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THE USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF MEDICINAL PLANTS

BY

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DECLARATION

I declare that the thesis “The use of high performance liquid chromatography for the analysis of medicinal plants” has not been previously submitted for a degree at this or any other university, and that it is my own work in design, execution and that all reference material that I used or quoted has been acknowledged.

Titus Machuene Boloko November 2007

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ABSTRACT

The process of investigating plants to identify chemical substances is of great interest to natural product scientists because there is a need to discover new drugs for treating diseases. In our study, plant extracts were prepared from the bulbs of *Crinum macowanii, Boophane disticha* as well as *Eucomis autumnalis* and further experiments were made on the extracts. High performance liquid chromatography with other instruments (ultra-violet detector, mass spectrometer) coupled to it, were used in the search for the active ingredients in the extracts prepared. Old methods of separation and identification such as flash column chromatography and thin layer chromatography also played an important role in the investigation of these extracts. Other techniques such as nuclear magnetic resonance (NMR), helped in the structural elucidation once the compounds had been purified.

The use of analytical techniques (HPLC-MS, NMR) was found to be important in the process of investigating the extracts and the presence of various active ingredients was confirmed. The methods used traditionally for extract preparation (boiling plants in water for certain amount of time) were investigated and the important relationship between the boiling time and concentration of the active components was established. It was found that the increase in boiling time of the plants during preparation decreases the concentrations of the active components. The experiments conducted provide some scientific evidence which motivates that the traditional preparations of the plants are related to the dosage.

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High performance liquid chromatography (HPLC)

Mass spectrometry (MS)

Ultraviolet (UV)

High Performance Liquid chromatography – Mass spectrometry (HPLC-MS)

Thin layer chromatography (TLC)

Electrospray ionization (ESI)

Atmospheric pressure chemical ionization (APCI)

Nuclear magnetic resonance (NMR)

CHAPTER 1

INTRODUCTION

1.1. Background

All over the world scientists investigate plants, micro-organisms and many other forms of life for biologically active compounds. Research is directed towards interactions between organisms that can be attributed to a chemical substance present in at least one of the species concerned. Of greatest interest is the effect of extracts from flowering plants on human physiology and human pathology, since this is very relevant to the discovery of new drugs for treating diseases of human beings and other mammals. Most of the earliest pharmaceuticals were plant materials[1]. The effect on human health and activity following the ingestion or application of plant products is known in most societies and the use of plants for treating diseases started before written history[1].

The investigations have evolved into a search for new biochemical targets, the development of bio-assays, and high throughput screening of as many compounds as possible to find chemical structures for drug development[2]. Natural products fall into several different categories:

> Steroids from marine animal, plant and fungal sources; alkaloids from plants and some bacteria; protein, amino acids, antibiotics from microbes; purines from microbes; and terpenes, carbohydrates, fats and other macromolecular products from other organisms[2].

Natural products gained prominence through antibiotics and today they have been developed for a variety of medicinal uses such as immunosuppressive agents, hypocholesterolemic agents, enzyme inhibition, antimigrane agents, herbicides, antiparasitic agents; and ruminant growth promoters as well as bioinsecticides[2]. Natural products are also among the most important anticancer agents[2].

1.2. Sampling and sample preparation

Any plant species displays a variation within its features including its extract composition due to differences in its genetic characteristics, the environmental conditions under which it is grown and the period in its life history when collection took place. Variation in extract composition can also occur due to treatment after collection[1]. The amount of a particular active ingredient in a plant can vary due to one or more of the above factors and this is of great concern when a particular dose of a crude extract is applied. If the amount present is higher than normal, toxic effects may be experienced or; if the amount is considerably lower than normal, the desired therapeutic effect may not occur[1]. Clearly it is important to be able to measure the amount of the active substances or substances present, so that some degree of efficiency and safety can be assured. This can only be done once the identity of the 'active' compound is known.

There are several ways in which extracts are prepared in traditional medicine. The most common involves drying plant materials and boiling in water. One of the problems associated with this is that many medicinal compounds are not soluble in water and others are very sensitive to pH changes. Water insoluble medicinal compounds are normally dissolved in ethanol. One of the chemicals that is popular in

a laboratory for compound extraction in plants is Dimethyl Sulfoxide (DMSO). It is therefore important to investigate the appropriate solvent for compound extraction for each plant because different plants possess different compounds that might need special attention. Some extraction methods involve pressing the sap and using the resultant liquid for further experiments. There have been great developments in extraction procedures, with the time consuming, solvent-rich techniques such as liquid-liquid extraction, Soxhlet extraction and hydrodistillation being replaced by faster procedures that require less solvent and are less susceptible to sample losses. These include the solid phase extraction (SPE), solid-phase micro extraction (SPME) and single-drop micro extraction (SDME), also known as liquid phase micro extraction and solvent micro extraction.

