.

Thanks go to my family, especially my mother, for their prayers and support throughout the course of the project. I really appreciate all they did and continue to do for me.

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.

- John Addotey, 2009

ABSTRACT

Griffonia simplicifolia is a stout, woody, climbing shrub growing to about 3m with greenish flowers and inflated black pods. The seeds contain substantial quantities of the amino acid, 5-hydroxytryptophan (5-HTP). Today, the seed of this climbing shrub is used in the treatment of **fibromyalgia, headaches, insomnia, depression and stomach problems**.

Griffonia seeds continue to be exported from Ghana. Therefore it was considered prudent to develop a simple method of production of 5-HTP from the seeds of Griffonia in reasonable purity prior to export. This will add value to the export.

This project, is to develop enhanced extraction and purification methods in the production of 5-HTP from the seeds of *Griffonia simplicifolia.* In addition, the kinetics of the degradation of the 5-HTP obtained was studied in order to predict the most the stable conditions of storage prior to export.

Griffonia seeds that had been harvested and stored for 10 months were used for the project. The solvent system used for all extractions was water- methanol (50-50). This has been determined to be the optimum solvent for the extraction (Lemaire and Adosraku 2002).

The amount of 5-HTP present in the seeds was determined by High Pressure Liquid Chromatography. Using the HPLC method for the direct assay of 5-HTP from the seeds of Griffonia developed by Lemaire and Adosraku, the content of the seeds was found to be 6.37 %w/w. However, a modified method which employs the use of solvent at 80 °C gave 5-HTP content of 8.98 %w/w.

The effect of varying conditions on the extraction of 5-HTP from the seeds was investigated. The conditions investigated were temperature of extracting solvent, volume of extracting solvent and the particle size of the powdered seeds. In all instances, the amount of 5-HTP extracted was determined by HPLC using pure 5-HTP as reference.

Generally it was observed that an increase in the temperature and volume of solvent provided an increase in yield of 5-HTP extracted. A reduction in particle size of the powdered seeds by crushing the seeds, also increases the amount of 5-HTP extracted. A combination of the effects of the above factors was found to yield the highest amount of 5-HTP.

The optimum solvent composition for the recrystallisation of the extracted 5-HTP crystals was also investigated. The limitation here is that the solvents used must non-toxic and readily available in Ghana. Thus, various compositions of water and ethanol were used to recrystallise the 5-HTP. The composition found to be useful was water-ethanol (20-80). Using this solvent mixture, 5-HTP was obtained as approximately 92% pure. The percentage yield of 5-HTP(92% pure) was 6.14 %w/w of seed taken.

The purified 5-HTP crystals were subjected to stability studies, assuming first order kinetics. The rate constant for the degradation was 1.059 E-03 day^{-1} at refrigerator temperature (approx. 5°C) as compared to 1.195 E-03 day^{-1} for that kept at room temperature. The t_{90} values for refrigerator temperature and room temperature were 99.62 days and 88.28 days respectively. The difference in shelf life was thus 11 days. This is considered economically not significant considering the cost of refrigeration.

The ether insoluble constituents of the aqueous- methanolic seed extract were separated using preparative thin layer chromatography and studied. Two constituents apart from 5-HTP were isolated. One of the constituents is postulated to be amino methoxy indole-3-acetic acid from the spectroscopic data obtained. The other constituent could not be identified with certainty and further work will have to be done.

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1 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

In recent years, there has been a renewed interest in obtaining biologically active compounds from natural sources. Contributing to this worldwide attention towards formulations based on natural products are their low toxicity, their complete biodegradability, their availability from renewable sources, and, in most cases, their low-cost compared with those of compounds obtained by total chemical synthesis. A further drive to the study of compounds from natural sources is the increasing consciousness that destruction or severe degradation of rain forests and other wild habitats, including seas and oceans, will unavoidably result in the loss of unexamined species and consequently of potentially useful compounds. Owing to this renewed attention to pharmaceuticals, agrochemicals and nutraceuticals (functional foods) obtained from natural sources, the study of bioactive secondary metabolites, traditionally carried out mainly by chemists, has increasingly attracted the attention of pharmacologists, biologists, botanists, agronomists, etc. stimulating co-operative work.

The past decade has witnessed a tremendous resurgence in the interest and use of medicinal plant products, especially in North America. Surveys of plant medicinal usage by the American public have shown an increase from just about 3% of the population in 1991 to over 37% in 1998 **(Brevoort, 1998)**. The North American market for sales of plant medicinals has climbed to about $3 billion/year **(Glaser, 1999)**. Once the domain of health-food and specialty stores, phytomedicines have clearly re-emerged into the mainstream. This is evidenced by their availability for sale at a wide range of retail outlets, the extent of their advertisement in the popular media, and the recent entrance of several major pharmaceutical companies into the business of producing phytomedicinal products. **(Brevoort, 1998; Glaser, 1999)**

The past decade has also witnessed intense interest in "nutraceuticals" (or "functional foods") in which phytochemical constituents can have long-term health promoting or medicinal qualities. Although the distinction between medicinal plants and nutraceuticals can sometimes be vague, a primary characteristic of the latter is that nutraceuticals have a nutritional role in the diet and the benefits to health may arise from long-term use as foods (i.e. chemoprevention) **(Korver, 1998)**. In contrast, many medicinal plants exert specific medicinal actions without serving a nutritional role in the human diet and may be used in response to specific health problems over short- or long-term intervals.

For many of the medicinal plants of current interest, a primary focus of research to date has been in the areas 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. Research in the pharmacognosy of medicinal plants has also involved assays of bio-activity, identification of potential modes of action, and target sites for active phytomedicinal compounds. Horticultural research on medicinal plants has focused on developing the capacity for optimal growth in cultivation. This has been especially pertinent as many medicinal plants are still harvested in the wild, and conditions for growth in cultivation have not been optimized.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. It can be seen that plant secondary metabolites have been employed in a wide variety of uses. Herbal remedies have been used for centuries but more recently the compounds that are active have been identified and this has enabled them to be extracted and purified, synthetic organic chemists have then been able to produce the molecules in vitro and so produce them on larger scales. The technologies used in this process are quite complex and beyond the scope of small-scale producers. Techniques that are more refined would involve the use of chromatography to separate the different molecules present and a combination of spectroscopic techniques to correctly identify the molecules.

In phytochemical production, it is important that the scale of the production is matched to the system for which it is intended (small, medium or large scale). The sustainability of the production and the technology involved in the production of the product must be gauged. The end user will want to pay a price that makes the use of the product beneficial. The producer will want to ensure that the product is sold at a price that will ensure good return on time and investment. In the case of local, small-scale production of 5-hydroxy tryptophan from the seeds of griffonia simplicifonia, processes that are less cumbersome and involve the use of less expensive materials will have to be developed. In addition, the stability of the products will have to be monitored.

1.2 LITERATURE REVIEW

1.2.1 GRIFFONIA SIMPLICIFOLIA

Botanical Name: *Griffonia simplicifolia*

Common Names: Griffonia

Ghanaian Names: Kagya (Akan)

Active Constituents: 5-Hydroxytryptophan, (5-HTP); *Lectin i-b4*

Family**:** *Leguminosae* (legume)

Botanical description: Griffonia simplicifolia

Griffonia simplicifolia is a stout, woody, climbing shrub growing to about 3 m with greenish flowers and inflated black pods. Griffonia's main habitat is in West & Central Africa. Found mostly in thickets, usually associated with mounds of the termite Macrotermes on plains, in forests, in secondary vegetation and on old farms. G simplicifolia is an evergreen and has wide adaptability; it produces fair quantities of highly palatable herbage of good nutritive value and fair quantities of large, viable seeds.**) (shamanica.com, 2004)**

1.2.1.1 PHYSIOLOGY, BIOCHEMISTRY OF *LEGUMINOSAE* .

Nitrogen-fixing root nodules may be present, or absent.

Cynogenic constituents may be tyrosine-derived, or phenylalanine-derived, or leucine-derived. Alkaloids present (commonly), or absent.
Proanthocyanidins present, or absent; when present, cyanidin, or delphinidin.
Flavonols present (mostly), or absent; kaempferol and quercetin, or quercetin and myricetin, or kaempferol, quercetin, and myricetin.
Ellagic acid consistently absent (from 54 species and 41 genera, representing all three subfamilies). Arbutin present, or absent. Aluminium accumulation not found. Sugars transported as sucrose (in numerous species and genera from all three subfamilies). **(Watson and Dallwitz, 1992)**

1.2.1.2 USES OF GRIFFONIA

In African folk medicine, Griffonia Seed is reputed to be an aphrodisiac, as well as an antibiotic and a remedy for diarrhea, vomiting, and stomachache. **(mdidea.com)** Traditional African uses for Griffonia include the utilization of the stem and roots as chewing sticks, leaves for wound healing, and leaf juice for the treatment of bladder and kidney ailments. **(HerbalExtractsPlus.com, 2005**) Today, the seed of this climbing shrub is used in the treatment of fibromyalgia, headaches, insomnia, depression and stomach problems. **(HerbalExtractsPlus.com, 2005**)

1.2.2 5-HYDROXY TRYPTOPHAN

5-HTP (5-hydroxytryptophan) is an effective replacement for tryptophan. It is a metabolic product of tryptophan, an essential amino acid present in most protein foods. 5-HTP is a precursor to serotonin and works essentialy the same as does tryptophan, and is one chemical step closer to serotonin. 5-HTP naturally increases the serotonin levels in the brain and body, whereas the antidepressants, Prozac, Zoloft, Paxil, Effexor, Serzone, Luvox, Remeron, etc, unnaturally increase serotonin levels and have many significant side effects.

Tryptophan was used by millions of people for many years and is safe. It was removed from the market because of problems caused by a contaminant from a Japanese supplying company. Political reasons have kept it off the over-the-counter market. It is available by prescription from many compounding pharmacies. L-Tryptophan is an essential amino acid that is converted into 5-HTP in the body. 5HTP is then converted into serotonin and melatonin. Low levels of serotonin have been associated with uneasiness, carbohydrate cravings and weight gain, mood, sleep disorders and substance dependence. 5-HTP (5 Hydroxytryptophan) is a natural substance already in our body that is made from tryptophan. **(mdidea.com)**

1.2.2.1 PHARMACOLOGY OF 5-HTP (5-HYDROXYTRYPTOPHAN)

Low serotonin levels are believed to be the reason for many cases of mild to moderate depression which can lead to symptoms like anxiety, apathy, fear, feelings of worthlessness, insomnia and fatigue. 5-HTP enhances the activity of serotonin, a hormone produced by the brain that is involved in mood, sleep and appetite. Decreased levels of this neurotransmitter have been associated with depression, insomnia, obsessive/compulsive disorders as well as eating disorders leading to obesity, or the reverse/bulimia. **(mdidea.com)** 5-HTP may be helpful in treating a wide variety of conditions related to low serotonin levels, including the following:

A. DEPRESSION

Low levels of serotonin in the brain can contribute to the development of depression. Many drugs prescribed for depression increase serotonin levels. Some studies indicate that 5-HTP may be as effective as certain antidepressant drugs in treating individuals with mild to moderate depression. Such individuals have shown improvements in mood, anxiety, insomnia, and physical symptoms. Over 19 studies, mainly conducted in Europe, have evaluated the clinical effects of 5HTP. A Swiss study found that a 300mg daily dose was slightly superior to fluvoxamine, a standard selective serotonin reuptake inhibitor (SSRI) with lower incidence of side-effects. 5-HTP has also been compared to tricyclic

antidepressants (clomipramine and imipramine) and has been found to be as effective as these prescriptive antidepressants. **(Camsi et al, 1990)**

B. FIBROMYALGIA

Although many factors can influence the stiffness, pain, and fatigue associated with fibromyalgia, evidence from several studies indicates that low serotonin levels may play a role in the development of this condition. 5-HTP has been shown to improve sleep quality and reduce pain, stiffness, anxiety, and depression in individuals with fibromyalgia. Several clinical trials have confirmed significant improvement in symptoms, including pain, morning stiffness, anxiety, and fatigue. An Italian study showed significant improvement following a total dose of 300mg for 30 days. A longer 12-month study using a daily dose of 400mg of 5-HTP found the supplement to be as effective as amitriptyline and/or phenelzine. No patient withdrew from the study. **(Camsi et al, 1990)**

C. INSOMNIA

Medical research indicates that supplementation with tryptophan before bedtime can induce sleepiness and delay wake times. Studies also suggest that 5-HTP may be useful in treating insomnia associated with depression. **(Attele et al, 2000)**

D. HEADACHES

Some studies suggest that 5-HTP may be effective in children and adults with various types of headaches including migraines. A Spanish study compared 5-HTP with the drug methysergide and found comparable improvement (over 70% of both groups) with considerably fewer side-effects in the 5-HTP group. The researchers concluded 5-HTP to be the preferred preventive measure against migraine attacks. An Italian study confirmed these results. **(Camsi et al 1990)**

E. OBESITY

There is some evidence that low tryptophan levels may contribute to excess fat and carbohydrate intake (which can result in weight gain). A study of overweight individuals

with diabetes suggests that supplementation with 5-HTP may decrease fat and carbohydrate intake by promoting a feeling of satiety. Nineteen obese female subjects with body mass index ranging between 30 and 40 were included in a double-blind crossover study aimed at evaluating the effects of oral 5-Hydroxytryptophan administration on feeding behaviour, mood state and weight loss. Either 5-Hydroxytryptophan (8 mg/kg/day) or placebo was administered for five weeks during which patients were not prescribed any dietary restrictions. The administration of 5-Hydroxytryptophan resulted in no changes in mood state but promoted typical anorexia-related symptoms, decreased food intake and weight loss during the period of observation. **(Ceci et al, 1989)**

1.2.2.1.1 ADVANTAGES OF 5-HTP OVER L-TRYPTOPHAN

First, because it is one step closer to serotonin, 5-HTP is more effective than L-tryptophan. 5-HTP is also inherently safer. Although L-tryptophan is safe if properly prepared and free of the contaminants linked to severe allergic reaction known as eosinophilia myalgia syndrome (EMS), L-tryptophan is still produced with the help of bacterial fermentation (a situation that lends itself to contamination). In contrast, 5-HTP is isolated from a natural source - a seed from an African plant (Griffonia simplicifolia). Evidence that this natural source of 5-HTP does not cause EMS is provided by researchers who have been using 5-HTP for over 25 years. They state that "EMS has never appeared in the patients of ours who received only uncontaminated L-tryptophan or 5-Hydroxytryptophan (5-HTP). Furthermore, researchers at the NIH studying the effects 5-HTP for various metabolic conditions have also not observed a single case of EMS nor has a case of elevated eosinophils been attributed to 5-HTP in these studies. **(mdidea.com)**

1.2.2.1.2 TOXICITY, SIDE EFFECTS, INTERACTIONS, AND CONTRAINDICATIONS

5-HTP may cause mild gastrointestinal disturbances including nausea, heartburn, flatulence, feelings of fullness, and rumbling sensations in some people. Large doses of 5-HTP may significantly increase serum levels of serotonin, and theoretically, this may result in the serotonin syndrome. Symptoms and signs of the serotonin syndrome, include confusion, agitation, diaphoresis, tachycardia, myoclonus and hyperreflexia. 5-HTP

should not be taken with antidepressants, weight-control drugs, other serotonin-modifying agents, or substances known to cause liver damage, because in these cases 5-HTP may have excessive effects. **(umm.edu)**

1.2.2.2 DIETARY SOURCES

5-HTP is not commonly available in food but the amino acid tryptophan, from which the body makes 5-HTP, can be found in turkey, chicken, milk, potatoes, pumpkin, sunflower seeds, turnip and collard greens, and seaweed. **(umm.edu)**

1.2.2.3 AVAILABLE FORMS

5-HTP can be obtained in the diet (from the conversion of tryptophan) or in supplement form. 5-HTP supplements are made from extracts of the seeds of the African tree Griffonia simplicifolia. 5-HTP can also be found in a variety of multivitamin and herbal preparations. **(healthandage.com)**

1.2.2.4 BIOSYNTHESIS OF 5-HYDROXYTRYPTOPHAN

5-Hydroxy-L-tryptophan (5-HTP) is the immediate precursor in the biosynthesis of 5-hydroxy-tryptamine (5-HT; serotonin) from the essential amino acid L-tryptophan.

Tryptophan is an essential amino acid. It is synthesized by plants and microbes. The tryptophan biosynthesis pathway in plants is essentially the same as it occurs in microbes. **(Radwanski, 1995).** The primary role of the pathway in microbes is to supply tryptophan for protein biosynthesis. In contrast, plants also use the pathway to provide precursors for the synthesis of plant hormone auxin and plant secondary metabolites including glucosinolates and alkaloids. Like its counterpart in microbes, the plant enzyme, anthranilate synthase, catalyzing the first step of the pathway is feed-back regulated by the end product tryptophan. **(Bernasconi, 1994)** Interestingly, naturally occurring feedback-insensitive anthranilate synthase has been reported in a few plants. **(Song, 1998; Ishihara, 2006)**

1.2.2.5 BIOSYNTHETIC PATHWAY

Phosphoenolpyruvate (PEP), a glycolytic intermediate, condenses with erythrose-4-phosphate, a pentose-phosphate pathway intermediate, to form 2-keto-3-deoxyarabinoheptulosonate-7-phosphate and inorganic phosphate. The enzyme involved is a synthase. This condensation product eventually cyclizes to chorismate as shown below

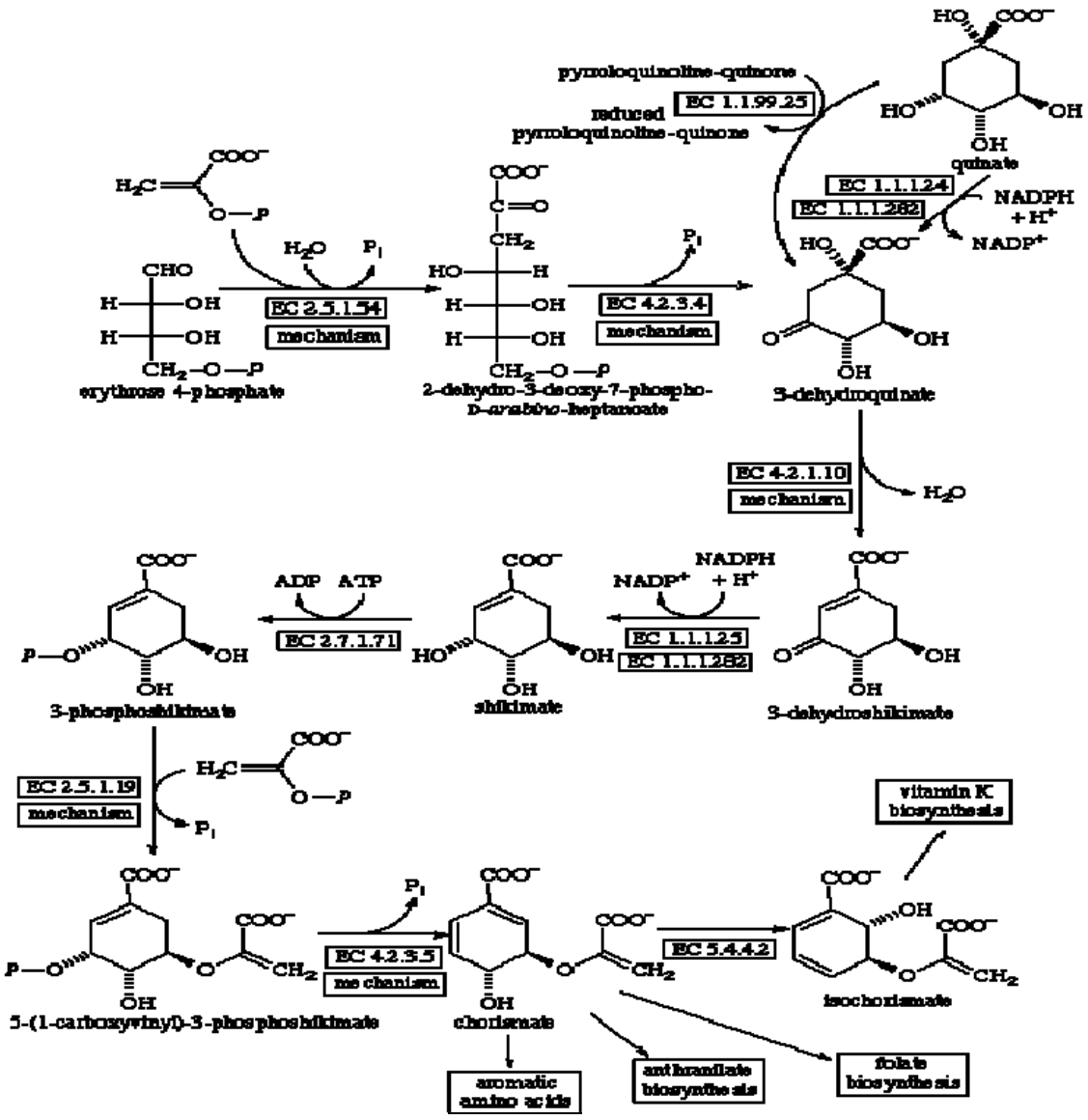


Figure 1.1 Biosynthetic pathway for chorismate

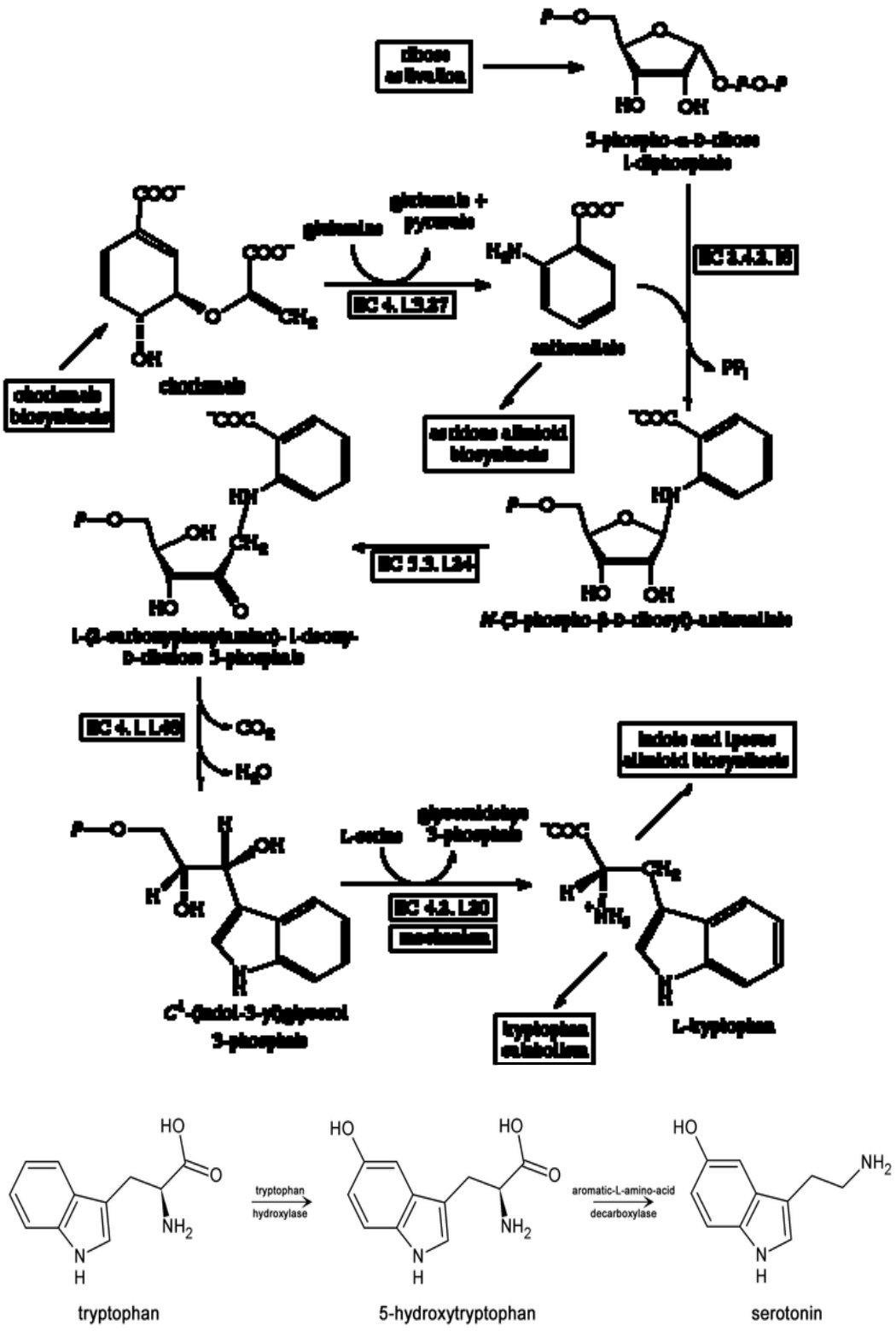


Figure 1.2 Biosynthetic Pathway for 5-hydroxytryptophan

1.2.3 RESEARCH UPDATE ON GRIFFONIA SIMPLICIFOLIA AND 5-HTP

5-Hydroxy-L -tryptophan (5-HTP) has been found in high concentration in the mature seeds of *Griffonia simplicifolia*, a West African legume of reputed physiological activity, and in lower concentration in other parts of the plant. An enzyme system capable of hydroxylating L-tryptophan has been identified in various tissues. 5-Hydroxytryptamine (5-HT or serotonin) has been found in concentrations of up to 0·2% (dry wt.) in the pods and in lower concentration in the leaves of mature plants. Indole-3-acetyl-aspartic acid (IAAA) and 5′-hydroxyindole-3-acetic acid (5-HIAA) were among other indoles detected. **(Fellows and Bell 1970)**