1.3. Role of chromatography in natural product chemistry

Many methods have been devised whereby the activity of a compound or extract can be tested scientifically[1]. Old chromatographic techniques such as column chromatography and thin layer chromatography (TLC) are still found to be the widely used means of extract clean-up/work-up and identification in natural product chemistry[3]. The analytical task of efficient detection and rapid characterisation of natural products plays an important role in natural product chemistry[4]. The achievement of structural elucidation of the constituents of an extract is essential for an efficient and selective isolation procedure. In order to perform efficient screening of the extracts, both biological assays and high performance liquid chromatography (HPLC) analysis with various detection methods are used[5]. Hyphenated techniques such as HPLC coupled to ultra violet (UV) photo-diode array detection or mass spectrometry (LC-UV or LC-MS) provide useful structural information on the

compounds prior to isolation. With such an approach, time-consuming isolation of common natural products is avoided and an efficient targeted isolation of compounds presenting interesting spectroscopic or biological features can be performed[6].

Nuclear magnetic resonance (NMR) spectroscopy is also useful in medicinal chemistry because of its powerful stereochemical information content but it has the disadvantage of the lower sensitivity in comparison to other methods, e.g. mass spectrometry. The combination of chromatographic separation techniques with NMR spectroscopy is one of the most powerful and timesaving methods for the separation and structural elucidation of unknown compounds and mixtures.

1.4. Objective of the study

The indigenous medicinal plant industry has considerable potential for creating an impact on South Africa's economy, welfare and biodiversity. In a country as biologically and culturally diverse as South Africa, it is not surprising that about 3000 of the plants do possess some medicinal applications[7]. Most of these plants do possess some pharmacological properties, even though only a small portion has been scientifically investigated[7] .

The objective of the study was to evaluate the use of high performance liquid chromatography–mass spectrometry (HPLC MS) for the detection and confirmation of the presence of reported biologically important or active chemical compounds in medicinal plants. This offers the advantage as it does not rely on tedious purification methods or the use of high quality standards. The study was also extended into establishing whether HPLC-MS can be applied to monitor changes in the chemical profile/s (if any) of medicinal plants when these are used as traditional preparations such as boiling over extended times. Such information would be of help to

understand the purpose of extensive boiling of plant material such as bulbs by traditional healers.

For the purpose of the study; three species were investigated to understand their chemical composition using this technology. The three bulbs that were studied are *Boophane disticha, Crinum macowanii* and *Eucomis autumnalis. Boophane disticha* and *Crinum macowanii* belong to the same family of plants known as *Amaryllidaceae* and are traditionally used for wound dressing, treatment of sores and septic cuts, while *Eucomis autumnalis* belongs to the family of *Hyacinthaceae* and is used for urinary diseases, stomach ache, fevers, colic, hangovers, syphilis and to facilitate childbirth. The three are reportedly toxic plants and the chemical compounds present in the plants are also reportedly toxic[7]. It is important to be able to show the presence of these compounds in the plant using rapid and robust analytical techniques. This technique, if proven to be valid, can be used for several purposes such as quality control and standardization of medicinal plants.

1.5. Procedures to achieve the goals of the study

Plant extracts were prepared by the addition of organic solvents (e.g. methanol) to the dried sliced portions of the bulb. These solvents were mixed in a given ratio with a more polar solvent (water, dilute acid, aqueous solution of salts) to aid the breaking of weak electrostatic bonds that bind some compounds to other substrate molecules.

The analyses of the components of each plant were done by HPLC-MS and thin layer chromatography (TLC). Thin layer chromatography (TLC) was used mainly for detection of compounds and it is also useful for separating the active compounds from

interfering compounds in the extract. A range of developing solvents was tried in order to separate the active ingredients from the interfering compounds.

For the confirmation of presence of the targeted compounds in the extracts, column-chromatography was used for purification as it has a very wide application, and is used in a number of regulatory or officially approved methods of sample clean up. A glass column was packed with one or more adsorbent materials and added the crude extract to the top of the column. The column was then eluted with a series of solvents or solvent mixtures which are designed to first wash off interfering compounds and then elute the desired compounds, whilst other interfering compounds remain strongly bound on the column.

After separating compounds from each extract by column chromatography and TLC monitoring, the analysis of semi-pure and pure fractions were conducted using HPLC-MS. Purified compounds of each bulb were analysed by NMR for structural identification. HPLC-MS was used to find previously reported compounds in the *Amaryllidaceae* family (Lycorine, Buphanidrine and Buphanisine). Confirmation of the compound, 3-(3,4-Dimethoxybenzyl)-5,7-dihydroxychroman-4-one reported in literature to be found in *Eucomis autumnalis,* was done.

The main preparation method of traditional medicines involves boiling plants in water over a period of time. This procedure was mimicked in a laboratory to observe the effect on the chemical profile as the boiling progresses. Most often the boiling of these plants is meant for bringing the active components into solution and this study was used to investigate the validity of this assumption.

1.6. Structure of this thesis

The first chapter introduces the project (what it's all about, what analytical techniques were used as well as the plants studied and what influenced the choice of these plants). The second, third and fourth chapters give respectively, detailed background on liquid chromatography, mass spectrometry and plants studied. Chapter five gives a detailed discussion of results while the conclusion is described in chapter six. The last chapter (seven) is the experimental section followed by the reference section.

CHAPTER 2

2. CHROMATOGRAPHY

2.1. Introduction to chromatography

Chromatography is a physical separation method in which the components to be separated are selectively distributed between two immiscible phases, a mobile phase flowing through a stationery phase bed. High performance liquid chromatography (HPLC) bears a close resemblance to gas chromatography (GC)[8]. It (HPLC) arose out of experience of GC and it complements the latter, by being able to separate substances that cannot be readily volatilized. HPLC is particularly suitable for the separation of compounds having one or more of the following characteristics: (a) high polarity (b) high molecular weight (c) thermal instability (d) a tendency to ionize in solution[8].

The operating conditions, namely, column temperature, inlet pressure and flow rate are controlled in HPLC, as in GC. The column is used repeatedly, the sample is injected directly by a syringe or a valve onto the column, the separated solutes are detected as they emerge from the column by a sensitive detector and the signal is recorded to give a quantifiable record of the chromatographic separation[9].

In contrast to GC, liquid chromatography (LC) refers to any chromatographic procedure in which the moving phase is a liquid. Examples of HPLC are traditional column chromatography (whether adsorption, partition, or ion-exchange), thin layer chromatography, paper chromatography and modern HPLC. The difference between HPLC and these other older procedures involves improvements in equipment, material, techniques and the application of theory[10, 11,12].

There are five basic components of an HPLC, which are shown in figure 1. These are:

a. Pumping system

b. Injection system

c. Column (Separating system)

d. Detecting system

e. Data collecting system

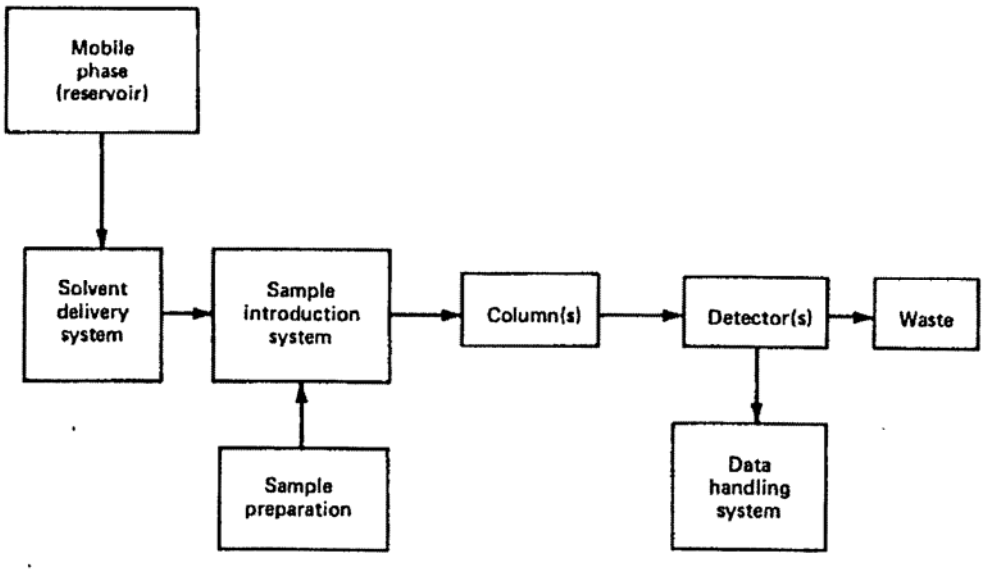


Figure 1: The basic components of an HPLC instrument[10]

In older liquid chromatography methods a column was often used once, and then discarded. Therefore the packing of the column had to be repeated for each separation, and this represented the expense of both manpower and material[12]. The different types of liquid chromatography are shown in figure 2.

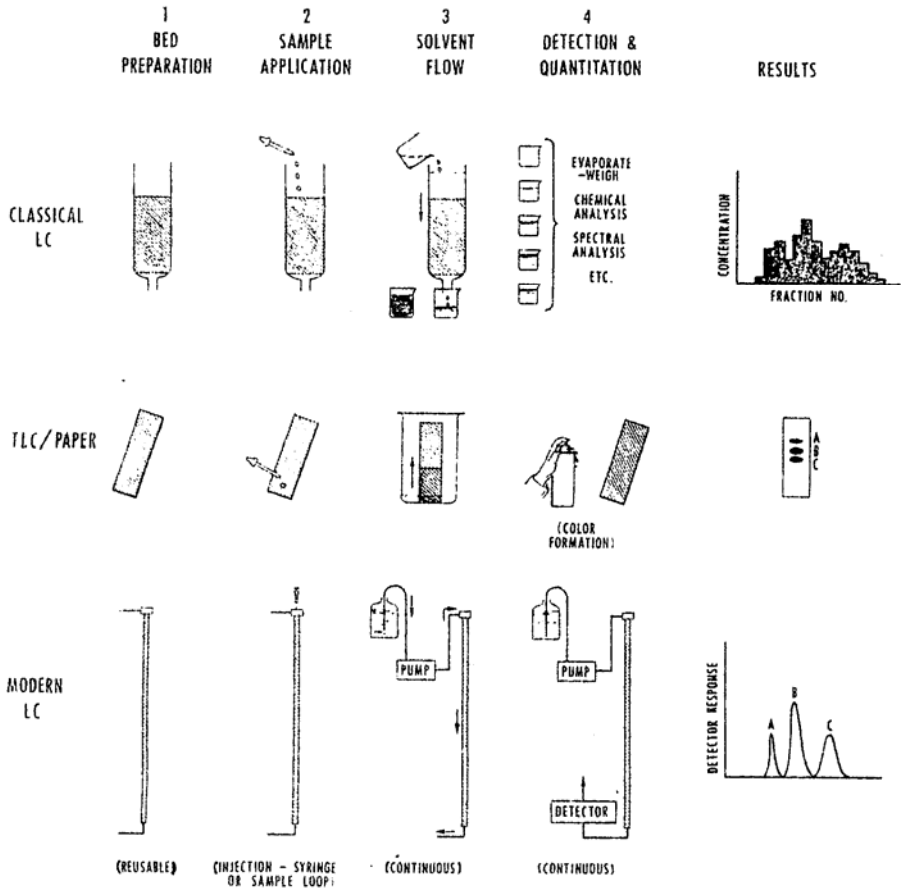


Figure 2: The different types of liquid chromatography[10].