- **Isolation and characterization of a Lewis b-active lectin from Griffonia simplicifolia seeds**

A fourth lectin (GS IV), a serologically Lewis b (Leb)-active binding lectin, was isolated from Griffonia simplicifolia seeds by affinity chromatography on an Leb (alpha-L-fucose-(1 leads to 2)-beta-D-Gal-(1 leads to 3)-[alpha-L-fucose-(1 leads to 4)]-beta-D-GlcNAc)-active fragment coupled to Synsorb, followed by elution with 0.1 N acetic acid. The purified lectin was shown to be homogeneous by polyacrylamide gel electrophoresis and analytical gel column chromatography on Sephadex G-200. **(Shibata et al, 1982.)**

- **Purification and Characterization of Griffonia simplicifolia Leaf Lectins**

Leaves from mature Griffonia simplicifolia plants were examined for the presence of leaf lectins possessing sugar binding specificities similar to the four known seed lectins (GS-I, GS-II, GS-III, GS-IV). Three (GS-I, -II, -IV) of the four known G. simplicifolia seed lectins were present in the leaves. Leaf G. simplicifolia lectins I and IV were similar to the respective seed lectins. Leaf GS-II, however, was composed of two types of subunits (M(r) = 33,000 and 19,000), whereas the seed lectin consists of only one type of subunit (M(r) 32,500). Seed and leaf GS-II lectins also had different isoelectric points. **(Lamb et al, 1983.)**

- **An insecticidal N-acetylglucosamine-specific lectin gene from Griffonia simplicifolia (Leguminosae)**

Griffonia simplicifolia II, an N-acetylglucosamine-specific legume lectin, has insecticidal activity when fed to the cowpea weevil, Callosobruchus maculatus (F.). A cDNA clone encoding G. simplicifolia II was isolated from a leaf cDNA library, sequenced, and expressed in a bacterial expression system. The recombinant protein exhibited N-acetylglucosamine-binding and insecticidal activity against cowpea weevil, indicating that glycosylation and multimeric structure are not required for these properties. These results support the hypothesis that genes of the legume lectin gene family encode proteins that function in plant defense against herbivores. **(Zhu K et al, 1996.)**

- **5-Hydroxytryptophan: a clinically-effective serotonin precursor**

5-Hydroxytryptophan (5-HTP) is the intermediate metabolite of the essential amino acid L-tryptophan (LT) in the biosynthesis of serotonin. Intestinal absorption of 5-HTP does not require the presence of a transport molecule, and is not affected by the presence of other amino acids; therefore it may be taken with meals without reducing its effectiveness. Unlike LT, 5-HTP cannot be shunted into niacin or protein production. 5-HTP is well absorbed from an oral dose, with about 70 percent ending up in the bloodstream. It easily crosses the blood-brain barrier and effectively increases central nervous system (CNS) synthesis of serotonin. In the CNS, serotonin levels have been implicated in the regulation of sleep, depression, anxiety, aggression, appetite, temperature, sexual behaviour, and pain sensation. Therapeutic administration of 5-HTP has been shown to be effective in treating a wide variety of conditions, including depression, fibromyalgia, binge eating associated with obesity, chronic headaches, and insomnia.**(Birdsall, 1998).**

- **Isolectins I-A and I-B of *Griffonia* (*Bandeiraea*) *simplicifolia***

Seeds from the African legume shrub *Griffonia simplicifolia* contain several lectins. Among them the tetrameric lectin GS I-B_4 has strict specificity for terminal αGal residues, whereas the closely related lectin GS I-A_4 can also bind to αGalNAc. These two lectins are commonly used as markers in histology or for research in xenotransplantation. To elucidate the basis for the fine difference in specificity, the

amino acid sequences of both lectins have been determined and show 89% identity. (**Lescar et al, 2002)**

- **An HPLC method for the direct assay of the serotonin precursor, 5-hydroxytrophan, in seeds of Griffonia simplicifolia**

An HPLC method has been developed for the direct assay of 5-HTP in seeds of G. simplicifolia which overcomes the problems associated with previous techniques. By optimising the solvent extraction procedures and the HPLC conditions, levels of 5-HTP could be estimated following a single-step seed extraction. The chromatographic conditions, solvent system and the extraction technique developed make this method relatively simple, fast and efficient. Using the described methods, the highest ever levels of 5-HTP (namely, 20.83% on a fresh weight basis) have been determined in seeds of G. simplicifolia obtained in Ghana. **(Lemaire and Adosraku, 2002)**

- **Fatty acids and sterols of Griffonia**

Lipids, fatty acids and sterols of Griffonia simplicifolia seeds oil were studied. The main sterol is ß-sitosterol -60%, stigmasterol is 29%, and campesterol is 11%. Linoleic acid can be relatively simply enriched to 95% separating the other fatty acids as urea adducts. (**Petkov and Ramazanov 2003)**

- **Spectrophotometric Estimation of L- 5-Hydroxytryptophan in *Griffonia simplicifolia* Extracts and Dosage Forms**

A simple, sensitive and precise spectrophotometric method has been developed for the determination of 5-Hydroxytryptophan in *Griffonia simplicifolia* seed extracts 9and dosage forms. 5-Hydroxytryptophan gives a blue colored chromogen with Folin-Ciocalteu reagent under alkaline conditions with absorption maxima at 736 nm. The chromogen obeys Beer's law in the concentration range 1-10 µg/ml. The proposed method is reproducible and statistically validated and recoveries range between 98.95% and 99.96%**. (Gottumukkala, 2005)**

- **Estimation of 5-hydroxytryptophan in rat serum and *Griffonia* seed extracts by liquid chromatography–mass spectrometry**

The application of liquid chromatography–mass spectrometry (LC–MS) was investigated for the analysis of 5-hydroxytryptophan (5-HTP) in rat serum. The chromatographic elution was carried out using C_{18} column (YMC, 250 mm × 2 mm, 5 μm) with a mobile phase water: acetonitrile (93:7, v/v). Quantitative analysis was performed by negative ion mode using selective ion monitoring (SIM) method with electrospray ionization interface. The serum samples were de-proteinized with acetonitrile. The limit of detection (LOD) and limit of quantification (LOQ) in the serum was found to be 2 and 6 ng and calibration curve ranging from 5 to 17 ng μl^{-1} showed to be linear with correlation coefficient 0.994 ± 0.0006. The method developed was also found to be applicable for the analysis of *Griffonia simplicifolia* seed extracts and dosage forms with LOD and LOQ, 0.02 and 0.05 ng, respectively and linearity range 3–20 ng μl^{-1} with a correlation coefficient of 0.9992 ± 0.0004. (**Koppisetti et al, 2005)**

- **5-HTP is a more potent in vitro hydroxyl radical scavenger than melatonin or vitamin** C.

Hydroxyl radicals are involved in direct damage of important biomolecules. Potent radical scavengers such as vitamin C and indoles of the tryptophan family can avert the potential damage. Melatonin and its precursor 5-hydroxytryptophan (5-HTP) were compared with water-soluble vitamin C. 5-HTP showed highest hydroxyl radical scavenging effects, more than vitamin C, which was more than melatonin. **(J Pineal Res. 2005)**

JUSTIFICATION

Griffonia simplicifolia seed has also been found to contain several lectins in addition to 5-HTP. In addition, the seed oil contains fatty acids and sterol. The pharmacological activity of 5-HTP has been well investigated over the years.

Various methods of quantifying the amount of 5-HTP in the seeds of *Griffonia simplicifolia* have been developed. A study on regional variation of content of 5-HTP in seeds of *Griffonia simplicifolia* showed a range of 14.18- 18.24%w/w. Levels of 5-HTP as high as, 20.83% on a fresh weight basis have been determined in seeds of G. simplicifolia obtained in Ghana **(Lemaire and Adosraku, 2002)**. This represent substantial amount of the phytochemical.

Furthermore, Stability studies carried out on the seeds showed that heating the seeds at 60 ºC for 30 min was enough to preserve 5-HTP in the seeds, possibly through enzyme inactivation. **(Asiamah, 2003).** Using 50% methanol in water as extracting solvent, crystals of purity 59.16 % 5-HTP have been obtained in Ghana **(Asiamah, 2003).**

The seeds continue to be exported from Ghana. This study, hopes to develop enhanced extraction and purification methods in the production of 5-HTP from the seeds of *Griffonia simplicifolia.* This should make it possible to produce it locally in reasonable purity. The kinetics of the degradation of 5-HTP will be studied in order to predict most stable conditions of storage prior to export. *Griffonia simplicifolia* is a legume and as such is likely to contain other pharmaceutically important constituents. As such, an attempt would be made to isolate and characterise other constituents of the seeds.

OBJECTIVES

- To investigate the principles involved in the extraction of 5-HTP from the seeds of *Griffonia simplicifolia* using various solvents. (i.e. effect of factors such as temperature, time and volume of solvent on amount of 5-HTP extracted.)
- To determine the most effective recrystallisation conditions for the purification of the extracted 5-HTP.
- To carry out stability studies on the purified 5-HTP
- To isolate and characterize any other components in the aqueous methanolic seed extract

1.2.4 THEORY OF EXPERIMENTAL WORK

1.2.4.1 SOLVENT EXTRACTION

The process is generally applied to the removal of natural products from dried tissue originating from plants, fungi, seaweed, mammals etc. The steam-volatile natural products (e.g. those occurring in the essential oils) such as the alcohols, esters and carbonyl compounds of the aliphatic(both acyclic and alicyclic) and the simpler aromatic systems, are removed by steam distillation. The non-steam-volatile compounds may be removed by solvent extraction using a batch or continuous process. Frequently, a comprehensive study of the range of organic substances in a particular tissue requires extraction with a succession of solvents starting with light petroleum (b. p. 40ºC) for the removal of the least polar components (e.g. higher homologues of terpenes, steroids, etc) progressing through to more polar solvents such as diethyl ether, acetone ethanol and finally water for the sequential removal of the more polar compounds (e.g. amino acids, carbohydrates, etc.)

The batch process, which tends to be less efficient than the continuous extraction 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. The combined solvent extracts are then evaporated, usually under reduce pressure and the residue submitted to appropriate fractionation procedures.

For the continuous extraction by a hot solvent, it is better to use a Soxhlet apparatus. **(Furniss et al,1994)**

1.2.4.2 FILTRATION TECHNIQUES

Filtration of a mixture after extraction will be necessary either to isolate a solid product that has separated out or to remove insoluble impurities. 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 in a more complete removal of the mother-liquor than filtration under atmospheric pressure.

Some modification of the general process of filtration may become necessary in the light of the chemical nature of the mixture, or 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 acidic mixtures weaken cellulose filter papers. Acid hardened grades (e.g. Whatman filter papers) are more chemically resistant, but for maximum chemical resistance, glass fibre paper or a glass fitted with a fixed sintered glass plate may be used. Filtration aids such as high grade diatomaceous earth may be added to a suspension when the particle size is too small. The selection of a funnel appropriate to the amount of solid rather than total volume of liquor to be filtered is also important. **(Furniss et al,1994)**

1.2.4.3 RECRYSTALLISATION TECHNIQUES

Solid organic compounds when isolated from organic reactions are seldom pure; they are usually contaminated with small amounts of other compounds (impurities) which are produced along with the desired product. The purification of impure crystalline compounds is usually effected by crystallization from a suitable solvent or mixture of solvents.

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

(i) dissolving the impure substance in some suitable solvent at or near the boiling point of the solvent;

(ii) filtering the hot solution from particles of insoluble material;

(iii) allowing the hot solution to cool thus causing the dissolved substance to crystallize out; and

(iv) separating the crystals from the mother-liquor.

The resulting solid after drying is washed and tested for purity (usually by a melting point determination, spectroscopic methods, or by thin-layer chromatography). If found

impure, it is again recrystallised from fresh solvent. The process is repeated until the pure compound is obtained.

The most desirable characteristics of a solvent for recrystallisation are as follows;

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

Table 1-1 **Common solvents for recrystallisation (Furniss et al, 1994)**

SOLVENT	**b.p.(ºC)**	
Water(distilled)	100	To be used whenever possible
Methanol*****	64.5	Flammable; toxic
Ethanol	78	Flammable
Industrial spirit	77-82	Flammable
Rectified spirit	78	Flammable
Acetone	56	Flammable
Ethyl acetate	78	Flammable
Acetic acid (glacial)	118	Not very flammable, pungent vapours
Dichloromethane (methylene chloride)*****	41	Non-flammable; toxic
Chloroform*****	61	Non-flammable; vapour toxic
Diethyl ether	35	Flammable, avoid whenever possible
Benzene***Ï**	80	Flammable, vapour highly toxic
Dioxane*****	101	Flammable, vapour toxic
Carbon tetrachloride*****	77	Non-flammable, vapour toxic
Light petroleum	40-60	Flammable
Cyclohexane	81	Flammable

CAUTION:** *The vapours of these solvents are toxic and therefore recrystallisations involving their use must be conducted in an efficient fume cupboard;* ***Ï *Toluene is much less toxic than benzene and should be used in place of the latter whenever possible. Other fractions available have b.p. 60-80, 80-100 and 100-200ºC; when the boiling point exceeds 120ºC the fraction is usually called 'ligroin'. Pentane, b.p. 36ºC, and heptane, b.p. 98ºC, are also frequently used recrystallisation solvents.*

It is assumed, of course, that the solvent does not react chemically with the substance to be purified. If two or more solvents appear to be equally suitable for recrystallisation, the final selection will depend upon factors such as ease of manipulation, toxicity, flammability and cost.Some common solvents available for the recrystallisation are given in Table 1-1, broadly in the order of decreasing polarity.

If large quantities of hot solution are to be filtered, the funnel and fluted filter paper should be warmed externally during the filtration. The heating mantle is particularly suitable, using the lower heating element; no flames should be present while flammable solvents are being filtered through this funnel. When dealing with considerable volumes of aqueous or other solutions which do not deposit crystals rapidly on cooling, a Buchner funnel preheated in an oven may be used for filtration. (**Furniss et al,1994)**

1.2.4.3.1 USE OF DECOLOURISING CARBON

The crude extract of an organic material may contain impurities. Upon recrystallisation, these impurities may dissolve in the boiling solvent and be partly adsorbed by the crystals as they separate upon cooling, yielding a coloured product. Sometimes the solution is slightly turbid owing to the presence of a little resinous matter or a very fine suspension of an insoluble impurity which cannot always be removed by simple filtration. These impurities can be removed by boiling the substance in solution with a little decolouring charcoal for 5-10 minutes, and then filtering the solution while still hot as described above. The decolourising charcoal adsorbs the coloured impurity and holds back resinous, finely divided matter, and the filtrate is usually free from extraneous colour, and therefore deposits pure crystals. (**Furniss et al,1994)**

1.2.4.3.2 DIFFICULTIES ENCOUNTERED IN RECRYSTALLISATION

Occasionally, substances form supersaturated solutions and the first crystals separate with difficulty; this is sometimes caused by the presence of a little tar or viscous substance acting as a protective colloid. The following methods should be tried in order to induce crystallization:

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

Occasionally, conversion into a simple crystalline derivative is applicable; subsequent regeneration of the original compound will usually yield a pure, crystalline solid. Instances will occur however when assessment by thin layer chromatography of the number of probable impurities in the isolated reaction mixture and of their relative amounts is advisable. It may then be judged whether some prior purification by suitable preparative chromatography or by solvent extraction should be performed before crystallization is attempted.

If the solvent constituting the crystallization medium has comparatively high boiling point, it is advisable to wash the solid with a solvent of low boiling point in order that the ultimate crystalline product may be easily dried. **(Furniss et al,1994)**

1.2.4.3.3 RECRYSTALLISATION AT VERY LOW TEMPERATURES

This technique is necessary when the solubility of the compound in the requisite solvent is too high. It is also applicable when handling compounds which are liquid at room temperatures but which may be crystallized from a solvent maintained at much lower temperatures (say, 10 to 40ºC). In this latter case, after several successive low temperature recrystallisation, the compound will revert to a liquid on storage at room

temperature, but the purification process by recrystallisation will have been achieved. (**Furniss et al, 1994)**

1.2.4.3.4 RECRYSTALLISATION IN INERT ATMOSPHERE

Substances that decompose, or otherwise undergo structural modification, on contact with air must be recrystallised in an indifferent atmosphere, which is usually nitrogen or carbon dioxide. In those cases where even a short exposure to the atmosphere is harmful, the recrystallisation and filtration processes may be carried out in a nitrogen-filled manipulator glove box (available, for example, from Gallenkamp, Miller-Howe) which has been adapted to accommodate the services required for a normal recrystallisation procedure. The size of the glove box itself and the dimensions of the outlet panels will naturally limit the scale on which recrystallisation can be carried out in this manner. **(Furniss et al, 1994)**

1.2.4.4 DRYING OF RECRYSTALLISED MATERIAL

The conditions for drying recrystallised material depend upon the quantity of product, the nature of the solvent to be removed and the sensitivity of the product to heat and to the atmosphere.

With large-scale preparations of stable compounds, moist with non-toxic solvents which are volatile at room temperature (e.g., water, ethanol, ethyl acetate, acetone), the Buchner funnel is inverted over two or three thicknesses of drying paper (i.e. coarse-grained, smooth-surfaced filter paper) resting upon a pad of newspaper, and the crystalline cake removed with the aid of a clean spatula; several sheets of drying paper are placed on top and the crystals are pressed firmly. If the sheets become too damp with solvent, the crystals should be transferred to a fresh paper. The crystals are then covered by a piece of filter paper perforated with a number of holes or with a large clock glass or sheet of glass supported upon corks. The air drying is continued until only traces of solvent remain (usually detected by smell or appearance) and final drying is accomplished by placing the solid in an electric oven controlled at a suitable temperature. The disadvantage of this

method of drying is that the crystallized product is liable to become contaminated with the filter-paper fibre.

With smaller amount (e.g. 1-20g) of more valuable recrystallised material the filter cake is transferred to a tared watch glass, broken down into small fragments without damaging the crystalline form, and air dried under another suitably supported watch glass before being placed into a temperature-controlled oven.

With low melting solids, the best method of drying is to place the crystals on a watch glass in a desiccator charged with an appropriate substance to absorb the solvent. For general purposes, water vapour is absorbed by a charge of granular calcium chloride, concentrated sulphuric acid or silica gel. Methanol and ethanol vapours are absorbed by granular calcium chloride or silica gel. Vapours from diethyl ether, chloroform, carbon tetrachloride, benzene, toluene, light petroleum and similar solvents are absorbed by a charge of freshly cut shavings of paraffin wax; since the sample may contain traces of moisture, it is advisable to insert also a dish containing a suitable desiccant. (**Furniss et al, 1994)**

1.2.4.5 ISOLATION AND PURIFICATION TECHNIQUES

The adoption of a particular isolation procedure will depend largely upon the physical and chemical properties of the product. Some guidelines for useful general approaches may however be given with regard to the physical state at ambient temperature of the crude mixture, i.e. whether it is *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. Removal of the solvent after drying leads to the recovery of the purified solid for recrystallisation from a suitable solvent. Continuous extraction of the solid (i.e. in a Soxhlet apparatus) may be necessary if the required product is only sparingly soluble in convenient organic solvents.

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

The one-phased liquid system 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 crystallization 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.

Direct filtration would also be employed when the solid consists of unwanted 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.

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 solid; 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 based on 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, 1994**)

1.2.4.6 CHARACTERIZATION OF ORGANIC COMPOUNDS

The organic chemist is frequently faced with the problem of characterizing and ultimately elucidating the structure of unknown organic compounds. The worker in the field of natural products, for example, has the prospect of isolating such compounds from their sources in a pure state and then of determining their structures. The elucidation of the structure requires the identification of the molecular framework, the nature of the functional groups which are present and their location within the skeletal structure, and finally the establishment of any stereochemical relationships which might exist.

Preliminary investigations should be carefully carried out, particularly the study of solubility. Accurate classification of a compound as acidic, basic or neutral greatly assists the subsequent search to identify the characteristic functional group or groups present. This involves a careful consideration of the results of selected classifying chemical tests in conjunction with the recognition of adsorptions of diagnostic value in the appropriate spectra.

Having established the functionality, the spectroscopic information may reveal information of skeletal features, and the compound may then be converted into an appropriate solid derivative, which if known, may confirm the identity of the compound, or provide further compounds on which reactivity and spectroscopic studies could be relevant.

Derivative preparation involves the application of concisely described general procedure to a specific compound, and its success may well depend upon the use of intuition and initiative in the preparative and purification stages.

Physical constants

The most widely used physical constants in the characterisation of organic compounds are melting and boiling points **(Furniss et al, 1994)**. In general, a sharp melting point (say, within 0.5ºC) is a characteristic property of a pure organic compound. The purity should not, however, be assumed but must be established by observation of any changes in the melting point (or range) when the compound is subjected to purification by recrystallisation. If the melting point is unaffected by at least one recrystallisation, the

purity of the substance may be regarded as established. Confirmation of purity may be obtained by thin layer chromatography.

1.2.4.7 CHROMATOGRAPHIC TECHNIQUES

Chromatography is the key to obtaining pure compounds for structure elucidation, for pharmacological testing or for development into therapeuticals **(Marston, 2007)**. It also plays a fundamental role as an analytical technique for quality control and standardisation of phytotherapeuticals.

1.2.4.7.1 THIN LAYER CHROMATOGRAPHY

Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or on combinations of these mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase)**(British Pharmacopoeia 2005).**

1.2.4.7.1.1 STATIONARY PHASES

Silica gel is the most important stationary phase for TLC, with other inorganic oxide adsorbents, such as alumina, kieselguhr (a silica gel of low surface area) and Florisil (a synthetic magnesium silicate), of minor importance. For silica gel, silanol groups are the dominant adsorption sites.