2.2. Pumps and injection systems

The function of the pump in HPLC is to pass the mobile phase through the column at high pressure and at a controlled flow rate. One class of pump (constant pressure pump) does this by applying constant pressure through the mobile phase, the flow rate is determined by the column and any other restrictions between the pump and the detector outlet. Another type (constant flow pump) generates a given flow of liquid, so that the pressure developed depends on the flow resistance[13,14,15]. The flow resistance of the system may change with time; this can be caused by swelling or settling the column packing, small changes in temperature, or the build up of foreign

particles from the samples, pump or injector. If a constant pressure pump is used, the sample flow rate will change if the flow resistance changes, but for the constant flow pumps changes in flow resistance are compensated for by a change in pressure. Small flow changes are undesirable, as they will cause retention data to lack precision, and may cause an erratic baseline on the recorder. In addition to being able to pump the mobile phase at high pressure and constant flow, the pump should also have the following characteristics:

(a) The interior of the pumps should not be corroded by any of the solvents that are to be used

(b) A range of flow rates should be available, and it should be easy to change flow rates

(c) The solvent flow should be non-pulsing

(d) It should be easy to change from one mobile phase to another

(e) The pump should be easy to dismantle and repair[15]

2.2.1. Constant pressure pumps

The earliest form of constant pressure pump in HPLC (the coil pump) used pressurized gas from a cylinder to directly drive mobile phase from a holding coil through the column[13,14].The operating principle of a more sophisticated constant pressure device, a the pneumatic amplifier pump, is shown in figure 3. Compared to syringe type or reciprocating pumps, pneumatic amplifier pumps are cheap. They are difficult to dismantle for repairs, and some types are very noisy in operation.

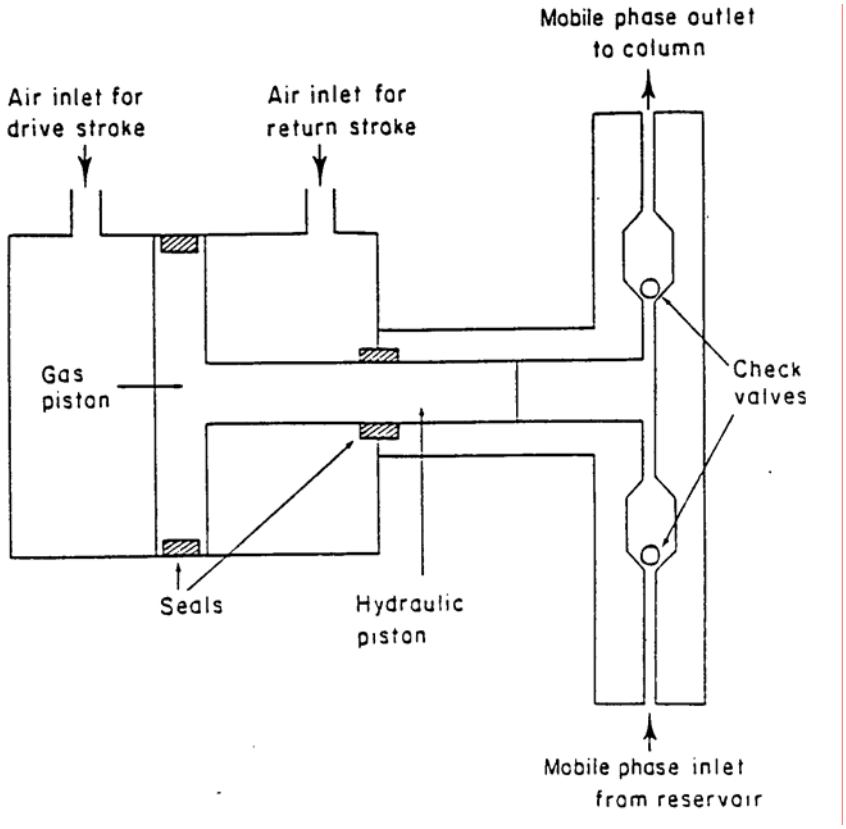


Figure 3: A pneumatic amplifier pump[13]

2.2.2. Constant flow pumps

Two types of constant flow pumps have been used in HPLC. One of these is a syringe type pump, shown in Figure 4. Mobile phase is displaced from a chamber by using a variable speed stepper motor to turn a screw that drives a piston[13]. The flow is pulseless and can be varied by changing the motor speed. The type of pump used in most instruments is a reciprocating pump, shown in figure 5. The piston is driven in and out of a solvent chamber by an electric cam or gear. Unlike syringe pumps, reciprocating pumps have an unlimited capacity, and their internal volume can be made very small[13].

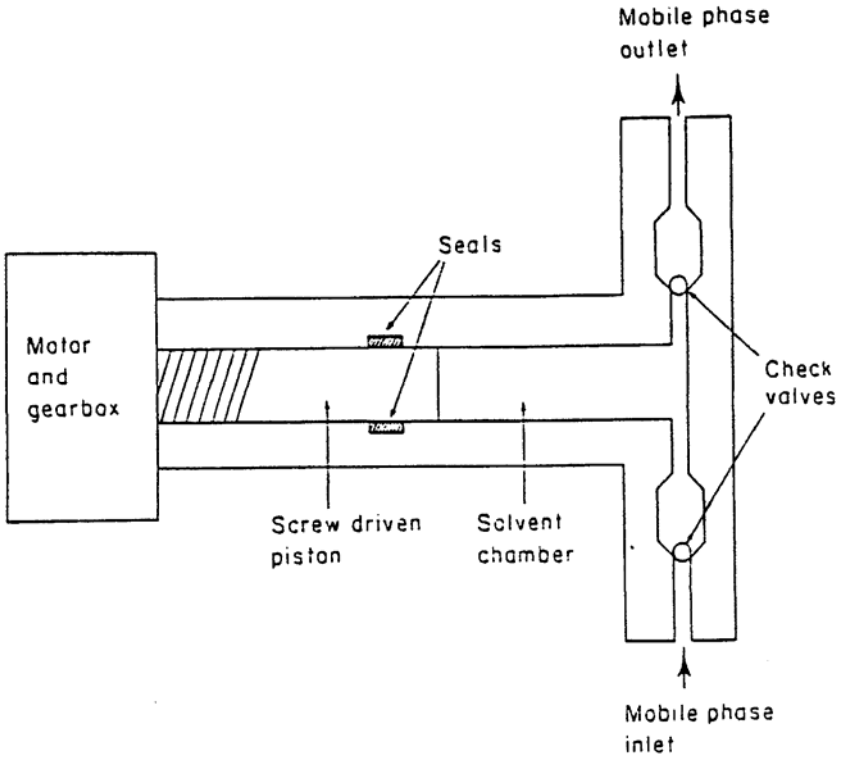


Figure 4: Syringes pump[14]

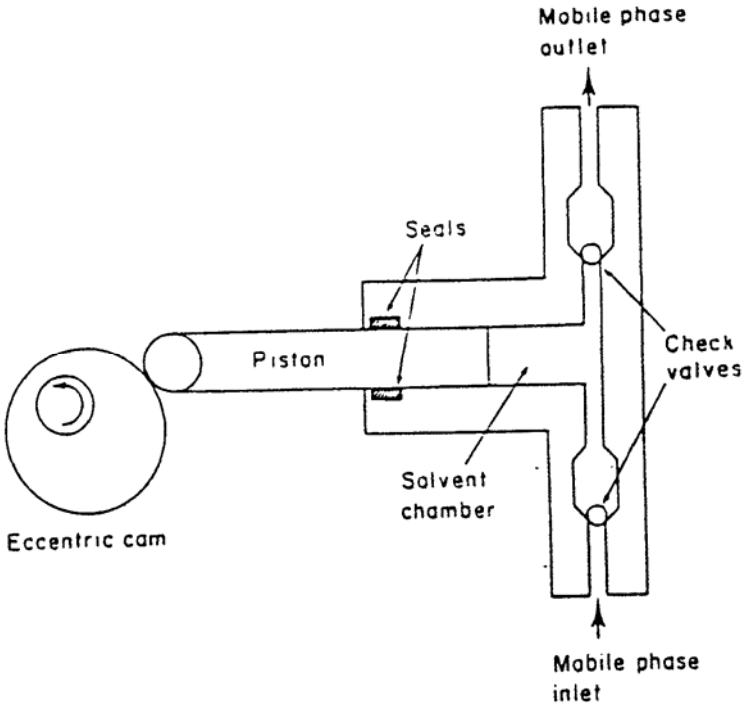


Figure 5: The reciprocating pump[14]

In a single headed reciprocating pump shown in figure 5, the mobile phase is delivered to the column for only half of the pumping cycle [9, 13,14,16]. During the drive stroke of the piston, the flow rate is not constant (because the speed of the piston changes with time). The representation of output from the reciprocating pumps outlined in figure 6 is as follows.

(i) Single headed pump.

(ii) Twin headed pump, heads 180^{o} out of phase.

(iii) Single headed pump with constant speed. Modern twin-headed pumps use two pistons driven by a cam or gear that is shaped so as to make the piston speed constant. Ideally the output of one such head should be as shown in figure 6(iii)

(iv) Single headed pump with constant piston speed

(v) Twin headed pump, head 180^{o} out of phase with different constant speeds on the drive and refill strokes[17].