1.2.4.7.1.2 LAYER PRETREATMENTS

Prior to chromatography, it is common practice to prepare the layers for use by any or all of the following steps: washing, conditioning and equilibration. Newly consigned precoated layers are invariably contaminated. To remove contaminants, single or double

immersion in a polar solvent, such as methanol or propan–2–ol, for about 5 min is generally superior to predevelopment with the mobile phase.

Physically adsorbed water can be removed from silica gel layers by heating at about 120° for 30 min. Afterwards, the plates are stored in a grease–free desiccator over blue silica gel. Heat activation is not normally required for chemically bonded layers.

1.2.4.7.1.3 Development

The principal development techniques in TLC are linear, circular and anticircular, with the velocity of the mobile phase controlled by capillary forces or forced–flow conditions. In any of these modes continuous or multiple development can be used to extend the application range.

For linear (or normal) development, samples are applied along one edge of the plate and the separation developed for a fixed distance in the direction of the opposite edge.

In continuous development the mobile phase is allowed to traverse the layer under the influence of capillary forces until it reaches some predetermined position on the plate, at which point it is evaporated continuously.

In unidimensional multiple development, the TLC plate is developed for some selected distance, then either the layer or the mobile phase is withdrawn from the developing chamber, and adsorbed solvent evaporated from the layer before repeating the development process. Multiple development provides a very versatile strategy for separating complex mixtures, since the primary experimental variables of development distance and composition of the mobile phase can be changed at any development step, and the number of steps varied to obtain the desired separation. Multiple development provides a higher resolution of complex mixtures than does normal or continuous development, can easily handle samples of a wide polarity range

For drug mixtures that span a wide retention range, some form of gradient development is required to separate all the components either in a single chromatogram or in separate chromatograms for successive developments.

In two–dimensional TLC, the sample is spotted at the corner of the layer and developed along one edge of the plate. The solvent is then evaporated, the plate rotated through 90° and redeveloped in the orthogonal direction. Using two solvent systems with complementary selectivity is the simplest approach to implement in practice, but it is often only partially successful.

1.2.4.7.1.4 Detection

About 1 to 10 μg of coloured substances with a quantitative reproducibility rarely better than 10–30% can be detected by visual inspection of a TLC plate. This may be adequate for qualitative methods, but for reliable quantification, *in situ* spectrophotometric methods are preferred, as they are more accurate and far less tedious and time consuming than excising zones from the layer for determination by conventional solution spectrophotometry. The fluorescence–quenching technique enables visualisation of UV-absorbing drugs on TLC plates that incorporate a fluorescent indicator. The zones of UV-absorbing substance appear dark against the brightly fluorescing background of a lighter colour when the plate is exposed to UV light of short wavelength. The method is not universal, since it requires overlap between the absorption bands of the indicator ($\gamma_{max} \approx 280$ nm with virtually no absorption below 240 nm) and the drug, but in favourable cases, it is a valuable and non–destructive method for zone location.

1.2.4.7.1.5 Preparative thin–layer chromatography

Preparative TLC is used mainly to purify drugs or to isolate drug metabolites and impurities in amounts of about 1 to 100 mg for subsequent use as reference materials, structural elucidation, biological activity evaluation and other purposes. Scale up from analytical TLC is achieved by increasing the thickness of the layer (loading capacity increases with the square root of the layer thickness) and by increasing the plate length used for sample application..

Sample application is a critical step in preparative TLC, and if performed improperly can destroy all or part of the separation. The sample, usually as a 5 to 10 % (w/v) solution in a volatile solvent, is applied as a band along one edge of the layer to give a maximum sample load of about 5 mg/cm for each millimetre of layer thickness. Sample loads are

usually lower for difficult separations and for cellulose and chemically bonded layers. A short predevelopment, of about 1 cm with a strong solvent, is often useful to refocus manually applied bands. In all cases, it is important that the sample solvent is evaporated fully from the layer prior to the start of the separation to avoid the formation of distorted separation zones. It is usual to leave a blank margin of 2 to 3 cm at each vertical edge of the layer to avoid uneven development.

After development, physical methods of zone detection are used to identify the sample bands of interest. Layers that contain a UV indicator for fluorescence quenching or the adsorption of iodine vapours are useful for this purpose. If a reactive spray reagent is used for visualisation, it should be sprayed on a small strip of the chromatogram only, so as not to contaminate the remainder of the material. Once the bands of interest are located, the zones are scraped off the plate carefully with a spatula or similar tool. A number of devices based on the vacuum–suction principle for removing the marked zones from the plate are available also. Soxhlet extraction, liquid extraction or solvent elution with a polar solvent is used to recover drugs from the sorbent. For solvent extraction, water is often added to dampen the silica gel prior to extraction with a water–immiscible organic solvent. Chloroform and ethanol (methanol is less suitable because of its higher silica solubility) are widely used for solvent elution. Colloidal silica can be removed by membrane filtration prior to vacuum stripping of the solvent.

1.2.4.7.2 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a method of chromatographic separation based on the difference in the distribution of species between two non-miscible phases, in which the mobile phase is a liquid, which percolates through a stationary phase contained in a column. **(British Pharmacopoeia 2005)**

The ability to separate and analyze complex samples is integral to the biological and medical sciences. Classic column chromatography has evolved over the years, with chromatographic innovations introduced at roughly decade intervals. A technique was needed which could separate water-soluble, thermally-labile, non-volatile compounds with speed, precision and high resolution Modern HPLC techniques became available in

1969; however, they were not widely accepted in the pharmaceutical industry until several years later.

It is presently used in pharmaceutical research and development:

- To purify synthetic or natural products.
- To characterise metabolites.
- To assay active ingredients, impurities, degradation products and in dissolution assays.
- In pharmacodynamic and pharmacokinetic studies.

Improvements made in HPLC in recent years include:

- Changes in packing material, such as smaller particle size, new packing and column materials.
- High–speed separation.
- Micro-HPLC, automation and computer–assisted optimisation.
- Improvements in detection methods, including the so–called hyphenated detection systems.

1.2.4.7.2.1 Chromatographic mechanisms

The systems used in chromatography are often described as belonging to one of four mechanistic types: adsorption, partition, ion exchange and size exclusion. *Adsorption chromatography* arises from interactions between solutes and the surface of the solid stationary phase. Generally, the eluents used for adsorption chromatography are less polar than the stationary phases and such systems are described as 'normal phase'. *Partition chromatography* involves a liquid stationary phase that is immiscible with the eluent and coated on an inert support. Partition systems can be normal phase (stationary phase more polar than eluent) or reversed–phase chromatography, referred to as RPC (stationary phase less polar than eluent).*Ion–exchange chromatography* involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. *Size–exclusion chromatography* involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, with the large molecules unable to enter the pores elute first.

Other types of chromatographic separation have been described. *Ion–pair chromatography* is an alternative to ion–exchange chromatography. It involves the addition of an organic ionic substance to the mobile phase, which forms an ion pair with the sample component of opposite charge. This allows a reversed–phase system to be used to separate ionic compounds. *Chiral chromatography* is a method used to separate enantiomers, which can be achieved by various means. In one case, the mobile phase is chiral and the stationary phase is non–chiral. In another, the liquid stationary phase is chiral with the mobile phase non–chiral or, finally, the solid stationary phase may be chiral with a non–chiral mobile phase.

1.2.4.7.2.2 Hyphenated techniques

The recent development of the so–called hyphenated techniques has improved the ability to separate and identify multiple entities within a mixture. These techniques include HPLC–MS, HPLC–MS–MS, HPLC–IR and HPLC–NMR. These techniques usually involve chromatographic separation followed by peak identification with a traditional detector such as UV, combined with further identification of the compound with the MS, IR or NMR spectrometer.

MS as a detector for an HPLC system has gained wide popularity over the past several years. The process of mass analysis is essentially the same as in any other mass spectrometric analyses that utilise quadrupole or ion–trap technology. Modern mass spectrometers commonly utilise a technique known as atmospheric pressure ionisation (API) to accomplish this.

HPLC–MS–MS is commonly used in the pharmaceutical industry and in forensic science to analyse trace concentrations of drug and/or metabolite. MS–MS offers the advantage of increased signal–to–noise ratio, which in turn lowers the limits of detection and quantification easily into the sub ng/mL range. MS–MS is also a very useful technique in the qualitative identification of previously unidentified metabolites of drugs, which thus makes MS–MS a very powerful technique in research laboratories. Several recently published studies have utilised MS–MS as a high–throughput analytical technique in the pharmaceutical industry.

HPLC–IR has proved to be an effective method to detect degradation products in pharmaceuticals. IR provides spectral information that can be used for compound identification or structural analysis. The IR spectra obtained after HPLC separation and IR analysis can be compared to the thousands of spectra available in spectral libraries to identify compounds, metabolites and degradation products. An advantage of IR spectroscopy is its ability to identify different isomeric forms of a compound based on the different spectra that result from alternative locations of a functional group on the compound.

HPLC–NMR is also growing in popularity for the identification of various components in natural products and other disciplines. The miniaturisation of the system and the possibility of measuring picomole amounts of material are both areas currently attracting a large amount of attention.

1.2.4.8 STABILITY TESTING OF DRUGS

A wide range of environmental factors such as temperature, light, and oxygen may cause drug deterioration. **(Florence and Attwood, 1988)** The effect of any particular factor on the drug breakdown under conditions of normal storage may be evaluated from a number of experiments in which this factor is greatly exaggerated. Such testing methods, referred to as accelerated storage tests, have for many years proved an effective replacement at the development stage, for the original time-consuming practice of storing the product at room temperature for periods corresponding to the normal time the products would likely remain in stock.

1.2.4.8.1 Accelerated stability testing

Instabilities in modern formulations are often detectable only after considerable storage periods under normal conditions. To assess the stability of a formulated product it is usual to expose it to high stress conditions to enhance its deterioration and therefore reduce the time required for the testing. This enables more data to be gathered in a shorter time which, in turn, will allow unsatisfactory formulations to be eliminated early within a study and will also reduce the time for a successful product to reach the market. It must be emphasised that extrapolations to ‘normal’ storage conditions must be made with care and that the

formulator must be sure that such extrapolations are valid. It is advisable therefore to run concurrently a batch under expected normal conditions to confirm later that these assumptions are valid.

The objectives of such accelerated tests may be defined as

- the rapid detection of deterioration in different initial formulations of the same product – this is of use in selecting the best formulation from a series of possible choices;
- the prediction of shelf-life
- the provision of a rapid means of quality control, which ensures that no unexpected change has occurred in a stored product.

All these objectives are based on obtaining a more rapid rate of decomposition by applying to the product a storage condition that places a higher stress or challenge to it when compared with normal storage conditions. **(Aulton, 1988)**

1.2.4.8.2 Common challenges

1.2.4.8.2.1 Temperature

An increase in temperature causes an increase in the rate of chemical reactions. The products are therefore stored at temperatures greater than room temperature. The nature of the products often determines the range covered in the accelerated test. Samples are removed at various time intervals and the extent of decomposition is determined by analysis. Sensitive analytical methods should be used in all stability tests of this nature since small changes may be detected after very short storage periods. **(Aulton, 1988)**

Effect of temperature on stability

The effect of temperature on a rate constant, k, is indicated by the Arrhenius equation

$$k = Ae^{-EaRT} \qquad (1.1)$$

$$\log k = \log A - \frac{E_a}{2.303RT} \qquad (1.2)$$

From the Arrhenius equation a plot of log k versus 1/T should be linear. The rate constant at any selected storage temperature may thus be extrapolated from measurements at a series of elevated temperatures where the reaction proceeds at an

accelerated rate. The time taken in conducting the stability tests is considerably reduced compared to that for a simple experiment in which the product is maintained at the required storage temperature and sampled over a period corresponding to the normal storage time. **(Florence and Attwood, 1993)**

Figure 1.3Graph showing the plot of the Arrhenius equation

An alternative method of data treatment is to plot the logarithm of the half- life, $t_{0.5,}$ as a function of reciprocal temperature since, from the relation, $t_{0.5} = 0.693/k$.

Therefore $\log k = \log 0.693 - \log t_{0.5}$
And substituting into equation 1.12 gives
$\text{Log } t_{0.5} = \log 0.693 - \log A + Ea/ 2.303RT$ (1.3)

Once the rate constant is known at the required storage temperature, it is a simple matter to calculate a shelf – life for the product based on an acceptable degree of decomposition. The usual decomposition level is taken as 90 percent of the initial concentration of the drug, although this may vary, depending, for example, on whether the decomposition products produce discolouration or have undesirable side effects.

Although accelerated storage testing based on the use of the Arrhenius equation has resulted in a very significant saving of time, it still involves the time – consuming step of the initial determination of the order of the reaction for the decomposition. Whilst most investigators have emphasised the need for knowledge of the exact kinetic pathway of degradation, some have bypassed this initial step by assuming a particular decomposition

model. Londi and Scott have indicated that at less than 10 per cent degradation and within the limits of experimental error involved in stability studies, it is not possible to distinguish between zero-, first- or simple second – order kinetics using curve – fitting techniques; consequently these authors have suggested that the assumption of first order kinetics for any decomposition reaction should involve minimum error. Even with the modification suggested above, the method of stability testing based on the Arrhenius equation is still time- consuming, involving as it does the separate determination of rate constants at a series of elevated temperatures. Experimental techniques have been developed which enable the decomposition rate to be determined from a single experiment. Such methods involve raising the temperature of the product in accordance with predetermined temperature-time programme and are consequently referred to as non – isothermal stability studies.

The advantages of this method over the conventional method of stability testing are that

(a) the data required to calculate the stability are obtained in a single one-day experiment, which may last for several weeks.

(b) no preliminary experiments are required to determine the optimum temperatures for the accelerated storage test, and

(c) the linearity of the plot of $\log f(c)$ against $\log(1 + t)$ confirms that the correct order of reaction has been assumed.

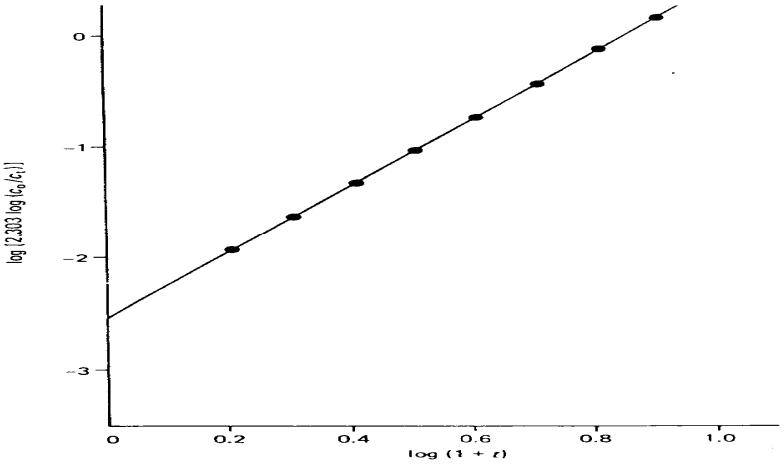


Figure 1.4 Accelerated Storage plot for the decomposition of a drug

2 EXPERIMENTAL

2.1 MATERIALS

- Thermostatically controlled Gallenkamp Ovens
- Buchi rotary evaporator
- Buchi recirculator chiller
- Buchi water bath
- Stuart Scientific Flask shaker
- Ceramic mortar and pestle
- Analytical Balance (Adams Instrument)
- HPLC Apparatus- LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied biosystems),
- Integrator- shimadzu CR 501 chromatopac
- Column -phenomenex hypersil 5micron C18 250 × 4.60 mm
- Blender
- Cecil CE 2041 single beam UV-VIS spectrophotometer
- Infra Red Spectrophotometer
- Refrigerator
- Melting point apparatus
- Precoated plates-silica gel Gf 254, 0.25mm Merck W. (Germany)
- IR spectra were obtained on a Shimadzu FTIR-8100A.
- Mass spectra were obtained on a JEOL JMS-DX 303 instrument.

2.2 REAGENTS

- Methanol
- Diethyl ether
- Distilled water
- Petroleum ether(B. P. 40-60°C)
- Chloroform
- Ethanol (96%)
- n-Butanol
- n-Propanol
- Glacial Acetic Acid
- Concentrated H_2SO_4

2.3 METHODOLOGY

2.3.1 PHYTOCHEMICAL ANALYSIS

2.3.1.1 EXTRACTION

Fifty grams of the powdered seeds was extracted with a 1:1 mixture of water and methanol at room temperature for 1 hour.

The suspension was then decanted and filtered using sterile Whatmann Paper No. 1. The filtrate was concentrated to dryness at 70 ^{0}C in a rotary evaporator. The residue obtained served as the seed extract.

The extract was screened for the presence of Alkaloids, Tannins, Terpenoids, Glycosides, Flavonoids, Saponins, and steroids.

1. **ALKALOIDS**: 1.5ml of 10% HCl was added to about 5ml of the extract in a test tube. The mixture was heated for 20 minutes. It was cooled and filtered 1ml of the filterate was tested with few drops (5 drops) of Mayer's and Dragendorff's reagents. A whitish yellow and reddish precipitate respectively observed in the extract tested as indication of the presence of alkaloids in the extracts.
2. **TANNINS**: 3 drops of 5% ferric chloride was added to 1ml of the extract. A greenish black precipitate observed in the extract was taken as indication of the presence of tannins in the extract.
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 (A and B) was added and the mixture was boiled for 5 minutes. A brick-red precipitate observed in the extract was taken as indication of the presence of glycosides in the extract.
4. **SAPONINS**: Frothing test: 2ml of the extract in a test tube was vigorously shaken for 2 minutes. The presence of frothing which persisted for 5 minutes and when warmed on water bath was taken as indication of the presence of saponin in the extract.
5. **STEROIDS**: Liebermann's Burchard test: 1ml of an aqueous methanolic solution of the extract was dissolved in 0.5ml of acetic anhydride and cooled well in ice. The mixture was mixed with 0.5ml of chloroform. Then 1ml of concentrated H_2S0_4 was then carefully added by means of a pipette. At the

separating level of the two liquids, a reddish-brown ring was formed, as indication of the presence of steroids.

6. **TERPENOIDS**: 0.5g of 2,4- dinitrophenylhydrazine was dissolved in 100ml of 2M HCl. 1ml of this mixture was added to 2ml of an aqueous methanolic solution of the extract. A yellow-orange colouration was taken as indication of the presence of a terpenoid.
7. **FLAVONOIDS**: Shibata's reaction: 3ml of extract was warmed with three pieces of magnesium turning's and mixed with 3 drops of concentrated HCl; An orange pink colouration was taken as indication of the presence of flavonoids. **(Ideoga et al, 2005)**

2.3.2 EXTRACTION OF 5-HTP PRIOR TO ASSAY

Approximately 1.0000g of Griffonia seeds was accurately weighed. This was transferred into a porcelain mortar, and triturated with a 1:1 mixture of water and methanol(solvent) into a smooth paste. The paste was quantitatively transferred into a 100 ml volumetric flask using solvent and additional solvent was added to the mark. The mixture was shaken with a shaker for 15 min. The mixture was filtered under pressure using a Buchner funnel and Whatman No.1 filter paper discarding the first 10 ml. The filtrate was conveniently diluted and subjected to analysis by HPLC. An appropriate concentration of pure 5-HTP was used as reference.

2.3.3 HPLC ANALYSIS OF CRUDE EXTRACT USING PURE 5-HTP

The complete chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied biosystems) and shimadzu CR 501 chromatopac Integrator. Column used was phenomenex hypersil 5micron C18 250 × 4.60 mm. the mobile phase consisted of 3% methanol in 0.05M KH_2PO_4 buffer at pH 4.8 eluted isocratically at 1.5ml/min. 20ul portions of a suitable concentration of pure 5-HTP as well as seed extract were loaded and injected in turn unto the column. The eluent was monitored at 275 nm and A.U.F. of 0.200. The peak areas were estimated from the chromatogram and used as a measure of concentration.

2.3.4 PREPARATION OF MOBILE PHASE (1L)

100 ml of 0.5M potassium dihydrogen orthophosphate was diluted to 800 ml with distilled water; the pH was adjusted to pH 4.8 with 0.1M sodium hydroxide and the resulting solution diluted to 1000 ml with distilled water. 970 ml of this was added to 30 ml of methanol to produce 1000 ml of the mobile phase.

2.3.5 PREPARATION OF 0.5 M POTASSIUM DIHYDROGEN ORTHOPHOSPHATE

Approximately 6.8050 g of potassium dihydrogen orthophosphate was weighed and dissolved with distilled water to 100 ml

2.3.6 DETERMINATION OF EFFECT OF VOLUME OF SOLVENT ON YIELD OF 5-HTP

1.0000g of powdered Griffonia seeds was weighed. This was then extracted with 10.00 ml of water-methanol (1:1) at room temperature. The amount of 5-HTP extracted was estimated by the HPLC method above. This was repeated using 20.00 ml and 30.00 ml of solvent. For each volume of solvent, triplicate determinations were made.

2.3.7 DETERMINATION OF EFFECT OF TIME OF EXTRACTION ON THE YIELD OF 5-HTP

1.0000 g of Griffonia seeds was weighed. This was then extracted with 15.00 ml of water-metanol(1:1) at room temperature. The amount of 5HTP extracted was estimated by the HPLC method above after 5 min. The estimation was repeated at 15 min, 30 min and 60 min. for each time point, triplicate determinations were made.

The above procedure was repeated using triturated seeds.

2.3.8 DETERMINATION OF THE EFFECT OF TEMPERATURE OF EXTRACTION ON THE YIELD OF 5-HTP

Two portions of about 1.0000g of powdered Griffonia seeds were weighed. These were extracted each with 10.00 ml of water-metanol(1:1) at room temperature (30 °C approx.).

The amount of 5-HTP extracted in each case was estimated by the HPLC method above. This was repeated using solvent at temperatures of 45 °C and 65 °C and 80 °C.

2.3.9 DETERMINATION OF OPTIMUM SOLVENT COMPOSITION FOR RECRYSTALLISATION OF 5-HTP

About 0.1g of the powdered substance was placed in a small test tube (75 × 11 mm) and the solvent was added a drop at a time with continuous shaking of the test tube. After about 1 ml of the solvent had been added, the mixture was heated to boiling. If the sample dissolved in 1 ml of cold solvent or upon gentle warming, the solvent was considered unsuitable. If the entire solid did not dissolve, more solvent was added in 0.5 ml portions and again heated to boiling after each addition. If 3 ml of solvent was added and the substance did not dissolve on heating, the substance was regarded as sparingly soluble in that solvent, and another solvent was sought. If the compound dissolved (or almost completely dissolved) in the hot solvent, the tube was cooled to determine whether crystallization occurs. If crystals separate, the amount was noted. The process was repeated with other possible solvents, using a fresh test tube for each experiment, until the best solvent was found; the approximate proportions of the solute and solvent giving the most satisfactory results were recorded.

2.3.10 RECRYSTALLISATION OF CRUDE 5-HTP

A specific quantity of crude 5-HTP was weighed and placed in a round bottomed flask of suitable size fitted with a reflux condenser. Slightly less than the required quantity of solvent was added together with a few pieces of porous porcelain to prevent ‘bumping’. The mixture was heated to boiling point on a water bath, and more solvent was added down the condenser until a clear solution, apart from insoluble impurities, was produced. The hot solution was rapidly filtered through a fluted filter paper supported in a relatively large funnel with a short wide stem. The funnel was warmed in an electric oven before filtration was started, and supported in a conical flask of sufficient size to hold all the solution. The conical flask was stood on a steam bath and the filtrate was kept boiling gently so that the warm solvent vapours maintained the temperature of the solution

undergoing filtration. This was to prevent premature crystallization. The filtered solution was covered with a watch glass and then set aside to cool undisturbed.

2.3.11 STABILITY STUDIES ON PURIFIED 5-HTP FROM GRIFFONIA SEEDS

Purified 5-HTP obtained from seeds of Griffonia was assayed monthly over three months. Conditions employed were refrigerator (approx. 5 °C), room temperature (approx. 30 °C), and 40 °C and 60 °C.

2.3.12 ASSAY OF PURIFIED 5-HTP CRYSTALS

An amount of purified 5-HTP (0.0200 - 0.0500 g) was weighed accurately. This was dissolved in 1:1 water-methanol to form 50.00 ml solution. 0.50 ml of this solution was diluted to 25.00 ml. The resultant solution was analysed by HPLC using pure 5-HTP as reference.

2.3.13 CHARACTERIZATION OF AQUEOUS METHANOLIC EXTRACT

2.3.13.1 THIN LATER CHROMATOGRAPHY

The water and methanol crude extract (1:1) was spotted and examined using TLC precoated plates. Solvent systems used included; n-butanol/n-propanol/ethanol, acetic acid, and water (12:3:5). From the chromatograms obtained, n-butanol containing system was selected due to acceptable separation obtained. Therefore the extract was developed using a mixture of butanol, water and acetic acid (12:3:5). After the development, the chromatogram was dried and viewed under UV lamp at 365 nm and 254 nm respectively. In addition, chromatograph was examined after spraying with concentrated H_2SO_4, anisaldehyde, dragendorff's reagent and also after exposure to iodine vapour.
Four components were observed and their corresponding R_f valves were noted.

2.3.13.2 GRAVITY COLUMN CHROMATOGRAPHY

The column (40 cm x 2 cm) was cleaned and dried.

A wad of cotton was placed on top of the glass wool. The adsorbent (silica gel) was measured into the column. The side of the column was tamped to ensure even packing. The solvent was carefully poured into the column. To eliminate air packets the column was gently tamped again. The solvent was drained until the level of the solvent was to the top of the adsorbent.

The sample (dried extract) was dissolved in the solvent and applied carefully and evenly unto the adsorbent by means of a pipette. Cotton wool was placed on the sample to prevent the sample from being disturbed. A pool of solvent was carefully added and the stopcock was opened.

The eluate was collected as 10 ml fractions at a flow rate of one drop per second.

2.3.13.3 PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC)

Glass plates (20 x 20 cm) coated with 0.75 mm with silica gel, (Gf 254, 60 mesh) were used. The solvent system used for the development was a mixture of butanol, water and acetic acid (12:3:5). After development, the plates were viewed under UV lamp. The observed bands were scraped having correlated their respective R_f values with the TLC Rf values before elution was done.

3 RESULTS

3.1 Phytochemical tests

The results below shows the results obtained from phytochemical tests on the aqueous methanolic extract of the seeds of Griffonia

The tests comfirmed the presence of Alkaloids, Tannins, Saponins, Glycosides, Steroids and Terpenoids.

Cardiac glycosides and Flavonoids were absent

3.2 ESTABLISHING THE FACTORS THAT AFFECT EXTRACTION

The following tables and graphs show the trends observed in the effect of selected factors on the amount of 5-HTP extracted from the seeds of Griffonia

Table 3-1 Effect of volume of solvent on peak area of the peak corresponding to 5-HTP

Volume/ml	Peak Area1	Peak Area2	Mean
10	7.9	7.9	7.9
20	9.4	9.2	9.3
30	10.0	10.2	10.1
40	10.3	10.3	10.3
50	10.25	10.35	10.3

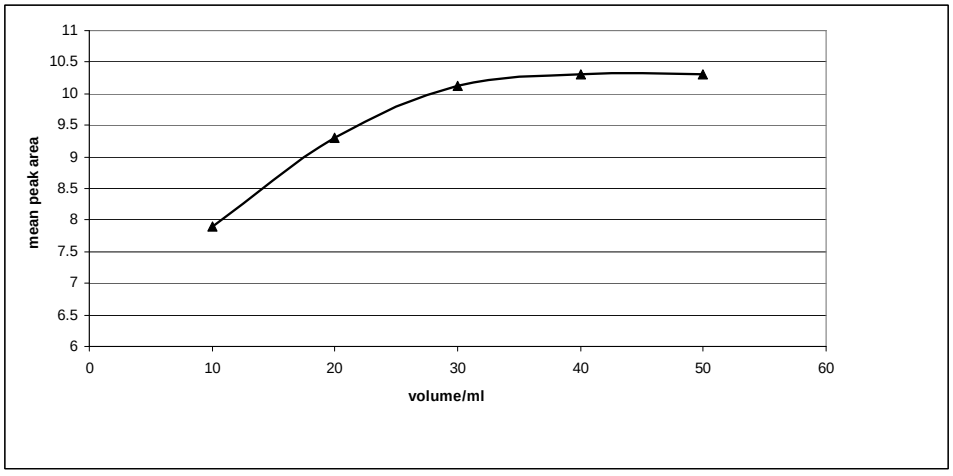


Figure 3.1 Graph showing the effect of volume on peak area of the peak corresponding to 5-HTP

Table 3-2 Effect of time on peak area of the peak corresponding to 5-HTP for triturated seeds

Time	Peak Area1	Peak Area2	Mean
15	18.881	19.9305	19.40575
30	18.887	16.337	17.612
60	17.841	15.141	16.491
90	17.991	15.738	16.8645

Table 3-3 Effect of time on peak area of the peak corresponding to 5-HTP for powdered seeds

Time	Peak Area1	Peak Area 2	Mean
5	4.291	4.291	4.2910
15	7.471	9.066	8.2685
30	9.883	10.063	9.9730
60	14.331	14.797	14.564

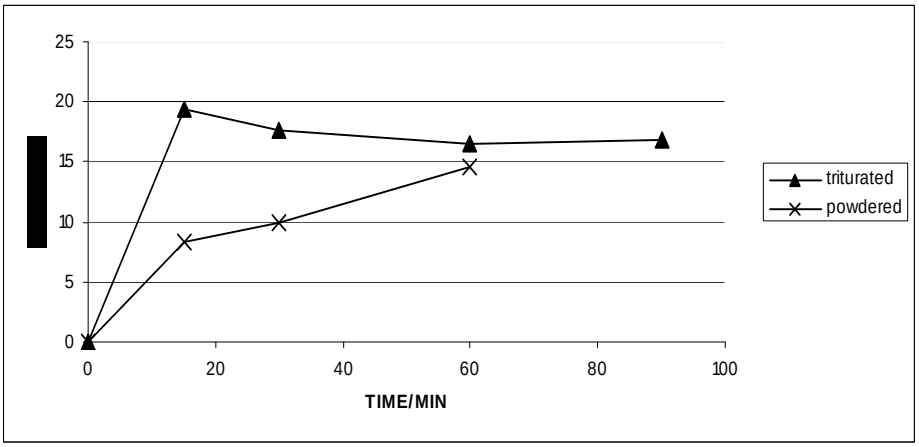


Figure 3.2 Graph showing effect of time on yield of 5-HTP for triturated and powdered seeds

Table 3-4 Effect of temperature on peak area of the peak corresponding to 5-HTP for powdered seeds

Temp/ºC	Peak Area1	Peak Area 2	Mean
Room(30)	10.2	9.3	9.75
45	12.3	13	12.65
65	14.4	13.8	14.1
80	17.3	16.2	16.75

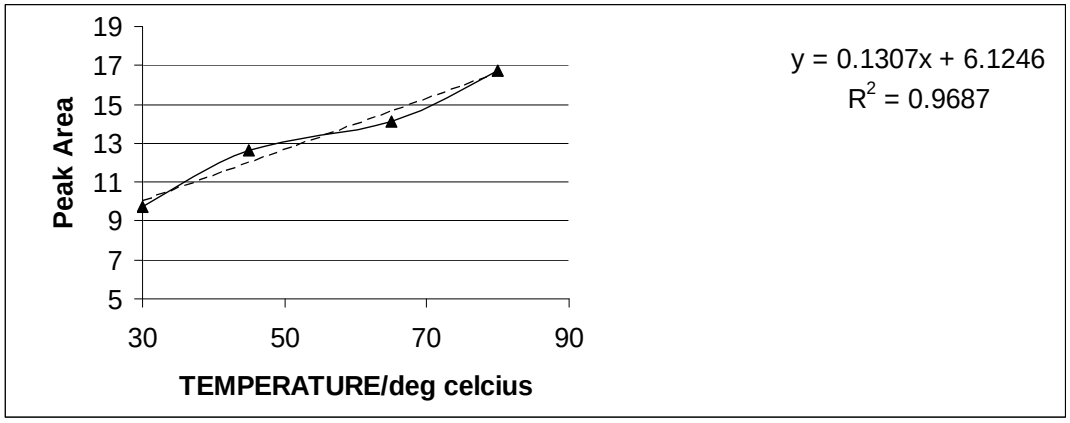


Figure 3.3 Graph (linear) showing the effect of temperature on peak area of the peak corresponding to 5-HTP

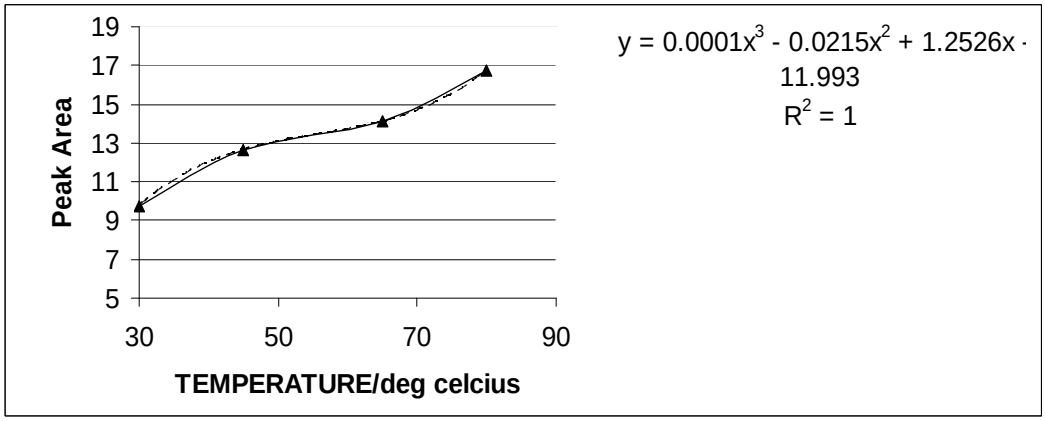


Figure 3.4 Graph (polynomial) showing the effect of temperature on peak area of the peak corresponding to 5-HTP

3.3 MEASUREMENT OF THE CONTRIBUTION OF THE FACTORS THAT AFFECT THE EXTRACTION

Percentage yield of 5-HTP obtained per seed weight was estimated. The contribution of a particular factor was estimated by calculating the difference between the percentage yield obtained in the absence of that factor and that obtained when that factor was introduced.

Table 3-5 Contribution of particle size (100 ml solvent) 30 ºC

	Peak area	%yield
Powdered	160.00	4.16
Triturated	244.73	6.37
Difference		**2.21**

Table 3-6 Contribution of Particle size (20 ml solvent) 30 ºC

	Peak area	%yield
Powdered	51.10	1.33
Triturated	72.99	1.90
Difference		**0.57**

Table 3-7 Contribution of particle size (100 ml solvent) 80 ºC

	Peak area	%yield
Powdered	250.11	6.51
Triturated	345.00	8.98
Difference		**2.47**

Table 3-8 Contribution of Particle size (20 ml solvent) 80 ºC

	Peak area	%yield
Powdered	86.82	2.26
Triturated	150.22	3.91
Difference		**1.75**

Table 3-9 Contribution of Temperature (for powdered seeds) 100 ml

Temperature/ºC	Peak area	%yield
Room temp (30)	160.00	4.16
80	250.11	6.51
Difference		**1.35**

Table 3-10 Contribution of Temperature (for triturated seeds) 100 ml

Temperature/ºC	Peak area	%yield
30	244.73	6.37
80	345.00	8.98
Difference		**2.61**

Table 3-11 Contribution of Temperature (for powdered seeds) 20 ml

Temperature/ºC	Peak area	%yield
30	51.10	1.33
80	86.82	2.26
Difference		**0.93**

Table 3-12 Contribution of Temperature (for triturated seeds) 20ml

Temperature/ºC	Peak area	%yield
30	72.99	1.90
80	150.22	3.91
Difference		**2.01**

Table 3-13 Contribution of volume (for powdered seeds)rt

Volume/ml	Peak area	%yield
20	51.10	1.33
100	160.00	4.16
Difference		**2.83**

Table 3-14 Contribution of volume (for triturated seeds)rt

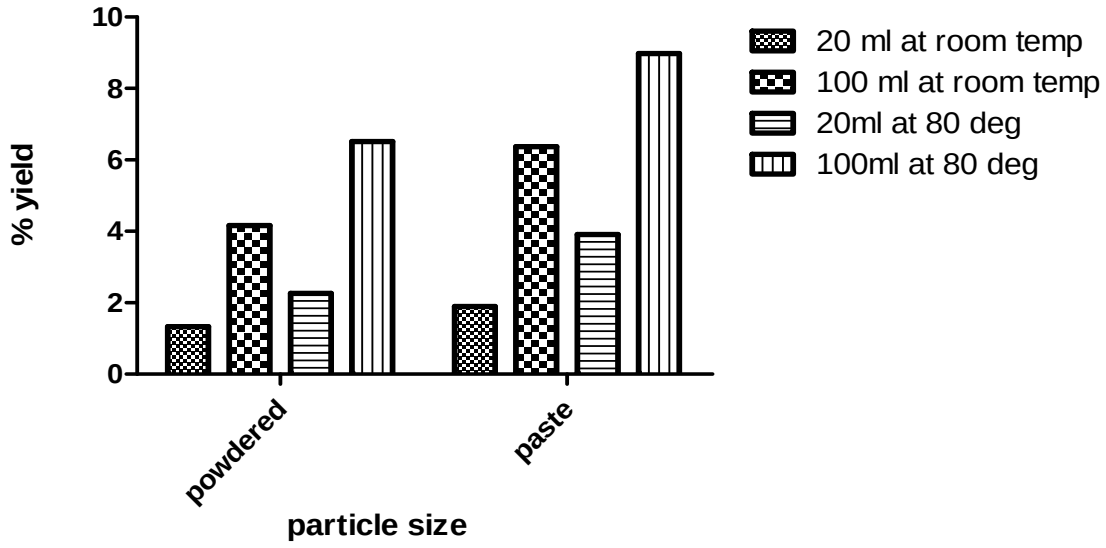
Volume/ml	Peak area	%yield
20	72.99	1.90
100	244.73	6.37
Difference		**4.47**

Table 3-15 Contribution of volume (for powdered seeds) 80 ºC

Volume/ml	Peak area	%yield
20	86.82	2.26
100	250.11	6.51
Difference		**4.25**

Table 3-16 Contribution of volume (for triturated seeds) 80 ºC

Volume/ml	Peak area	%yield
20	150.22	3.91
100	345.00	8.98
Difference		**5.07**



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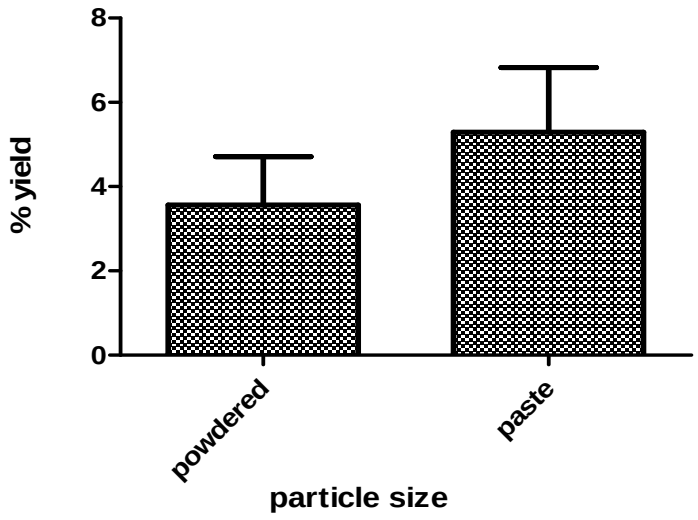
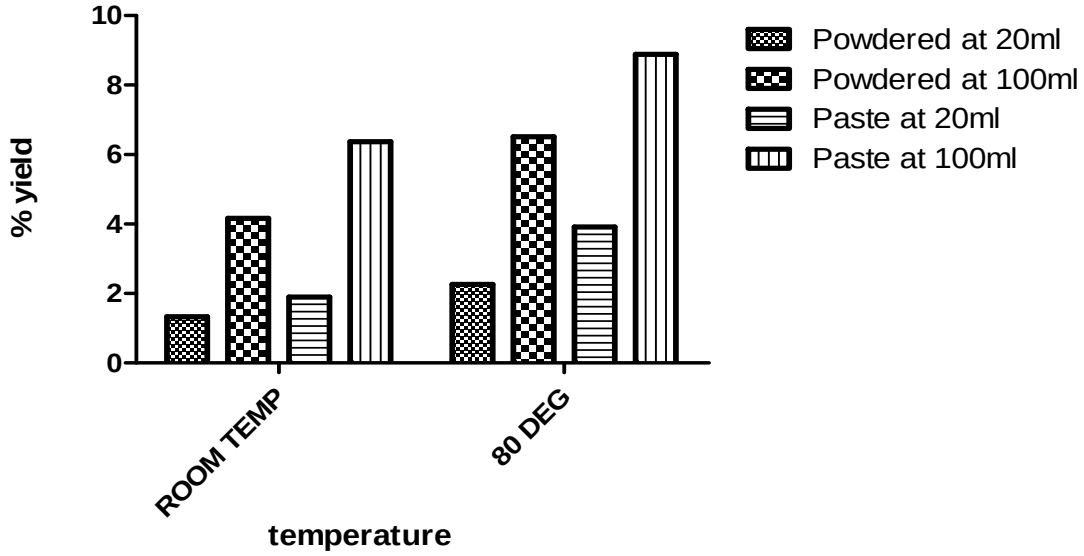


b)

Figure 3.5 Graphs showing the effect of particle size on yield of 5-HTP extracted

- %yield = weight of 5-HTP /weight of seed taken*100%
- 20ml at room temperature refers to yield obtained from 1g of powdered seeds which was extracted with 20ml of solvent at room temperature
- 100ml at room temperature refers to yield obtained from 1g of powdered seeds which was extracted with 100ml of solvent at room temperature
- 20ml at 80 deg refers to yield obtained from 1g of powdered seeds which was extracted with 20ml of solvent at 80°C
- 100ml at 80 deg refers to yield obtained from 1g of powdered seeds which was extracted with 100ml of solvent at 80°C



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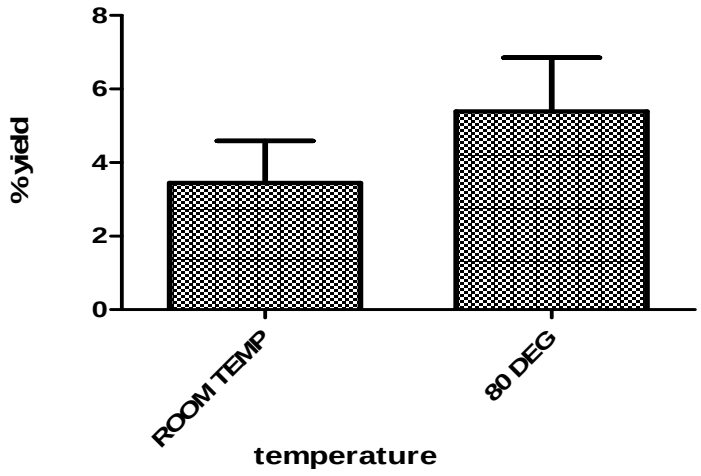
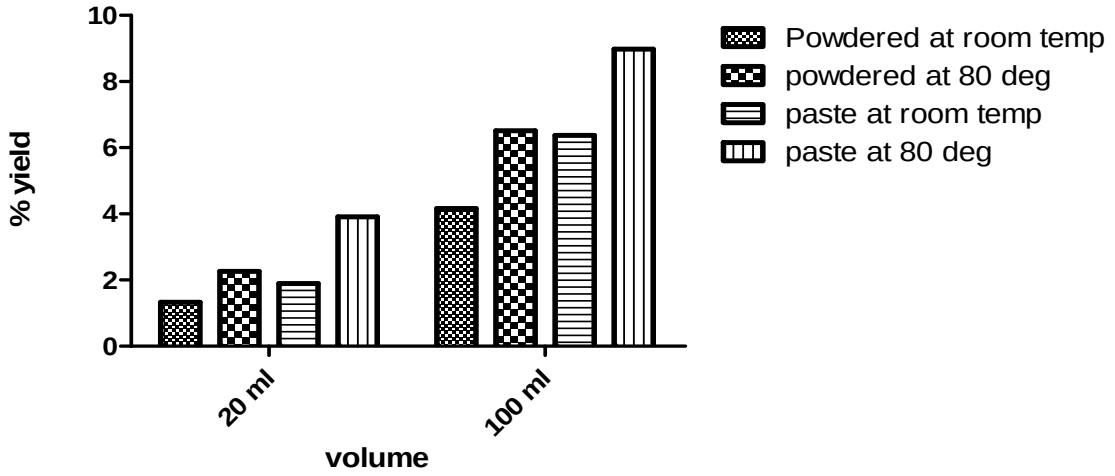


b)

Figure 3.6Graphs showing the effect of increase in temperature on yield of 5-HTP extracted

- %yield = weight of 5-HTP /weight of seed taken*100%
- Powdered at 20ml refers to yield obtained from 1g of powdered seeds which was extracted with 20ml of solvent
- Powdered at 100ml refers to yield obtained from 1g of powdered seeds which was extracted with 100ml of solvent
- Paste at 20ml refers to yield obtained from 1g of triturated seeds which was extracted with 20ml of solvent
- Paste at 100ml refers to yield obtained from 1g of triturated seeds which was extracted with 100ml of solvent



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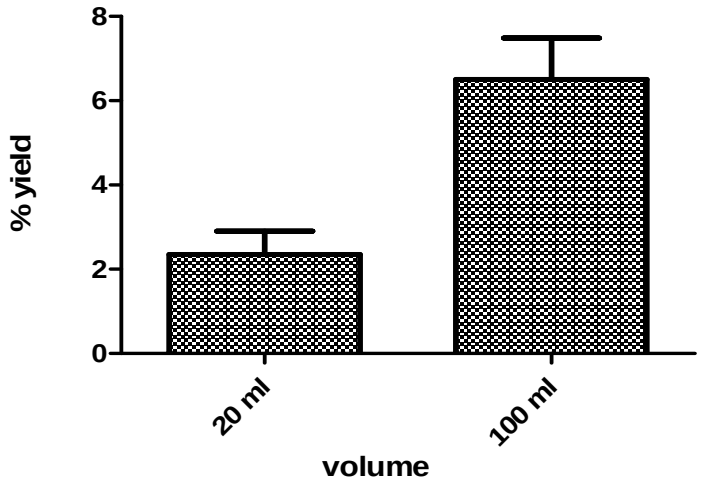


b)

Figure 3.7 Graphs showing the effect of volume of solvent on yield of 5-HTP extracted

- %yield = weight of 5-HTP /weight of seed taken*100%
- Powdered at room temp refers to yield obtained from 1g of powdered seeds which was extracted with solvent at room temperature
- Powdered at 80 deg refers to yield obtained from 1g of powdered seeds which was extracted with solvent at 80°C
- Paste at room temp refers to yield obtained from 1g of triturated seeds which was extracted with solvent at room temperature
- Paste at 80 deg refers to yield obtained from 1g of triturated seeds which was extracted with solvent at 80°C