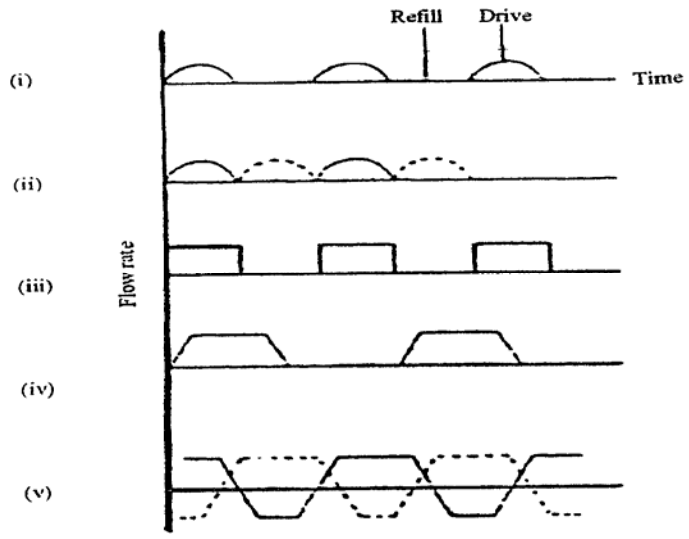


Figure 6: Output from reciprocating pump[17]

2.3. Sample preparation

Solid samples have to be dissolved before being introduced into the HPLC system. The choice of the solvent is critical and the sample should be dissolved in the mobile phase. This has several advantages. First, it minimizes the sample solvent peak at the void volume. This is critical with unknown samples where an impurity or peak of interest can be masked by the sample solvent peak[14,17]. Sample dissolution in an inappropriate solvent may speed up sample preparation but can later cause endless hours of troubleshooting and misinterpreted data due to the sample or a component of the sample precipitating on the column. This occurs when the sample slug dissolves and diffuses in the mobile phase. As the sample solute is dispersed, those components not readily soluble in the mobile phase can precipitate out. If the component of interest does not precipitate, interference from a precipitate may not be noticed until subsequent injections. If the precipitation occurs before the column, it is possible to observe unknown or random eluting peaks with later injections. All of these will hinder quantitative analysis [17].

2.3.1. Liquid samples

Liquid samples provide the chromatographer with the option of injecting the sample directly. However the solvent may or may not be compatible with the chromatographic system of choice. For samples not in a desired solvent or not concentrated enough, two simple procedures can be used. One is to evaporate the sample to dryness and reconstitute the sample with the mobile phase or a more suitable solvent [14].

2.3.2. Sample filtration

It is as important to filter the sample prior to injection, as it is to filter the mobile phase. The choice to filter a sample is determined by the nature of the sample, its solubility, and interferences or contaminants[17]. If not filtered and having contaminants, they can build up at the head of the column and lead to restriction of the mobile phase flow, increasing the column backpressure, decreasing the column efficiency and producing split peaks. The chromatographer can use classical filtration techniques, specialized equipment such as a syringe and a 5-micron filter pad in a Swinny adapter (Millipore Corporation) or a commercial sample clarification kit.

2.3.3. Solvent degassing

It is advisable to prepare samples with degassed solvents. This will reduce the possibility of degassing (bubble formation) occurring in the detector cell due to the sample solvent[17,18]. For quantitative analysis; the sample solution itself should not be degassed. Degassing causes solvent evaporation, which will change the sample concentration.

2.3.4. Sample injection

The earliest injection method in HPLC used a technique borrowed from GC in which a microlitre syringe is employed to inject the sample through a self-sealing rubber septum held in the injection unit at the top of the column. In another method (stopped flow), the flow of the mobile phase through the column was halted and when the column reached ambient pressure the top of the column was opened and sample introduced at the top of the packing[14,15,17]. Syringe injections through a septum into the mobile phase stream worked well, since many of the early instruments were not

operated at pressures much greater than 1000 Psi. Syringe injection in HPLC is pressure limited and is not useful over operating pressure greater than 1000 - 1500 Psi. The actual pressure limit will depend upon the type of septum, retaining nut, syringe needle, and so on.

2.4. Retention and peak spreading

The aim of the column technology in HPLC may be defined as the achievement of the optimum combination of resolution of solutes, speed of elution, and economic use of pressure[12]. The key to resolution in any form of chromatography is the proper combination of the differential migration of solutes and the control of band spreading.

2.4.1. Column dispersion mechanism

There are several mechanisms responsible for the dispersion of the solute as it travels through the column.

(a) *Multipath effect and lateral diffusion (flow dispersion)*. Multipath is the term for the dispersion produced by the existence of different flow paths, by which the solute species can progress through the column[9,13,14.] These path differences arise because the stationary phase particles may have irregular shapes, and also because the packing of the column may be imperfect. The smaller the particles and the narrower their size distribution the less the dispersion. If solute species travels at the same speed, those in different flow paths will travel different distances in the column in a given time. In fact, those in the wider flow path will move at different speeds while those in

midstream will be traveling faster than those close to the stationary phase particles.

(b) *Longitudinal diffusion*. Dispersion also arises because of diffusion of solute species in longitudinal (axial) direction in the column. Longitudinal diffusion will become more serious the longer the solute species spends in the column, so this effect, unlike flow dispersion, is reduced by using a rapid flow rate of the mobile phase[13,14].