Table 3-17Determination Of Suitable Solvent Composition For Recrystallisation Of 5-Htp

Solvent	Cold	Hot
Methanol	Soluble	very soluble
Water	Partially soluble	very soluble
Ethanol	Insoluble	Sparingly soluble
Water :ethanol 50:50	Insoluble	very soluble
Water :ethanol 25:75	Insoluble	Soluble
Water :ethanol 20:80	Insoluble	Soluble
Water :ethanol 15:85	Insoluble	Soluble
Water :ethanol 10:90	Insoluble	Sparingly soluble

Table 3-18 Table showing change in purity of 5-HTP during purification

Weight of seeds/g	Amount of 5-HTP expected/g	Weight of extract	%purity of extract
104.6240	9.3950	25.3754	45.27
100.3131	9.0081	22.2062	42.88

weight of crystals formed after washing and drying	% purity of crystals	weight of crystals formed after recrystallisation	% purity of crystals
8.964	78.34	6.4326	90.33
8.570	72.87	5.1520	92.54

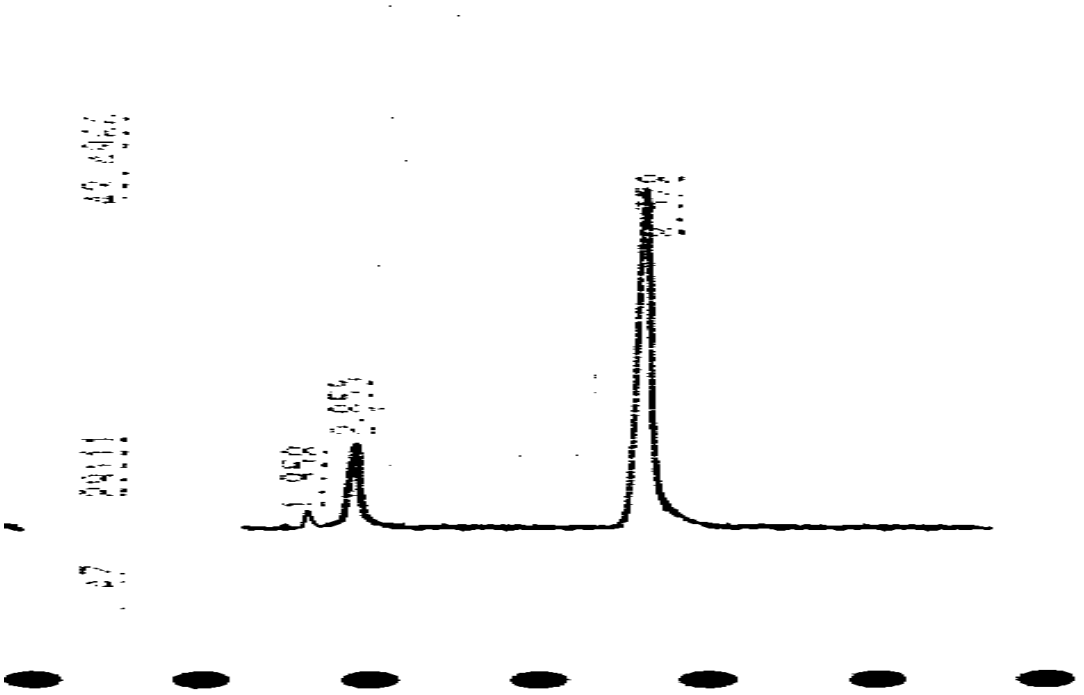


Figure 3.8 HPLC Chromatogram for unpurified water-methanol extract

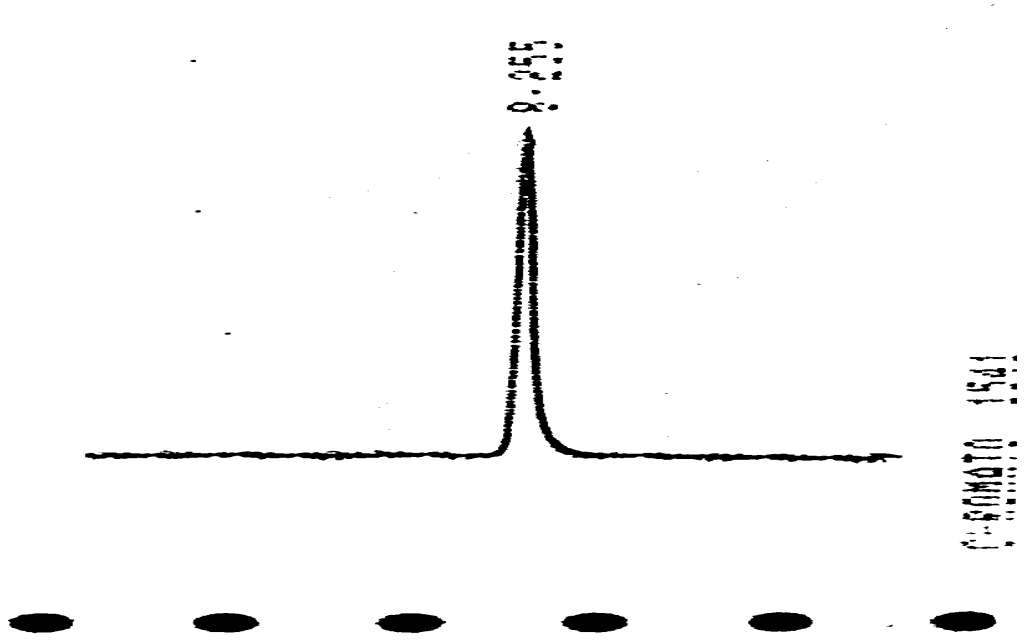


Figure 3.9 HPLC Chromatogram for 5-HTP crystals obtained after recrystallisation

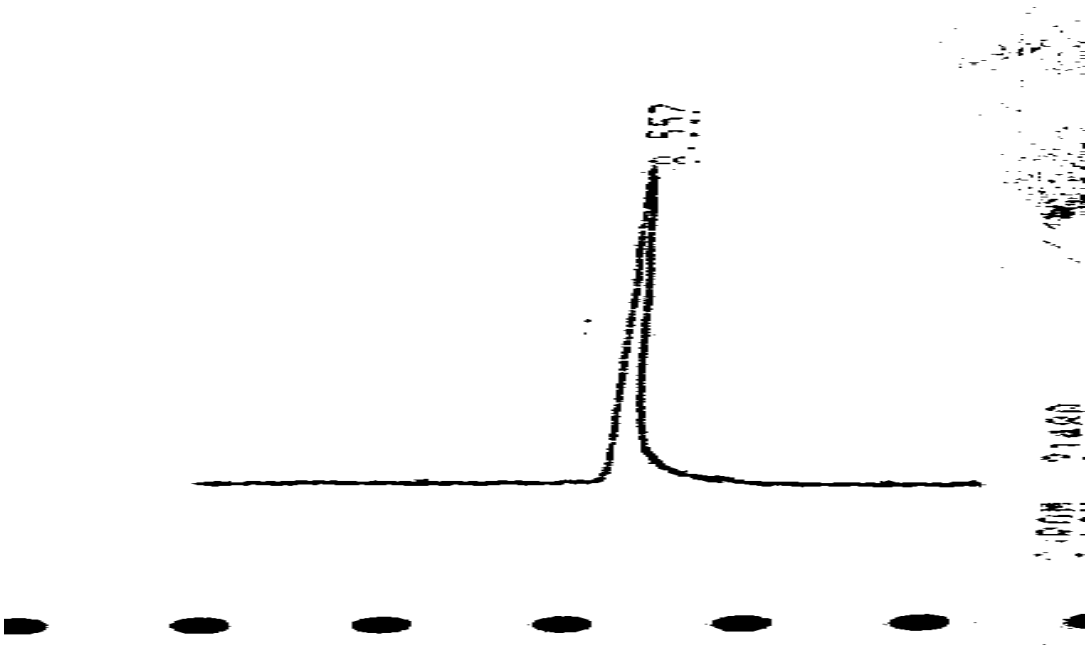


Figure 3.10 HPLC Chromatogram for Pure 5-HTP

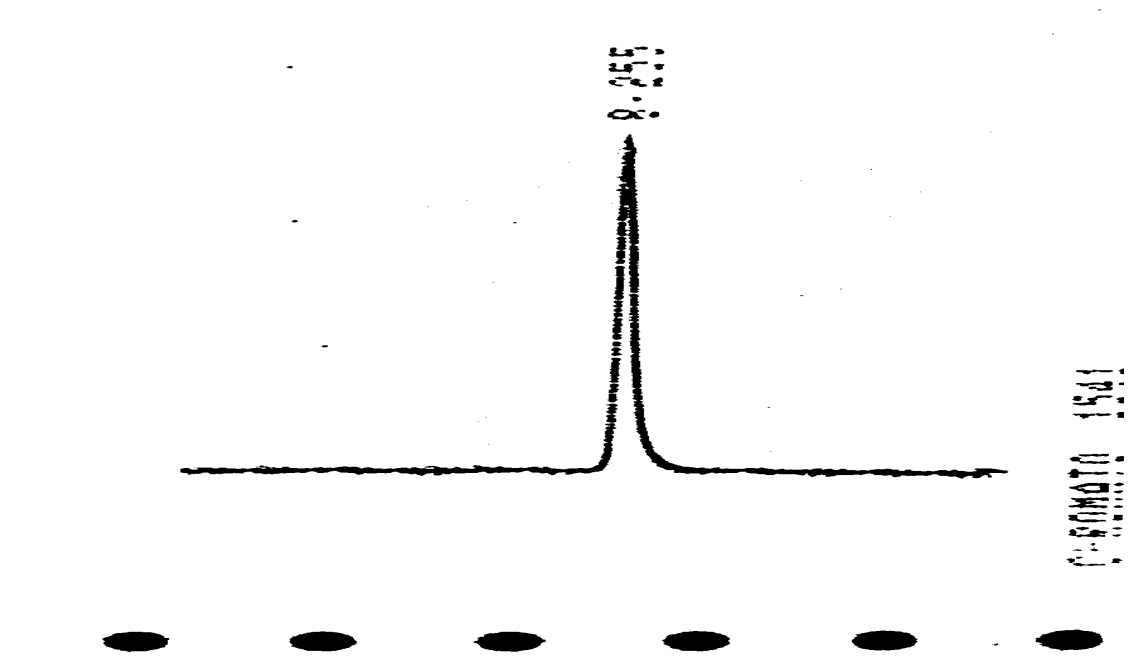


Figure 3.11 HPLC Chromatogram for 5-HTP crystals before stability studies

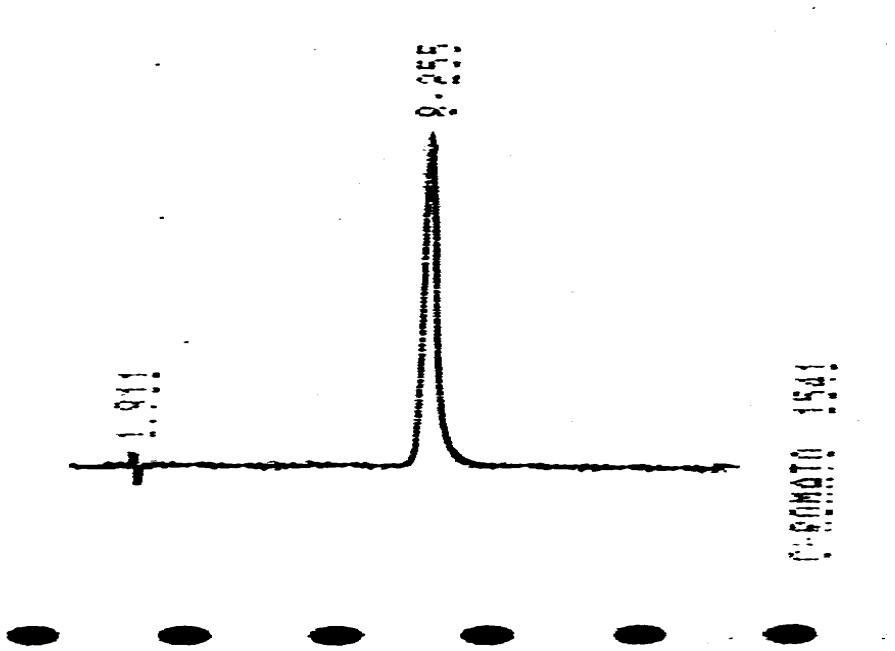


Figure 3.12 HPLC Chromatogram for 5-HTP crystals after stability studies (after 30days).

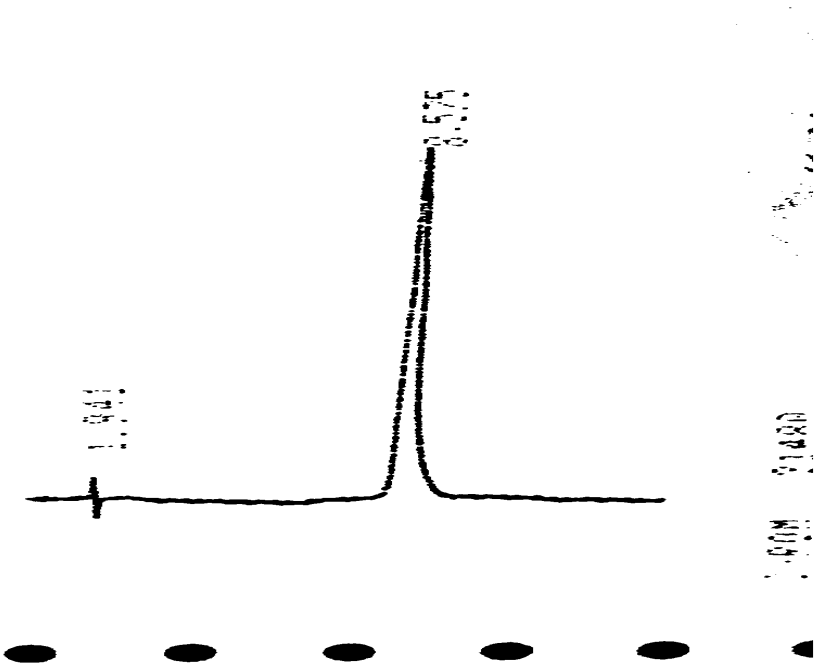


Figure 3.13 HPLC Chromatogram for 5-HTP crystals after stability studies (after 90days)

The peak at retention time of approximately 1.9 was determined to be due to solvent.

3.4 STABILITY STUDIES

Table 3-19 results for stability test at 5 ºC

Time/days	Concentration(%w/w)	log C	log C - log Co
0	90.96	1.65782	0
45	85.80	1.632457	-0.02536
90	83.14	1.61878	-0.03904

Table 3-20 Results for stability test at 30 ºC

Time/days	Concentration(%w/w)	log C	log C - log Co
0	90.96	1.65782	0
45	84.54	1.626032	-0.03179
90	82.48	1.615319	-0.0425

Table 3-21 Results for stability test at 45 ºC

Time/days	Concentration	log C	log C - log Co
0	90.96	1.65782	0
45	84.38	1.62521	-0.03261
90	80.96	1.607241	-0.05058

Table 3-22 Results for stability test at 60 ºC

Time/days	Concentration	log C	log C - log Co
0	90.96	1.65782	0
45	83.12	1.618676	-0.03914
90	80.10	1.602603	-0.05522

Table 3-23

TIME/days	5 ºC	30 ºC	40 ºC	60 ºC
0.	0.000000	0.000000	0.000000	0.000000
45.	-0.02536271	-0.03178775	-0.03261048	-0.03914446
90.	-0.03903997	-0.04250134	-0.0505795	-0.05521748

Graphs of logC- logC_o versus time were plotted for all temperatures and these were used to estimate the rate constants at the respective temperatures

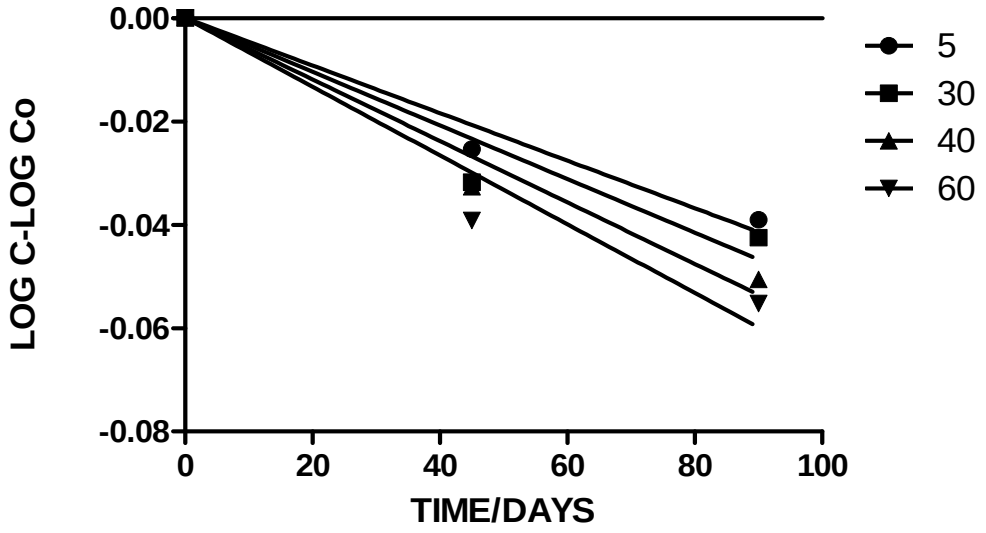


Figure 3.14 Plot of log C- log C_0 versus time

Table 3-24 Table For the Arrhenius Plot

SLOPE(S)	K(-2.303 × S)/day^{-1}	log k	t/ ºC	T/K	1/T
-0.00046	0.001059	-2.97523	5	278	0.003597
-0.000519	0.001195	-2.92249	30	303	0.0033
-0.000595	0.001369	-2.86356	40	313	0.003195
-0.000665	0.001531	-2.81502	60	333	0.003003

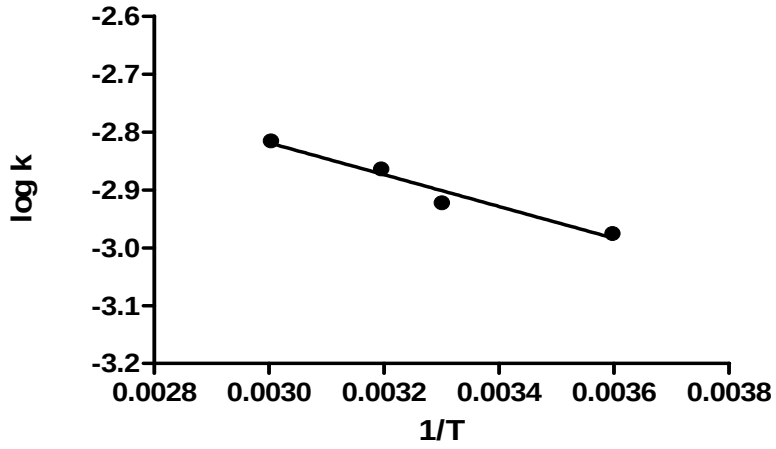


Figure 3.15Arrhenius Plot of experimental results

CALCULATION OF SHELF LIFE AT THE INVESTIGATED TEMPERATURES

At 30°C,

From graph of (log C – log C_0) against time, slope = -k/2.303

Therefore k = -2.303 × slope

k = -2.303 × (-0.000519) = 1.1954×10^{-3} day^{-1}

Shelf life, t_{90}

From the relation, log (C_0/C) = kt / 2.303

Where C_0 = initial concentration

C = concentration at time, t

K = rate of decomposition

T = [2.303/k] log (C_0/C)

T_{90} = [2.303/k] log (100/90)

= [2.303/1.1954×10^{-3}] × 0.0458

= 88.24 days.

= 2.94 months

Table 3-25 Shelf lives calculated at the various temperatures

TEMPERATURE (ºC)	**K (day^{-1})**	**t_{90} in days (months)**
5	1.059 E-03	99.62 (3.32)
30	1.195 E-03	88.28 (2.94)
40	1.369 E-03	77.06 (2.57)
60	1.531 E-03	68.91 (2.30)

3.5 RESULTS FOR THE SEPARATION OF THE COMPONENTS OF THE AQUEOUS METHANOLIC EXTRACT

3.5.1 THIN LAYER CHROMATOGRAHY

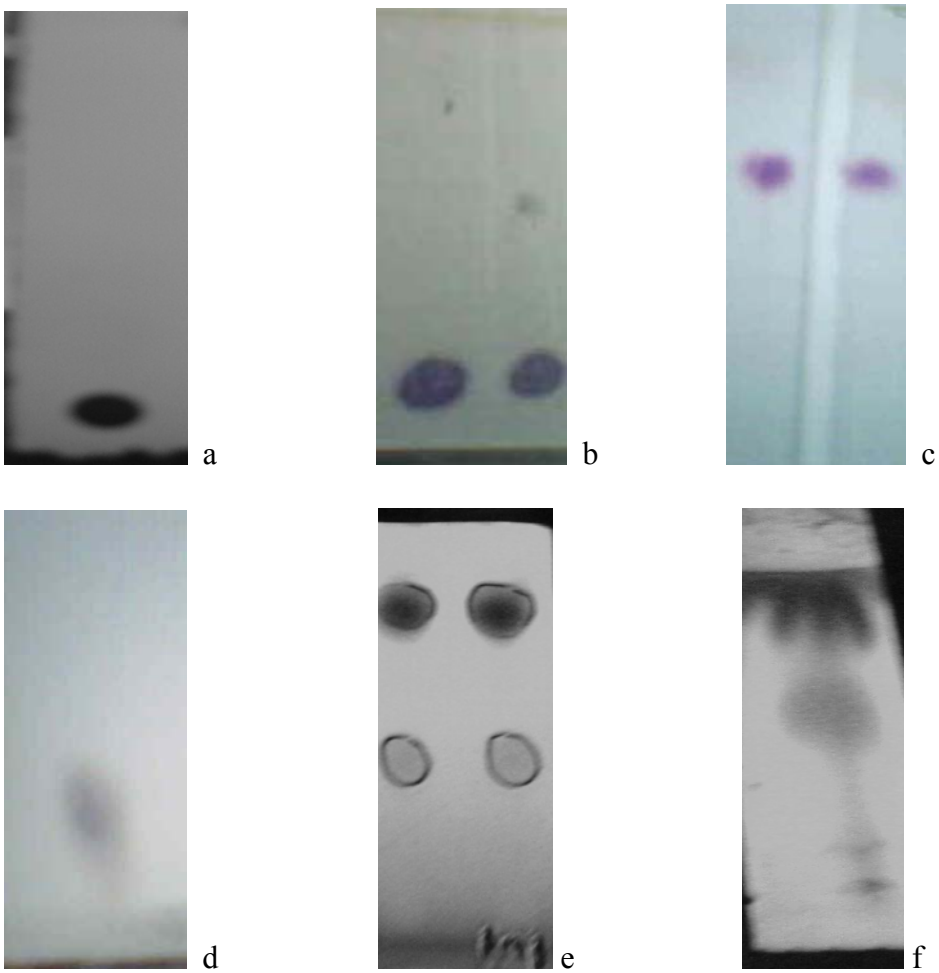


Figure 3.16 Chromatograms showing separations offered by various solvent compositions on a silica stationary phase a) $CHCl_3$ b) 5:95 methanol in $CHCl_3$ c) 20:80 methanol in $CHCl_3$ d) 20:80 ethanol in Chloroform e)n-propanol : water:acetic acid 12:5:3 f) ethanol : water :acetic acid 12:5:3

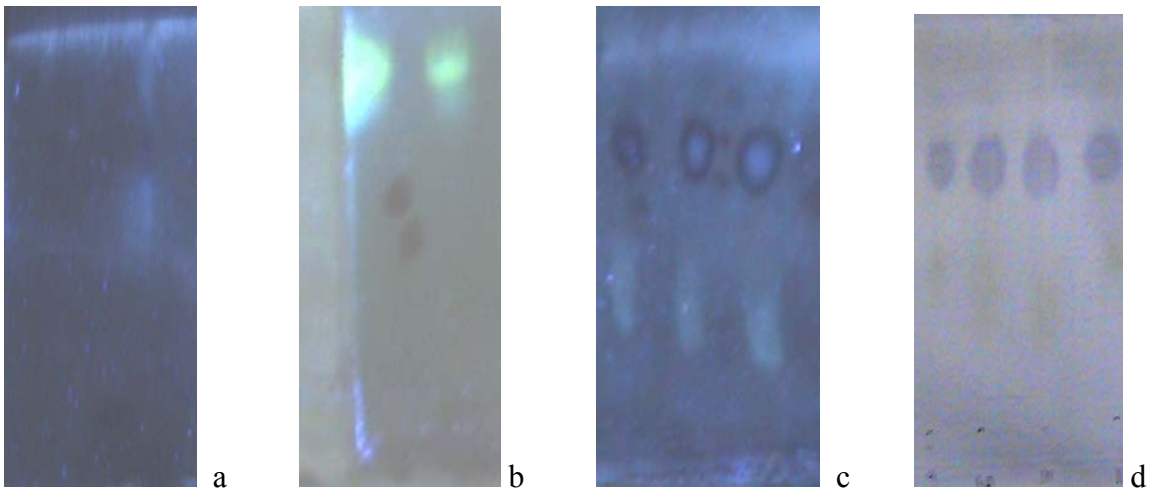


Figure 3.17 Chromatograms from eluates from column chromatography

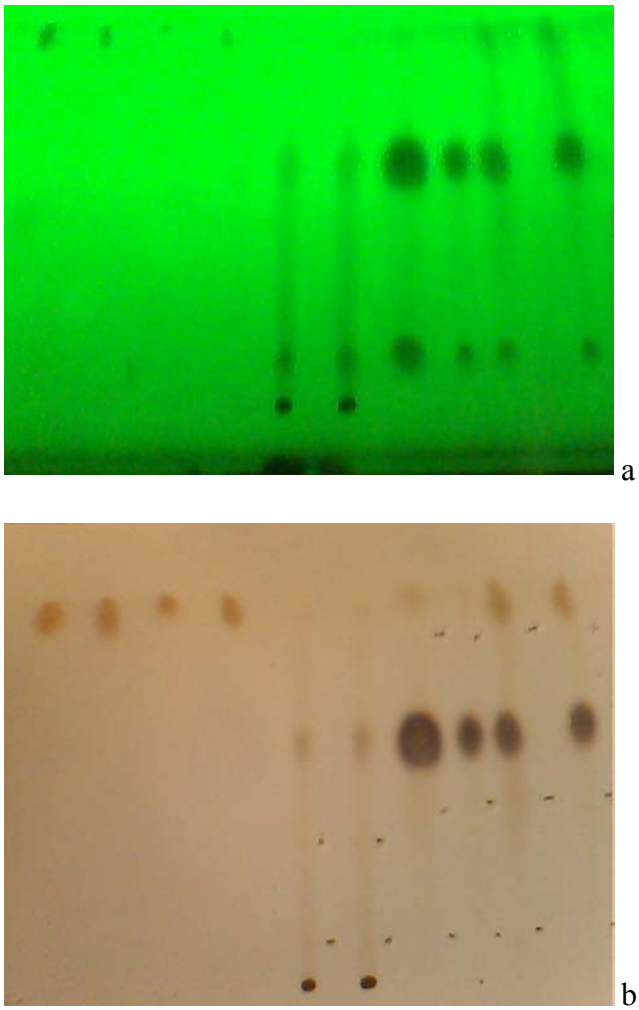


Figure 3.18 TLC Chromatograms for ether, chloroform, water, ethanol and methanol extracts of Griffonia seeds a) under 254nm b)white light

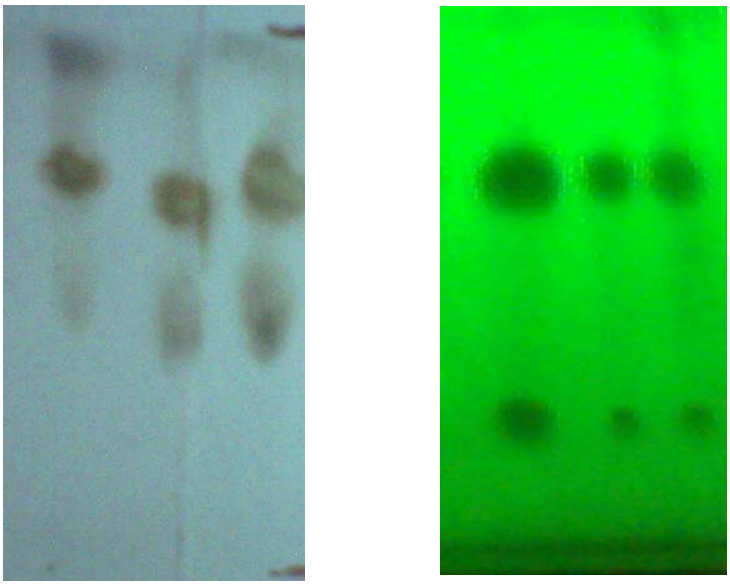


Figure 3.19 TLC chromatogram of aqueous methanolic extract of seeds of 5-HTP using butanol:water:acetic acid 12:5:3 as mobile phase. a)after spraying with 5:95 H_2SO_4 in methanol b)under 254nm

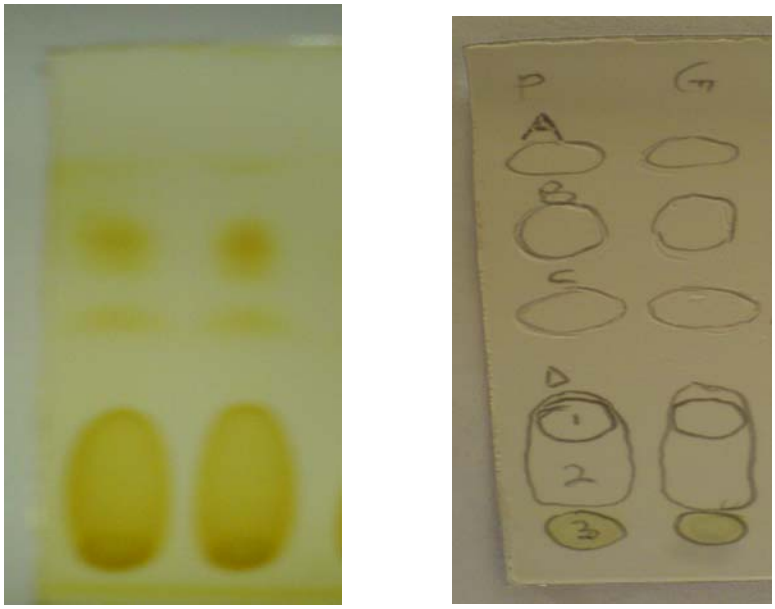


Figure 3.20 TLC chromatogram for pet ether extract using pet ether/chloroform 50:50 as mobile phase and silica as stationary phase and iodine vapour as detecting reagent

3.5.2 RESULTS OF PRELIMINARY CHEMICAL TESTS

Table 3-26 Preliminary chemical tests on sample GWM1

Test	Observation	Inference
Nature	Light brown Powder	Compound may be of high molecular weight
Solubility	Dissolved in water and methanol after warming	Polar groups may be present
Effect on litmus	Red litmus turned blue	Basic compound may be present
Action of dilute HCl	Soluble	Basic compound may be present
Action of NaOH solution	Soluble	Acidic compound may be present
2,4 DNP condensation	No ppt formed	Carbonyl may be absent
Primary aromatic amine	Intense brown ppt	Primary aromatic amine may be present
Test for phenols	No coloured ppt	Phenol may be absent

Table 3-27 Preliminary chemical tests on sample GWM2

Test	Observation	Inference
Nature	Cream powder	Compound may be of high molecular weight
Solubility	Dissolved in water and methanol after warming	Polar groups may be prent
Effect on litmus	Red litmus turned blue	Basic compound may be present
Action of dilute HCl	Soluble	Basic compound may be present
Action of NaOH solution	Soluble	Acidic compound may be present
2,4 DNP condensation	No ppt formed	Carbonyl may be absent
Primary aromatic amine	Intense brown ppt	Primary aromatic amine may be present
Test for phenols	No coloured ppt	Phenol may be absent

3.5.3 UV-VISIBLE SPECTROPHOTOMETRIC SPECTRA RESULTS

Table 3-28 Table showing the wavelengths of absorption of GWM1

Wavelength(nm)	0.1M HCl	0.1M NaOH	Distilled Water
1	264.5	265.5	265.5
2	357.5	359.0	358.5
3	407.0	394.5	395.5

Table 3-29 Table showing the wavelengths of absorption of GWM2

Wavelength(nm)	0.1M HCl	0.1M NaOH	Distilled Water
1	273.5	273.0	275.0
2	356.5	357.0	355.0
3	384.0	394.0	386.5

Table 3-30 Table showing the wavelengths of absorption of 5-HTP

Wavelength(nm)	0.1M HCl	0.1M NaOH	Distilled Water
1	275.0	275	276.0
2		321	
3	356.0	359	356.0

Table 3-31 Preliminary chemical tests on 5-HTP

Test	Observation	Inference
Nature	Buff Powder	
Solubility	Dissolved in water and methanol after warming	Polar groups may be present
Reaction with litmus	Red litmus turned blue	Basic compound may be present
Action of dilute HCl	Soluble	Basic compound may be present
Action of NaOH solution	Soluble	Acidic group may be present
2,4 DNP condensation	No ppt formed	Carbonyl may be absent
Sodium fusion	Blue stain observed on filter paper	Nitrogen may be present
Test for phenols	Intense coloured ppt	Phenol may be present