(c) *Mass transfer effects*. These effects arise because the rate of the distribution process of the solute species between mobile phase and stationary phase may be slow compared to the rate at which the species is moving in the mobile phase[13,14]. When solute species interact with the stationary phase they spend some time in or on the stationary phase before rejoining the mobile phase, and in this time they will have been left behind by those species that did not interact. The internal pores of the stationary phase particles will contain 'stagnant' mobile phase, through which the solute species have to diffuse before they can get at the stationary phase. Those that diffuse along the way into the porous structure will be left behind by those species that bypass the particles, or only diffuse short distance into it [14].

2.5. Reverse phase liquid chromatography

Reverse phase LC refers to systems where the stationary phase is non-polar and the mobile phase is polar. The most popular bonded phases are those that consist solely of aliphatic hydrocarbon chains bonded to the silica[12,13,17,18]. Non-polar stationary phases interact with solute molecules, and are employed with polar aqueous solvents or aqueous solvent mixtures such as methanol/water and acetonitrile / water mixtures.

The most commonly used phases contain aliphatic chains of C4, C8, and C18 and have been termed C4, C8, and C18 phases respectively. The C18 phases are mainly used for separating solutes having relatively low molecular weights whereas the C4 phases are used for the separation of very large molecules [12,13,17,18].

The C4 bonded phase is useful in the separation of materials of biological origin that may be chemically labile or easily denatured. Due to strong interactions that can take place between large polypeptide or protein molecules and the non-polar phase, such compounds are often denatured or de-conformed after interaction with the non-polar surface. In many instances, the de-conformation of the large macromolecules is accomplished by biological deactivation and is irreversible. It is clear that such deactivation must be avoided [17,18].

2.6. Detectors

There are various types of detectors for LC and the prevailing are UV detectors (fixed and variable wavelengths), the electrical conductivity detector, light scattering detector, the fluorescence detector and the refractive index detector[16,17,18].There are seven major detector specifications as follows:

Detector linearity, linear dynamic range, detector noise level, detector sensitivity, minimum detectable concentration, pressure sensitivity, flow sensitivity and temperature sensitivity.

2.6.1. The detector linearity and response index

A linear detector is one where the measured output of a detector is proportional to the concentration of an analyte. Detector linearity describes how close a given detector

matches this ideal property. The linearity of the detector influences the accuracy of the analysis and it is important to have a method for measuring detector linearity in numerical terms[17,19]. Scott and Fowlis[20] assumed that for a linear detector the response could be expressed by the following equation:

$$V = Rc\alpha$$

where V is the output from the detector, c is the concentration of the analyte inside the detector and R is a constant and α is the response index.

2.6.2. Linear dynamic range

As the linearity of the detector usually deteriorates at high solute concentration the linear dynamic range is defined as the range of concentration for which the detector output is proportional to concentration. The linear dynamic range of the detector is therefore also that range of the solute concentration over which the numerical value of the response index falls within a defined range[15,17].

2.6.3. Detector noise level

There are different types of detector noise, namely, short-term noise, long-term noise and drift[15,17].

Short-term noise consists of base line perturbations that have a frequency that is significantly higher than that of the eluted peaks. Its source is usually electronic, originating from either the detector sensor system or the amplifier. An appropriate noise filter can easily remove it without affecting the profiles of the peaks [15,17].

Long-term noise consists of baseline perturbations that have a frequency that is similar to that of the eluted peaks. The source of long term noise is due to the changes in either the temperature, pressure or flow rate in the sensing cell. This kind of noise can be controlled by detector cell design and ultimately limits sensitivity or the minimum detectable concentration[17].

Drift in a positive direction is an indication of contamination buildup. (Remedy: Flush the column, clean up the samples, and use fresh solvents). Negative drift is frequently caused by temperature fluctuation in the lab or the column compartment. (Remedy: Stabilize the room temperature, remove the instrument from drafts, and insulate the column and the capillaries). Negative drift can also be associated with non-HPLC grade solvents that are UV-absorbing[17].

2.6.4. Minimum detectable levels

This is the lowest concentration of the analyte in a sample that can be detected, but not necessarily quantified. This is defined to be the minimum concentration of an eluted solute that can be differentiated unambiguously from the noise[15]. The ratio of the signal to the noise for a peak that is considered decisively identifiable has been arbitrarily chosen to be two. The minimum detectable concentration is therefore that concentration that provides a signal equivalent to twice the noise level[15]. Minimum detectable concentration are sometimes chosen with S/N ratio of 3:1 while minimum quantifiable concentration most often refers to a S/N of 10:1.

2.6.5. Pressure and temperature sensitivity

The pressure sensitivity of a detector is one of the factors that determines the long-term noise and can thus be very important[15,17]. It is usually measured as the change in

detector output for unit a change in sensor-cell pressure. Both the sensing device of the LC detector and the associated electronics can also be temperature sensitive and cause the detector output to drift as the ambient temperature changes[15,17].

2.6.6. The UV detector

The UV detector is the most popular and most useful detector that is available[9,13,14,17] . Although these detectors have definite limitations, particularly with respect to the detection of non-polar compounds that do not possess a UV chromophore, it has the best combination of sensitivity, versatility and reliability of all the detectors so far developed for general LC analysis[21].

The majority of compounds absorb UV light in the range of 200 – 350 nm including all substances that have one or more double bonds and all substances that have unshared (non-bonded) electrons (e.g. all olefins, all aromatics, and all substances containing >CO, >CS, -N=O and -N≡N- groups) [21].