3.6 SPECTROSCOPIC DATA OBTAINED FROM ISOLATED CONSTITUENTS

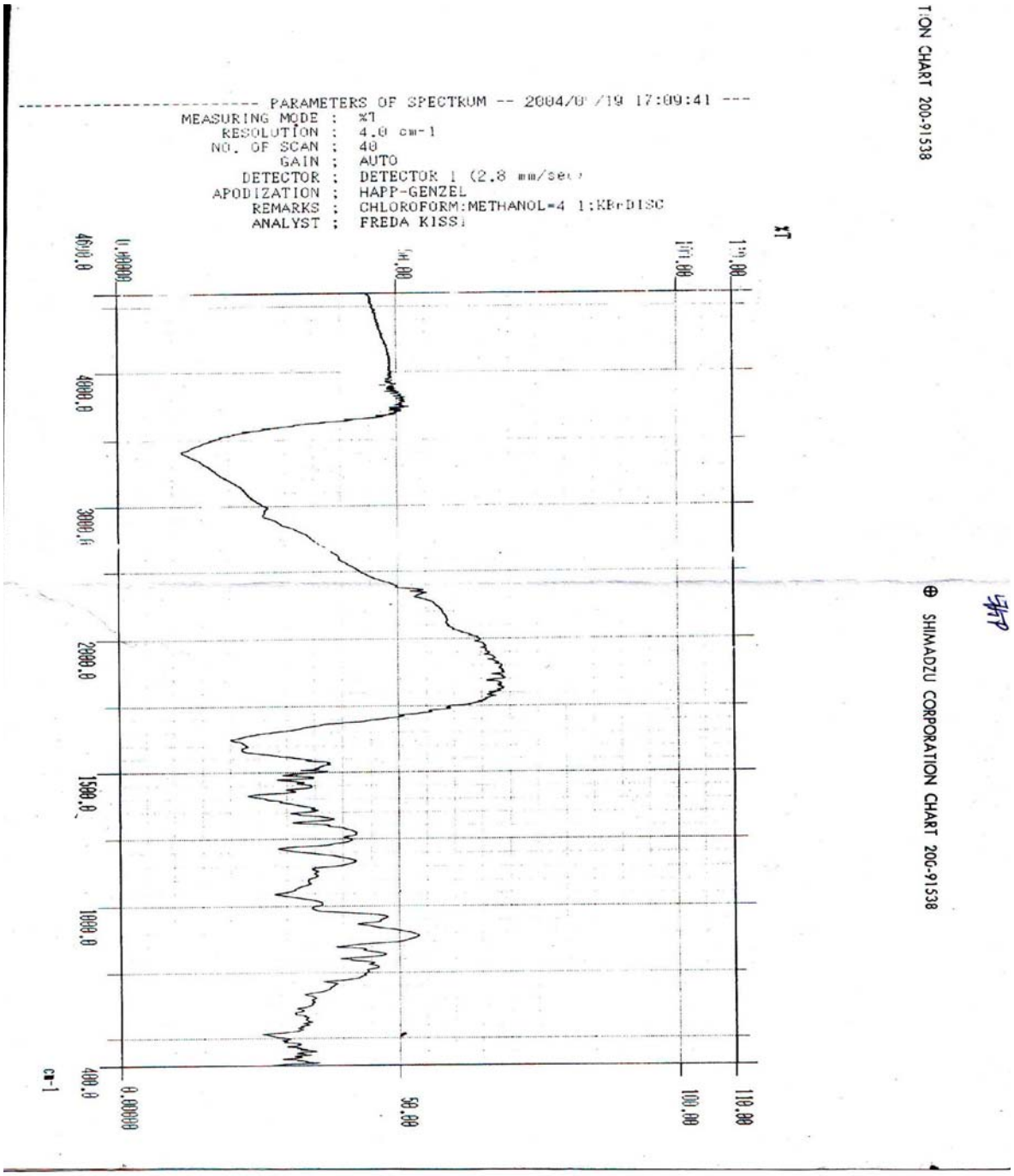


Figure 3.21 Infra Red Spectrum of 5-Htp Using KBr Disc

Figure 3.22Infra red spectrum of GWM1 using KBr Disc

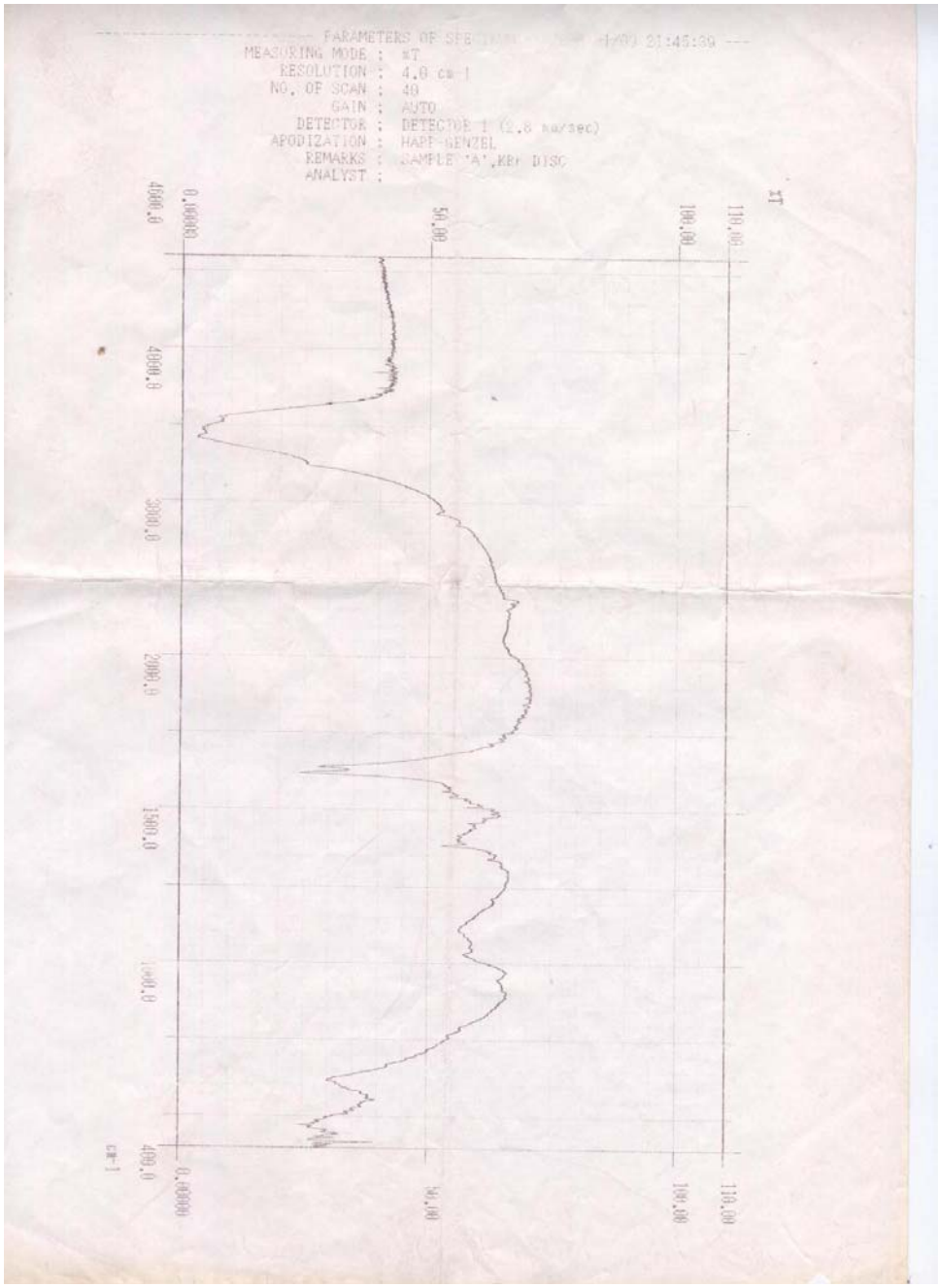


Figure 3.23Infra red spectrum GWM2 using KBr Disc

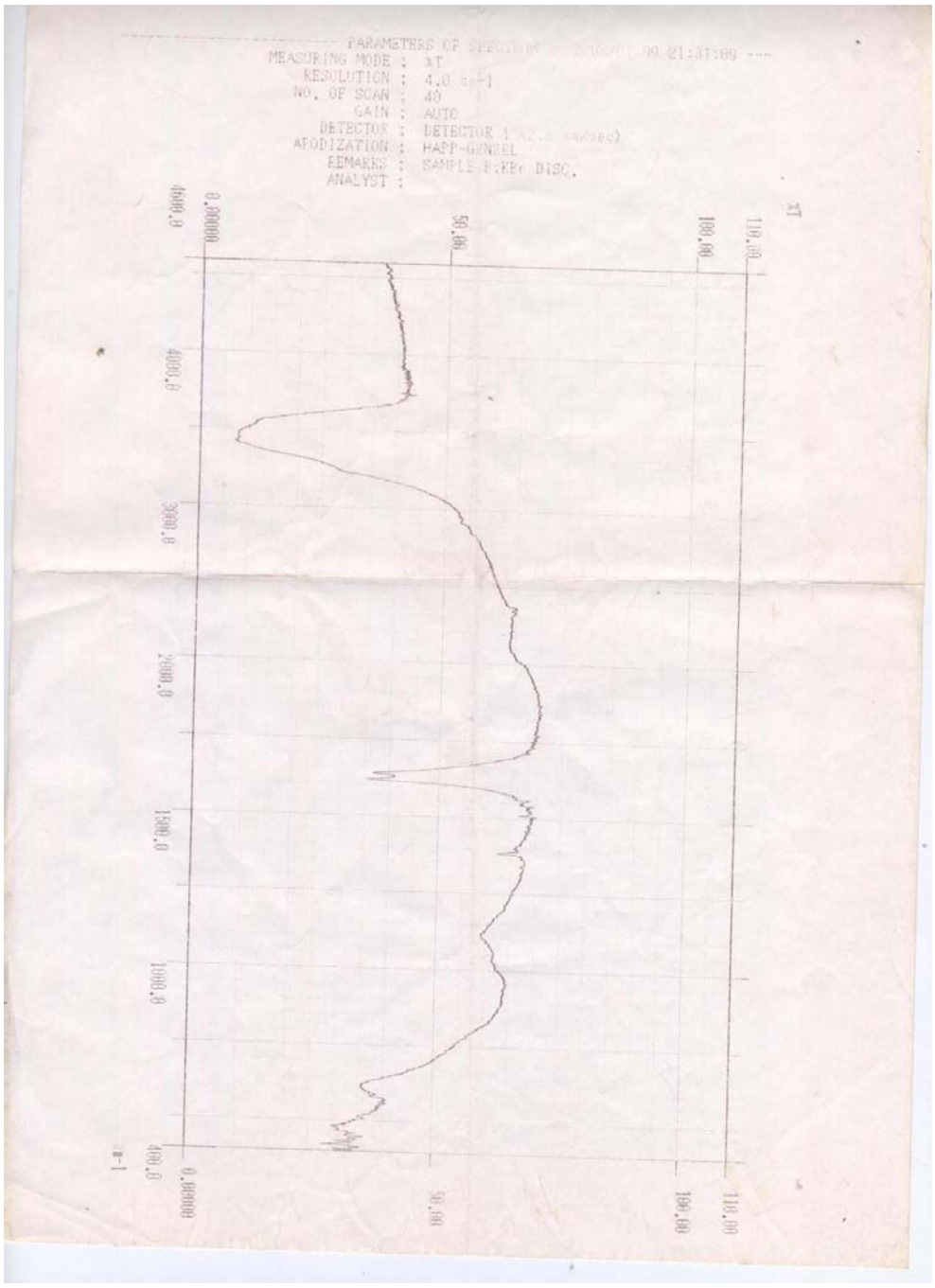


Figure 3.24Infra red spectrum of GWM1

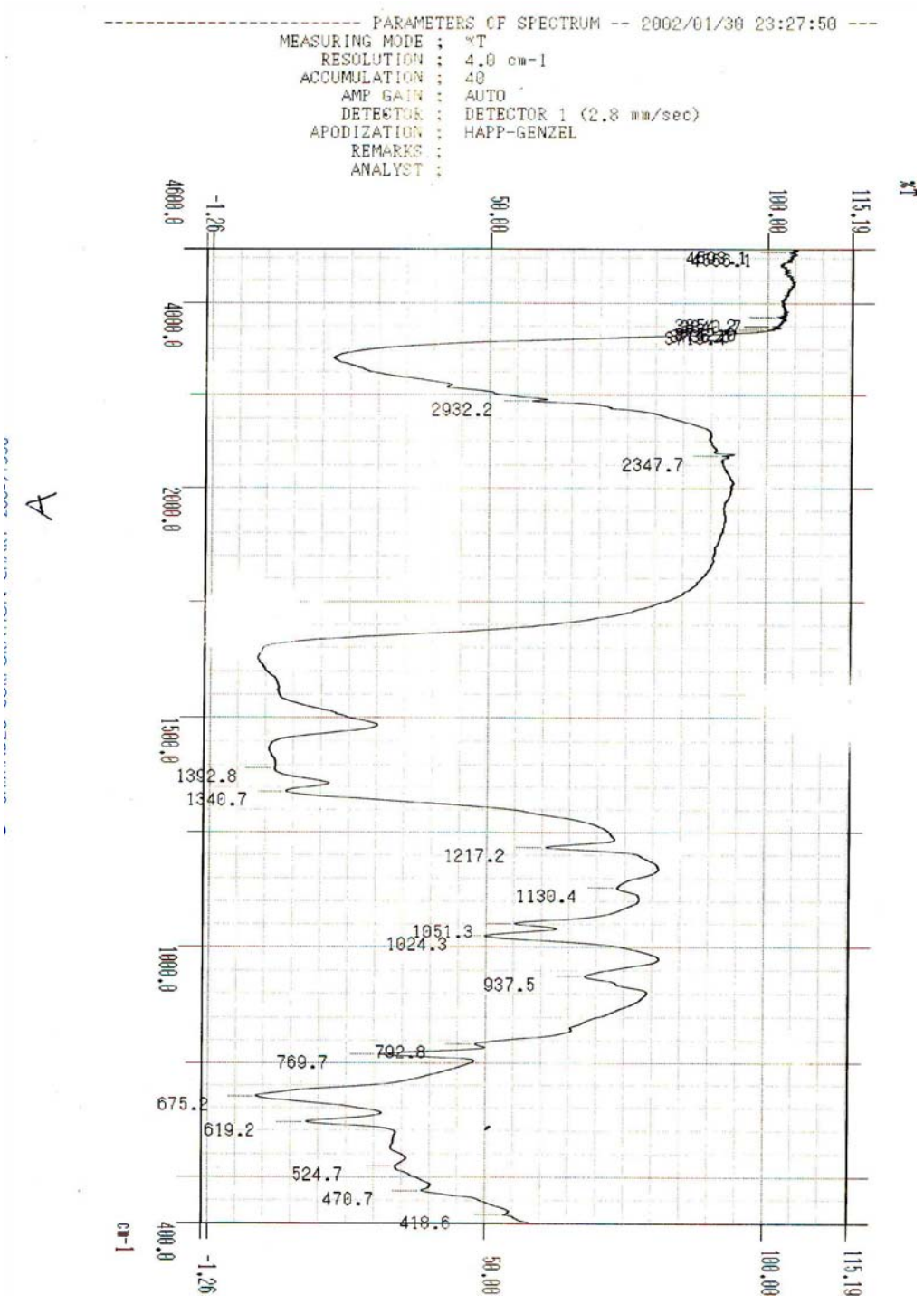


Figure 3.25Infra red spectrum of GWM2

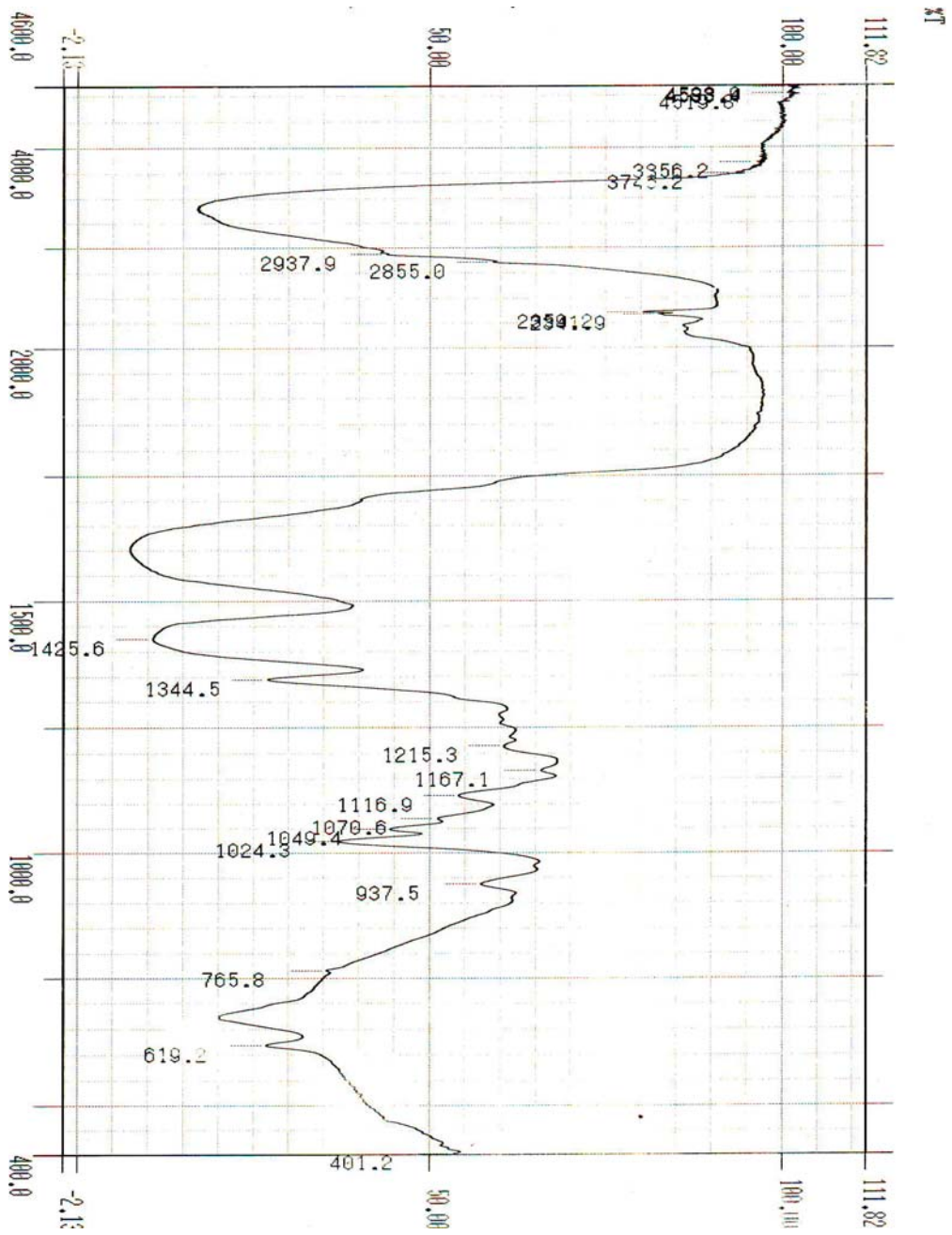


Figure 3.26Positive FAB-MS for GWM1

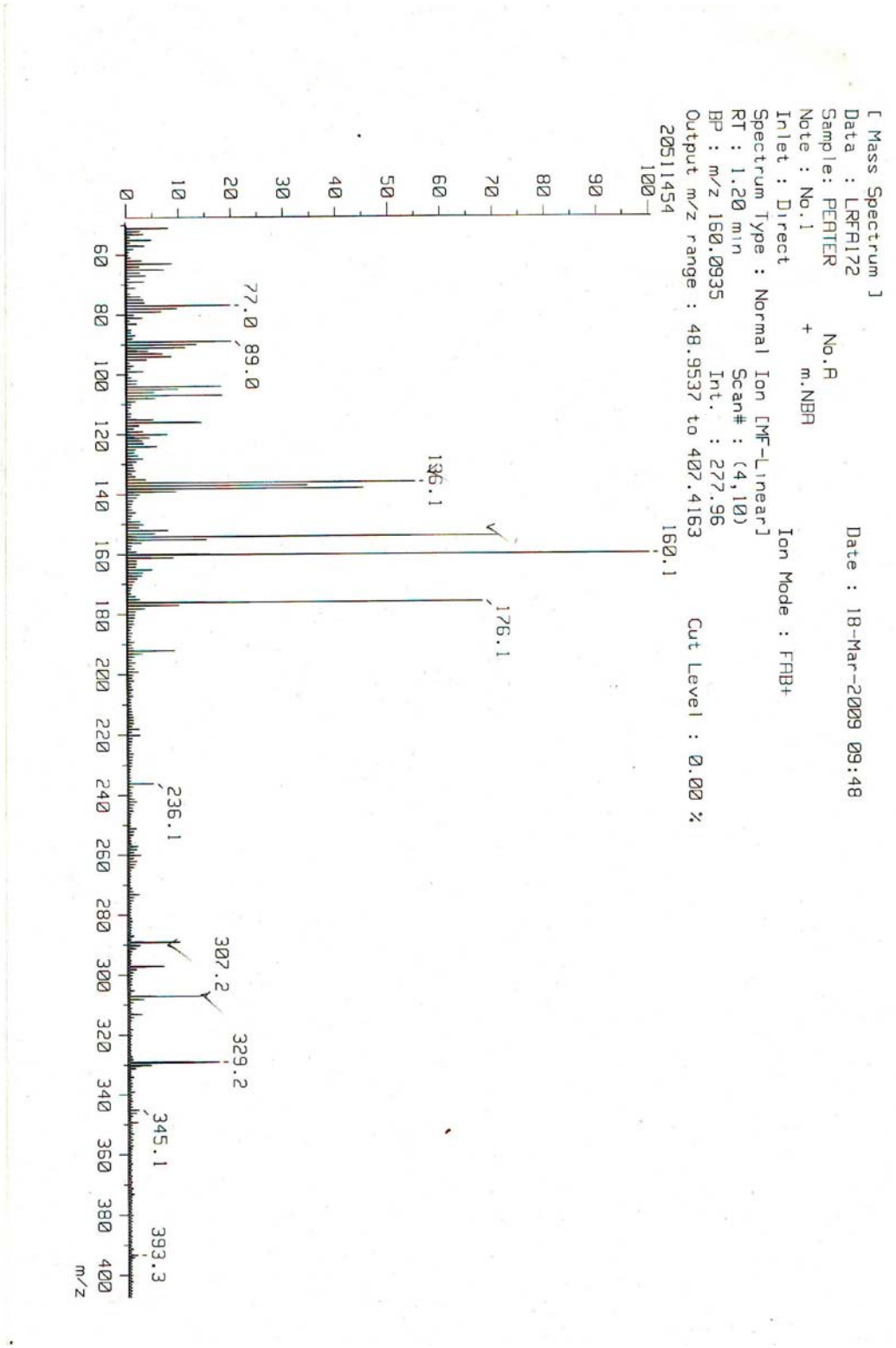


Figure 3.27Positive FAB-MS for GWM2

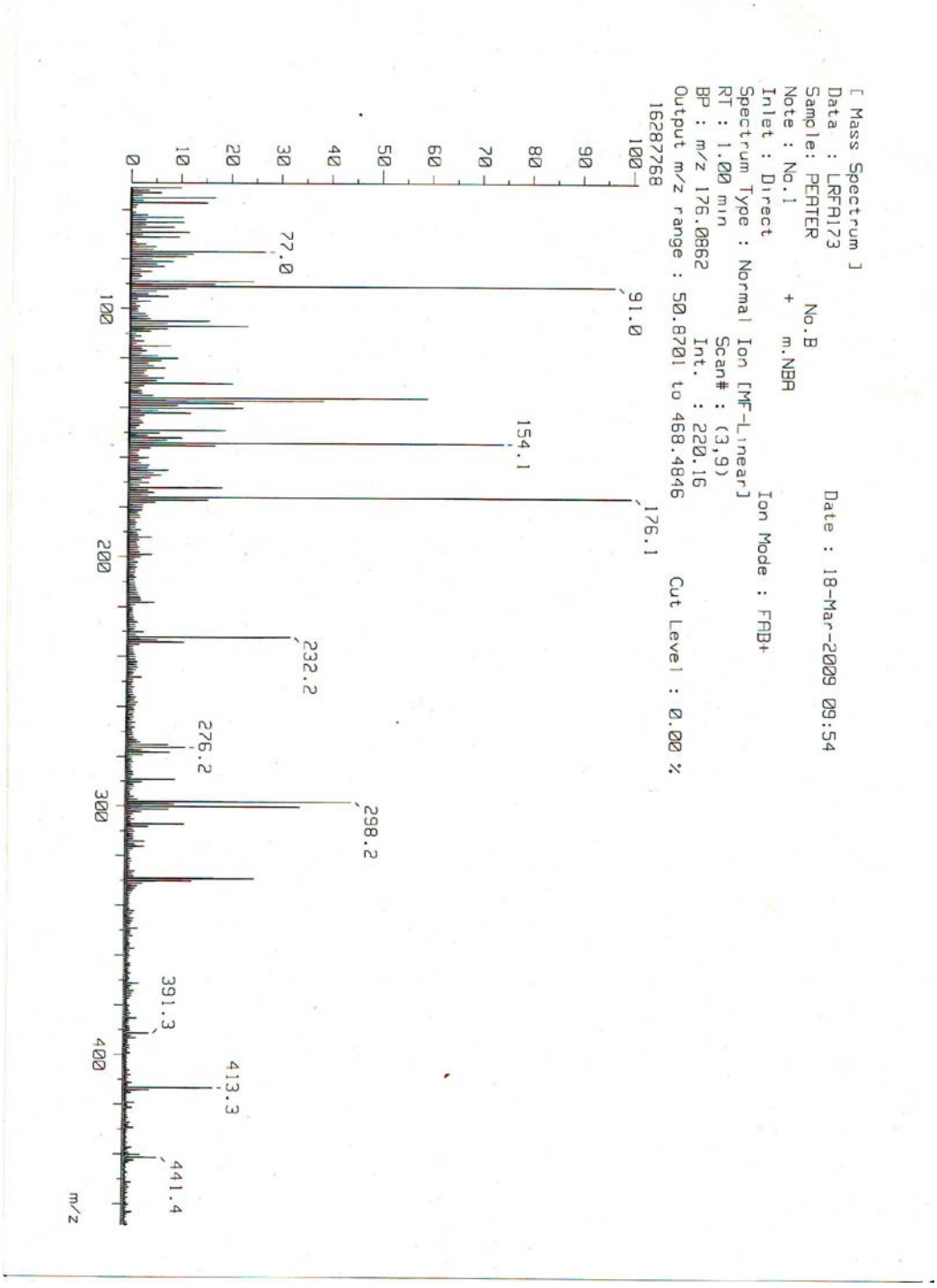


Figure 3.28 Shematic representation of proposed fragmentation of GWM1(A)

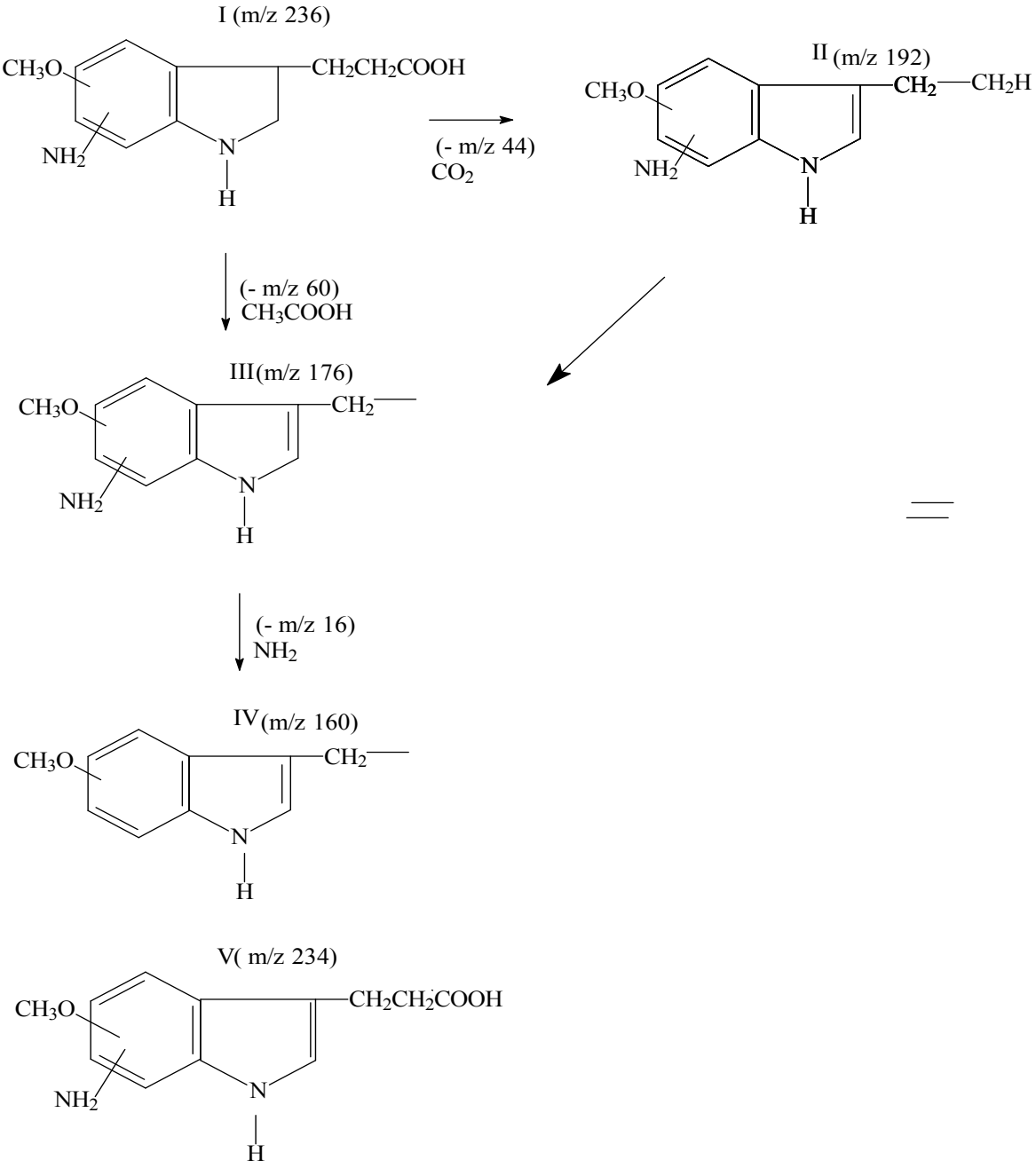
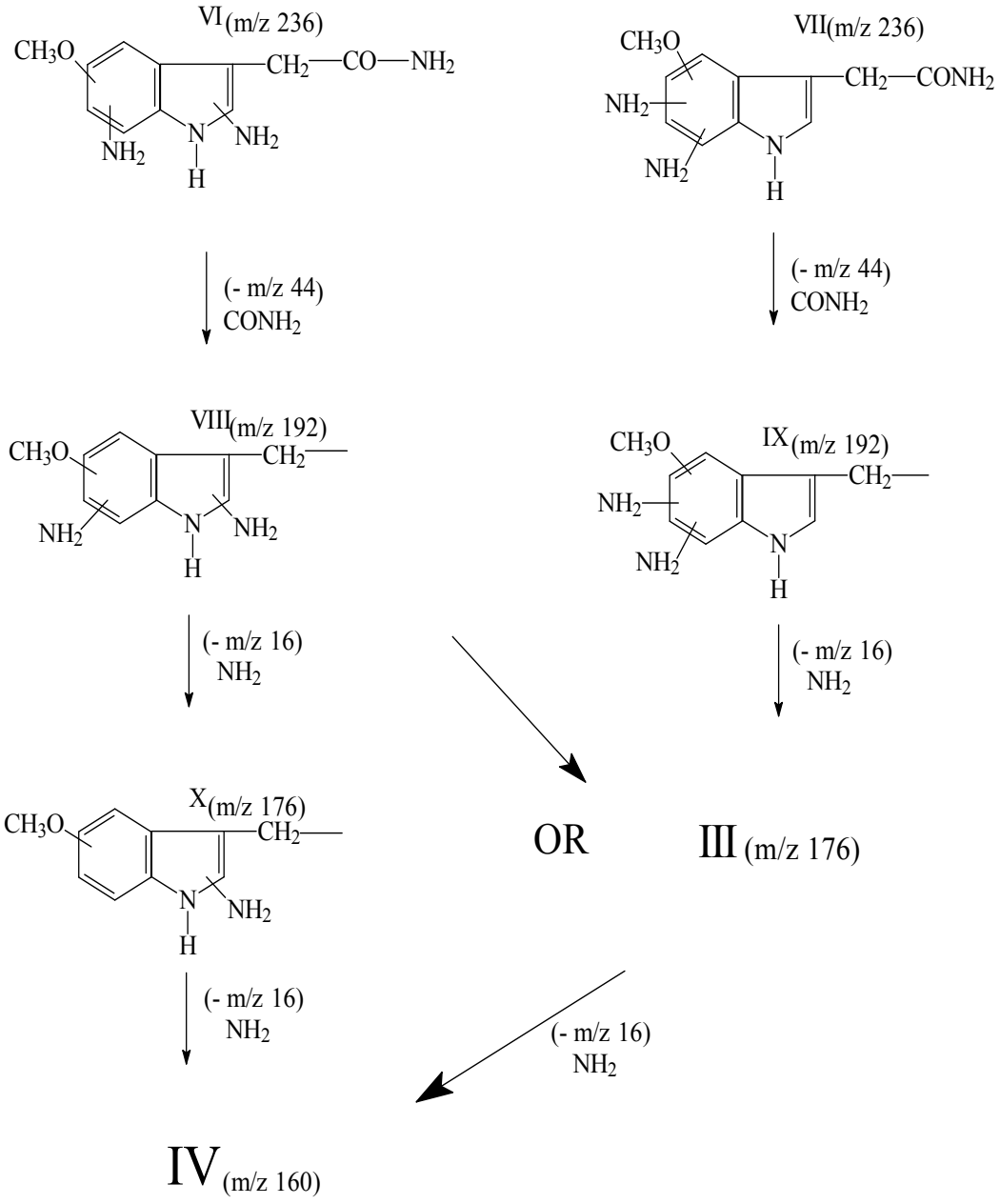


Figure 3.29Shematic representation of proposed fragmentation of GWM1(B)



4 DISCUSSION AND CONCLUSIONS

4.1 ASSAY OF SEEDS FOR 5-HTP CONTENT

The seeds used were obtained from dealers in Kumasi, Ghana. They were approximately 10 months old. The assay was a direct HPLC method. The rationale behind the assay was to quantitavely extract 5-HTP from the seeds of *Griffonia simplicifolia* and to analyse the extract directly by HPLC. The content of the extract was estimated by comparing the peak areas of the peak corresponding to 5-HTP from the seed extract to that of a prepared concentration of pure 5-HTP.

The assay method initially used to quantify the amount of 5-HTP in the seeds was that developed by Lemaire and Adosraku. This involved cold extraction of the powdered seeds with solvent. This method gave a percentage content of 6.37 %w/w of seed. However, upon increasing the temperature of extracting solvent, the amount of 5-HTP extracted was increased to 8.98 %w/w. The percentage yield of 5-HTP produced from the seeds was averagely 6.14% per seed taken.

4.2 INVESTIGATION OF FACTORS CONTROLLING EXTRACTION.

For the purpose of this project, the factors investigated were; *time, particle size, volume of solvent and temperature*.

Within the limits of the solubility of the substance the effect of the above factors on the extraction (estimated by change in yield of 5-HTP) could be determined. From the results obtained, it was observed that all these factors affected the extraction process in terms of yield.

Time

The effect of time on yield of 5-HTP was observable only in the case of powdered seeds. In the case of triturated seeds, after 10 minutes there was no change in the yield of 5-HTP. The near horizontal portion of the graph **(fig 3.2)** illustrates this. This observation can be explained in terms of the large surface area offered by trituration, thus allowing for more efficient extraction within reasonable time. Interestingly, after an hour the amount of 5-HTP extracted from powdered seeds was comparable to the average yield

obtained with crushed seeds **(fig 3.2)**. For the purposes of production, such time lapse may prove costly in the end.

Particle size

As mentioned above trituration enhances extraction. This observation measured at only one instance may however be deceptive. For example, the effect of particle size measured after an hour will be difficult to notice **(fig 3.2)**. Therefore, even though obvious, the effect of particle size must further be investigated under several other conditions to see whether this effect is constant and significant in the presence of concomitant conditions. As such, the contribution of particle size was investigated under varying conditions of volume of solvent and temperature.

The contribution of particle size to yield was **0.57 %** under conditions of room temperature. This increased to **1.75 %** when temperature alone was increased. The effect of particle size observed was **2.21 %** when only volume was increased and increased to **2.47 %** in the presence of an increase in both factors. This showed that the effect of particle size could be enhanced when temperature is increased. In addition, increasing the volume of extracting solvent within limits may enhance the effect of particle size reduction.

Temperature

There was an unmistakable increase in amount of 5-HTP extracted with increase in temperature **(fig 3.3)**. The highest temperature used was 80 ºC because it was close to the boiling point of the solvent used for the extraction. This trend can be attributed to the increase in migration and dissolution of 5-HTP with increase in temperature. An increase in temperature increases kinetic energy of molecules and hence will increase migration of 5-HTP from the seeds. It also increases rate of dissolution, which in turn draws more undissolved 5-HTP.

Monitoring the effect of temperature on yield at constant time (10 min) and volume of solvent **(100 ml)**, it was noticed that, increasing the temperature of solvent from 30 ºC to 80 ºC for powdered seeds yielded **(6.51 %)** of 5-HTP. This was only slightly higher than that obtained for triturated seeds at 30 ºC that was **(6.37 %)**. Thus it could be concluded

that the effect of particle size was somewhat balanced by the increase in temperature. A combination of the two factors yielded **(8.98 %)** 5- HTP. This shows an apparent additive effect.

Volume

The effect of increasing volume is as illustrated by **(fig 3.1)**. As long as the solubility of the substance is not exceeded, the effect of volume can be studied. However, because the amount of 5-HTP in a given amount of seed is limited, it will be expected that increasing the volume beyond a limiting value will not yield any further increase in the yield. Using 20 ml of solvent, the yield for triturated seeds at 80 ºC was **3.91 %**. Extrapolating an expected yield for 100 ml under the same conditions will give **11.73 %**. This was substantially above the actual yield of **8.98 %** obtained. This confirms that the limiting volume for extraction had been exceeded.

From **fig. 3.1,** it can be seen that above the volume of 40 ml, there was no further increase in mean peak area which is proportional to the amount of 5-HTP extracted. This implies that under optimum conditions, 40 ml of solvent will be enough to extract available 5-HTP from one gram of seed. However, for convenience, 100 ml was used as the optimum volume. It was assumed that for practical purposes all available 5-HTP in one gram of seed will be extracted using 100 ml of solvent.

The assay results carried out under the various conditions were analysed statistically using two-way ANOVA and the percentage contribution to the total variation observed were: volume, **69.26 %**; temperature, **15.55 %** and particle size, **11.94 %**. The total contribution from the three factors was 96.75 %. The remaining 3.25 % can be attributed to interaction of the above factors. This confirms that the combined effect is synergistic.

4.3 Determination of solvent for recrystallisation

The primary property which was taken into account in the selection of solvent for recrystall was safety. This is because, the 5-HTP produced was to be used orally and hence issues of toxicity had to be carefully considered. Based on availability, probable solvents were; water, methanol, ethanol (95%) and ether. 5-HTP was found to be

insoluble in ether and since methanol toxic, the list of suitable solvents reduces to water and ethanol (95%).

Experimentally, 5-HTP was found to be too soluble in water and insoluble in ethanol (95%). Thus, neither of the two could be used solely as a solvent for recrystallisation. It was therefore imperative that a mixture of the two solvents be used.

The most suitable combination was found to be water/ethanol 20:80. This combination

- Had a high solvent power for 5-HTP at elevated temperature and a comparatively low solvent power at the laboratory temperature.
- Dissolved the impurities to a very small extent. (The HPLC chromatograms for the crude and purified extracts clearly depicts this).
- Readily yielded well-formed crystals of the purified compound.
- Was easily removed from the crystals of the purified compound i.e. possess a relatively low boiling point.(This was possible due to the larger composition of ethanol as compared to water. Ethanol has a lower boiling point than water. **(Table 1-1)**

The effect of recrystallisation as a purification technique on the purity of 5-HTP obtained is illustrated in **tables 3-21** and **3-22**

Washing the crystals obtained from the crude extract with ice cold extracting solvent, yielded crystals with about **78 %** purity upon drying. The purity was enhanced to about **92 %** upon recrystallisation. The mass of 5-HTP so obtained was **6.4326 g**. This represents about **68.5 %** of extractable 5-HTP in the seeds and **6.14 %** of mass of seed taken.

4.4 STABILITY STUDIES

The effect of temperature on the stability of 5-HTP in the seeds of Griffonia had been previously investigated **(Asiamah, 2000)**. After the extraction and purification of the 5-HTP obtained from the Griffonia seeds, it was deemed prudent to carry out stability studies on the 5-HTP obtained. This was to enable collection of relevant data on the decomposition pattern and possible decomposition products as well as to predict storage conditions for the 5-HTP produced.

It is known that compounds containing the indole ring are photosensitive. Therefore, this challenge was not investigated. The temperature challenge was monitored instead.

The results from **table 3.2** clearly shows the increase rate of decomposition with increasing temperature. The rate constant for the degradation was **1.059 E-03 day^{-1}** at refrigerator temperature (approx. 5°C) as compared to **1.195 E-03 day^{-1}** for that kept at room temperature. The shelf life (t_{90}) of a pharmaceutical product is the length of time the product may safely be stored on the dispensary shelf before significant decomposition occurs **(Cairns, 2008)**. This is important since, at best, drugs may decompose to inactive products; in the worst case, the decomposition may yield toxic compounds.The t_{90} values for refrigerator temperature and room temperature were **99.62 days** and **88.28 days** respectively (table 3-26). The difference in shelf life was thus **11 days**. This is not economically significant considering the cost of refrigeration. There were no observable differences between the chromatograms for the 5-HTP samples analysed prior to the stability studies and that for the 5-HTP samples analysed during selected time intervals during the stability studies. This could be because the method of analysis was not sensitive to the degradation products.

4.5 CHARACTERIZATION OF ISOLATED COMPONENTS

From the phytochemical tests, it could be inferred that seeds of Griffonia contain diverse phytochemicals. Apart from 5-HTP other compounds previously known to be present include fatty acids and sterols such as *stigmasterol, campesterol* and *ß-sitosterol* (**Petkov and Ramazanov 2003).** This essentially represents the non-polar fractions.

The focus of the isolation and characterization was on the aqueous methanolic extract since this was the solvent used in the extraction of the 5-HTP. An idea of the compounds present in this extract would give an indication of possible contaminants of 5-HTP produced using this method.

A look at the TLC chromatograms for the extracts from several solvents shows varied number of spots. Using the solvent system of water, butanol and acetic acid, four components were separated, A, B, C and D with corresponding average retardation factor

(r.f.) values of 0.25, 0.45, 0.65 and 0.85 respectively component D contained predominantly the relatively non-polar compounds such as the terpenoids and steroids. This inference was supported by the r.f. value as well as the observation when sprayed with anisaldehyde (reddish-brown spots).

Component D was spotted using a mobile phase composition of pet ether/ether. Five spots were observed **(fig 3.20)**. This showed the diversity of the composition of constituent D. It is also worthy of note that component D was the only component present in the ether, pet ether and chloroform extract. It was unmistakably absent in the aqueous extract. This confirms that it is non-polar.

The components studied were thus A, B and C. Component C was confirmed to be 5-HTP based on the r.f. values obtained for pure 5-HTP.

Isolation of A and B from the seed extract was tried using gravity column chromatography. The results were as shown in fig 3.17 and fig 3.19 respectively. It can be seen from the TLC plate in fig 3.17a that no component was eluted in the first five fractions. When the polarity of the eluent was increased with methanol (10% methanol in chloroform), there was still no elution until solvent composition of 20% methanol in chloroform was used. At this stage, the most non-polar component, D, was eluted. After, this any further increase in the polarity of the mobile phase yielded more than a single component, making separation impossible.

The inability of the gravity column to effect separation was perhaps due to the particle size of the stationary phase. It is known that resolution is enhanced when particle size of the stationary phase is reduced. The particle size of the stationary phase used in the TLC was 60-120 mesh size. This was indeed smaller than that used in the column.

The choice of method of separation and isolation was made by taking the following factors into consideration

- Availability of reagents
- Efficiency of process
- Time

Based on the above factors Preparative TLC using silica gel as stationary phase and butanol, acetic acid and water (12:3:5) as mobile phase. This goes to buttress the fact that

polar solutes are best separated using a blend of polar solvents, such as n-butanol, with water and small amounts ethanoic acid. **(Kealy and Haines, 2002)**

Reasonable thin layer chromatographic separation was obtained with a mixture of butanol, acetic acid and water. This solvent separated the components based on their ionization properties. Compared to 5-HTP which had the greatest r.f. value, GWM1 can be said to possess more readily ionisable groups. The mobile phase used was slightly acidic and hence the more basic components were expected to be more ionized and hence more retained on the silica stationary phase.

Thus, component A (named GWM1 hereafter) is more basic than B (named GWM2 hereafter) which in turn is more basic than C (5-HTP).

4.5.1 STRUCTURE OF 5-HTP

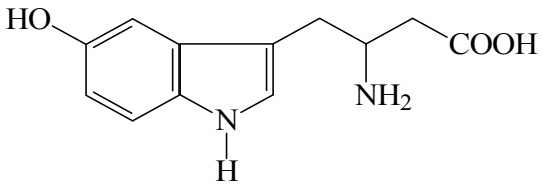


Figure 4.1 Structure of 5-HTP

4.5.1.1 EVIDENCE FOR THE STRUCTURE OF 5-HTP

The UV-VIS spectrum of 5-HTP showed a wavelength of maximum absorption of 275nm. This implies that there is a chromophore with auxochromes which extend the conjugation. Its solubility in both acidic and basic media suggested an amino acid. This was confirmed by the positive test with ninhydrin. The positive phenol test is also consistent with the structure. The sodium fusion test performed confirmed the presence of nitrogen, but ruled out the presence of sulphur and the halogens **(table 3-32)**.

The infra red spectrum showed a broad peak from 3700 to 2500 cm-1 (fig 3.21). This is characteristic of OH stretching vibration of the carboxyl group. From the HPLC chromatograms, the retention times for the pure 5-HTP and the extracted and purified 5-HTP were similar **(figs 3.9 and 3.10).**

4.5.2 STRUCTURAL ELUCIDATION OF GWM1

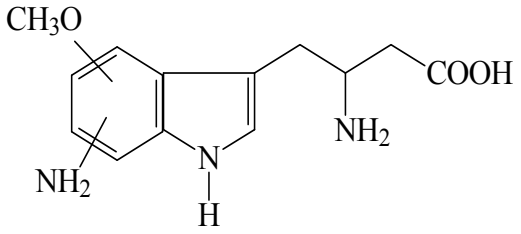


Figure 4.2 Amino methoxy indole-3-acetic acid

The structure elucidation of the above compound was carried out from first principles, taking into consideration the spectrophotometric as well as chromatographic data. The uv- vis data suggests the presence of a parent chromophore with at least an auxochrome. This is due to the fact that the wavelength of maximum absorption was approximately 265 nm which is greater than the benzenoid absorption wavelength of 254 nm.

Due to the similarities in the chromatographic and solubility behaviours of all the components and the fact that they are plant derived, it will be safe to assume that GWM1 is a closely related derivative of 5-HTP. It is also a fact that of the common amino acids, only three absorb in the UV region above 250nm and they all contain the benzene ring. They are; phenylalanine (~260 nm), tyrosine (~275 nm) and tryptophan (~280 nm) **(Finar)** . It is therefore expected that the structure of GWM1 will be within the limits of these structures. From the FAB mass spectrum of the compound, the ten most intense peaks were **77, 89, 104, 107, 136.1, 154.1, 160.1, 176.1, 307.2** and **329.2.**

However, the ions from the last two peaks could not be considered as molecular ion peaks because they failed to yield reasonable fragments (refer to figure). Their occurrence could be explained as having resulted from dimerization. In case of the 307.2 peak, it could be as a result of dimerization of the ion which contributed to the 154.1 peak. The 329.2 signal could have been due to dimerization of the 154.1 and 176.1 ions. This leaves the peaks at 236.1, 289 and 298. The latter two also did not yield reasonable fragments (refer to figure). Thus, 236.1 was assigned the molecular ion peak.

The base peak was 160.1, which could be attributed to the 5-methoxy indole-3-methylyl fragment (IV from fig 3.28). This peak was as a result of loss of 16 mass units from the 176.1 peak. This mass loss corresponds to the NH_2 group. The possible structures of the

176.1 fragment are shown in fig 3.28 and 3.29.The difference in mass between the 236.1 and the 176.1 peaks is 60 mass units. This correspond to the acetate group (CH_3COOH). Hence, the possible structures of the 236 peaks are shown in figure 3.28

Examination of the mass spectrum also shows a peak at approximately 192. This gives a mass difference of 44 when subtracted from 236. This could be from the loss of CO_2 or $CONH_2$. The structures in figure 3.28 support the loss of -CO_2 whereas the structures in figure 3.29 support the loss of -$CONH_2$

From the infra red specrum obtained the most intense peaks are **3400 cm^{-1}, 3100, 3000, 2932, 1650, 1625, 1550, 1440, 1392.8, 1340.7, 792.8, 675.2, 619.2**

1392 may be as result of CH_3 deformation, 2932 is CH_3- stretching and 1440 CH_3O-- deformation. The infra red confirmation of the presence of an ether group is in the presence of bands observed in the region between 1300 and 1000cm-1. However, C—O stretching of acids may also be observed in this region. 1217 may be from -OH bending in the carboxylic acid. 1625 may be stretching of conjugated aromatic C=C. Absoption around 1500 is masked due to the broadness of the peak around 1550. The peak at 1340.7 is due to C—N stretching of a primary aromatic amine. The corresponding N—H stretching for this group was masked by the broad O—H stretching between 3713 and 2500. The band at 937 is OH deformation of an acid dimmer **(Furniss et al,1994)**. The peaks at 792 and 675 show the substitution pattern of the aromatic structure. This does not tally with the correlation data due to the nature of the groups as well as the positions. It can be inferred that the substitution pattern does not allow for two or more adjacent hydrogen atoms on the ring. Chemical tests showed the presence of a primary aromatic amine and the absence of a phenol **(table 3-27)**. The presence of an α or β amino acid was ruled out because there was no reaction with ninhydrin. **(Furniss et al,1994)**

4.5.3 STRUCTURAL ELUCIDATION OF GWM2

The infra red and mass spectra of GWM1 and GWM2 appear remarkably similar. The broad peak from 3745 to 2500 is characteristic of bonded O—H stretching vibration. This may indicate the presence of a carboxyl. The C==O stetching vibration of 1700 can be seen as a shoulder on the broad band at 1600. The band around 3200 which appears as a

shoulder on the broad 3400 peak may be due to C==C in the aromatic ring structure. 2937 is CH_3 -- stretching and 1425 CH_3O— deformation. The band at 1344.5 is due to C—N stretching of a primary aromatic amine and 937.5 is OH deformation of an acid dimer **(Furniss et al,1994)**. Thus, it can be concluded that the two compounds have similar functional groups.

The principal peaks in the mass spectrum were **77, 91, 136,154.1 176.1, 232.2, 298.2, 329.2, 413.3**. The principal peaks in the infra spectrum were **3400, 3200, 2937, 2855, 1600, 1700, 1425, 1344.5, 765.8, 675 and 619.2**.

Despite these findings, the structure of GWM2 could not be deduced with any certainty. The presence of an intense peak at 91.0 is characteristic of benzyl compounds. **(Beckett and Stenlake)**. This gives an indication of the cleavage of the indole ring. However, there was also a peak at m/z of 130, which would suggest the indole-3-methylyl group **(Furniss et al, 1989).** The base peak was 176.1, but the structure shown could not be used to explain this peak since the 160.1 peak was absent. These discrepancies could be due to contamination. Therefore, GWM2 will have to be further purified and the mass spectrometric determination repeated for clarity.

4.6 CONCLUSIONS

The production of 5-HTP from the seeds of Griffonia can be optimized by exploiting the synergistic effect of increase in temperature of extracting solvent, increase in volume of solvent and reduction of particle size of seed. In the absence of these factors, the amount of 5-HTP extracted from 1g of seed was only 0.57 %. This was increased to 8.98% when the above factors were employed simultaneously in the extraction.

The amount of extractable 5-HTP in the seeds used was determined to be 8.98 % of the seed weight upon direct analysis by a modified HPLC method. This was higher than the 6.37 % obtained using the procedure developed by Lemaire and Adosraku. The increase in the assay was due to the fact that, the temperature of the extracting solvent was increased from ambient temperature (about 30 °C) to 80 °C.

Various combinations of water and ethanol were investigated for suitability as solvents for recrystallisation by observation of parameters such as solubility and ease of crystallization. A mixture of water and ethanol (20:80) was determined to be convenient in the purification the 5-HTP crystals produced. The purity of 5-HTP crystals which had been extracted and washed was increased from about 75 % to approximately 92 % upon recrystallisation. The amount of approximately 92 % pure 5-HTP obtained was 6.14 % of mass of seed taken. This represents about 68.5 % of extractable 5-HTP in the seeds.

An increase in the storage temperature resulted in an increase in the rate of decomposition of 5-HTP and therefore, storage of the 5-HTP under refrigerator conditions could be said to slow down degradation. The rate constant for the degradation was 1.059 E-03 day^{-1} at refrigerator temperature (approx. 5°C) as compared to 1.195 E-03 day^{-1} for that kept at room temperature. This implies that the degradation is temperature dependent. However, the difference in t_{90} values for room temperature and refrigerator temperature was only 11 days and hence it is not considered economically advantageous to store 5-HTP in the refrigerator for preservation.

Thus, 5-HTP can be produced in Ghana from the seeds of Griffonia in reasonable purity prior to export, using readily available solvents and unsophisticated apparatuses. The 5-HTP produced if stored under refrigerator conditions will remain within specifications for 3 months. That is the time taken for the active ingredient to decompose to 90 % of the original amount.

The aqueous methanolic extract of Griffonia seeds contain other components in addition to 5-HTP. The ether insoluble fraction (polar) contained at least three major components of similar chemical and spectroscopic properties. One of the polar constituents of the aqueous methanolic extract is suspected to be amino methoxy indole-3-acetic acid **(fig 4.2).**

4.7 RECOMMENDATIONS

- Determination of the true order of the thermal degradation of 5-HTP must be done since the stability studies performed was on the assumption that the degradation follows first order kinetics.

- Stability studies with other factors such as light have to be performed and the degredation products compared to that of the thermal challenge.
- The degredation products have to be isolated and characterized.
- Suitable methods have to be developed to detect possible degradation products and acceptable limits set for any toxic components that are discovered.
- Further spectroscopic studies have to be carried out to aid in the structural elucidation of GWM1 and GWM2. More advanced mass spectrometric techniques such as derivatization and variations of ionization procedures may also be employed.

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APPENDIX

STATISTICAL ANALYSIS

Table showing the % content of 5-HTP in seeds determined under varying conditions

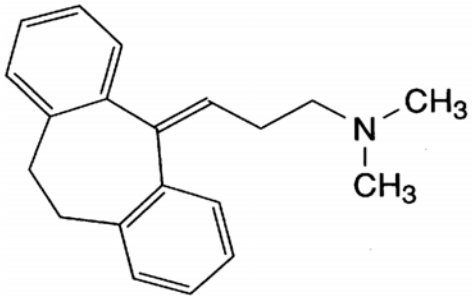
	20 ml at room temp	100 ml at room temp	20ml at 80 deg	100ml at 80 deg
powdered	1.33	4.16	2.26	6.51
paste	1.90	6.37	3.91	8.98

Two-way ANOVA				
Source of Variation	% of total variation	P value		
CONDITION	85.93	0.0064		
PARTICLE SIZE	11.94	0.0263		
Source of Variation	P value summary	Significant?		
CONDITION	**	Yes		
PARTICLE SIZE	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
CONDITION	3	42.84	14.28	40.23
PARTICLE SIZE	1	5.951	5.951	16.76
Residual	3	1.065	0.3550	
Number of missing values	0			

Two-way ANOVA				
Source of Variation	% of total variation	P value		
CONDITIONS	82.89	0.0042		
TEMPERATURE	15.55	0.0120		
Source of Variation	P value summary	Significant?		
CONDITIONS	**	Yes		
TEMPERATURE	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
CONDITIONS	3	40.65	13.55	53.18
TEMPERATURE	1	7.625	7.625	29.92
Residual	3	0.7644	0.2548	
Number of missing values	0			

Two-way ANOVA				
Source of Variation	% of total variation	P value		
CONDITIONS	28.03	0.0432		
VOLUME	69.26	0.0031		
Source of Variation	P value summary	Significant?		
CONDITIONS	*	Yes		
VOLUME	**	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
CONDITIONS	3	13.98	4.659	10.35
VOLUME	1	34.53	34.53	76.70
Residual	3	1.351	0.4502	
Number of missing values	0			

CHEMICAL SRUCTURES



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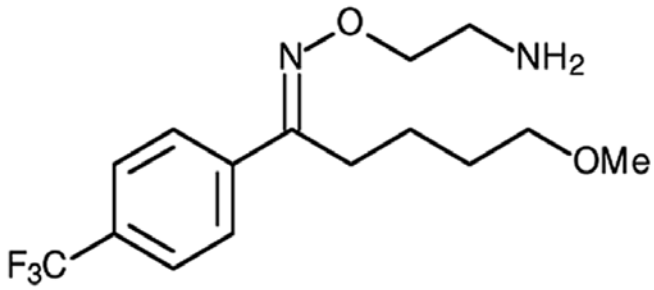

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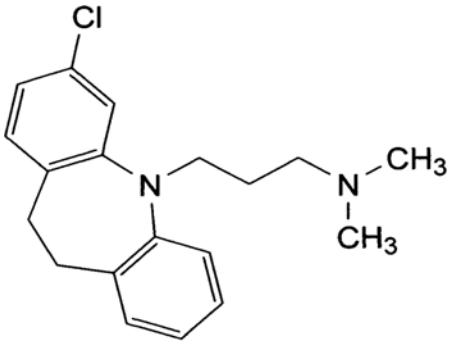

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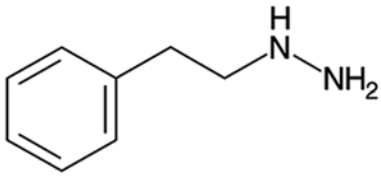

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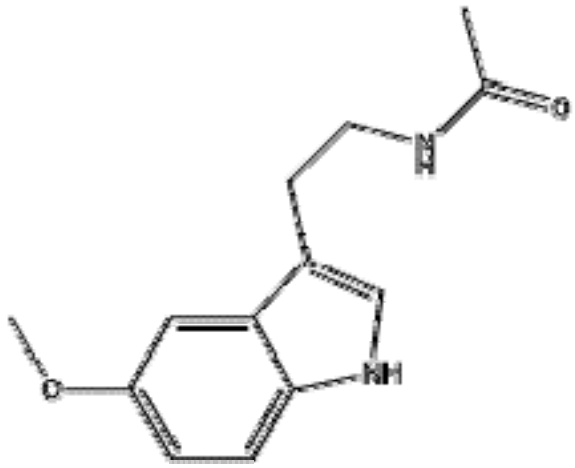
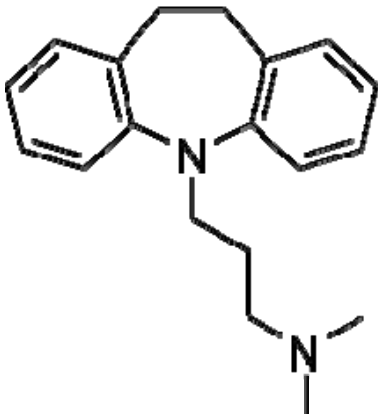


MELATONIN



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