2.6.6.1. The fixed wavelength detector

There are two types of UV detectors, the fixed wavelength detector and the multi-wavelength detector. The fixed wavelength detector shown in figure 7 is the least expensive and as all the light is emitted at specific wavelength(s), it has a higher sensitivity than the multi-wavelength detector [15,17].

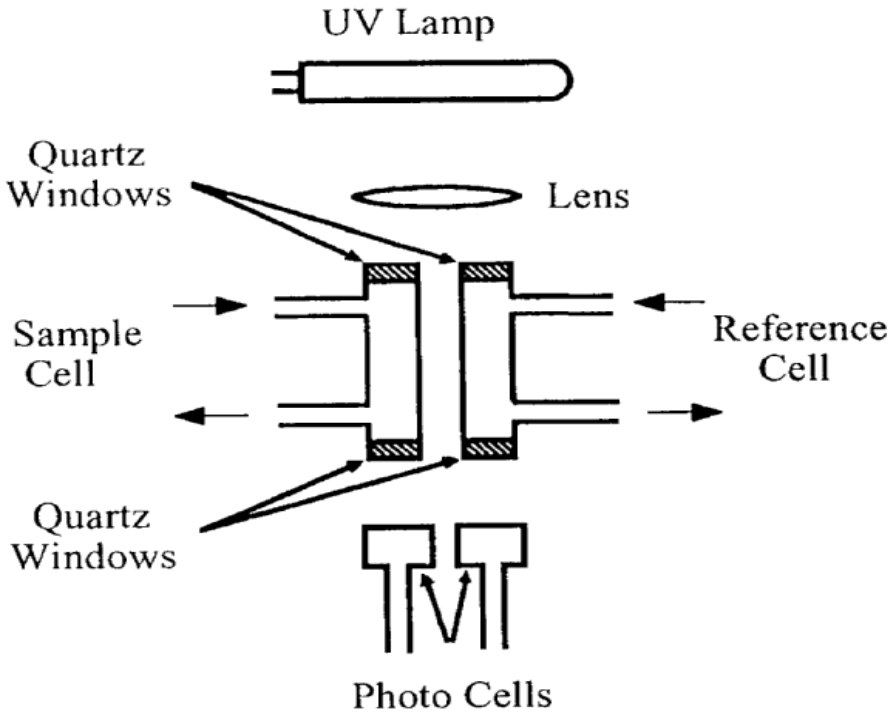


Figure 7: The fixed wavelength detector[17]

2.6.6.2. Multi-wavelength detector

These detectors can vary the wavelength selected to detect the solute. There are two types of multi-wavelength detectors, i.e., the dispersion detector (moving grating, shown in figure 8), that monitors the eluant at one wavelength at a time only, and the diode array detector, that simultaneously monitors the eluted solute over a range of wavelengths. Most moving grating detectors cannot produce a scan fast enough to obtain a full UV spectrum during one LC-peak [15,17].

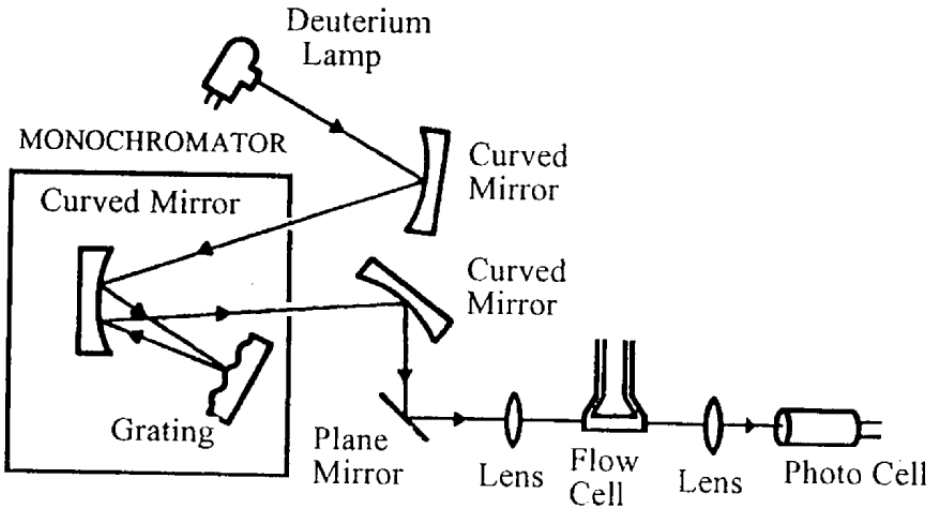


Figure 8: The multi-wavelength dispersive detector[17]

2.6.6.3. The diode array detector

The diode array detector, although offering detection over a range of UV wavelengths, functions in a slightly different way from the dispersion detector. A graphical description for this detector is shown in the figure 9. Light from the broad emission source such as deuterium lamp is collimated by an achromatic lens system so that the total light passes through the detector cell onto a holographic grating. In this way the sample is subjected to light of all wavelengths generated by the lamp. The dispersed light from the fixed grating is allowed to fall onto a diode array[14,15,17]. The array may contain many hundreds of diodes and the output of from each diode is regularly sampled by a computer and stored on a hard disc. At the end of the run the output from any of the diodes can be selected and a chromatogram produced employing the UV wavelength that was falling on that particular diode[15,17].

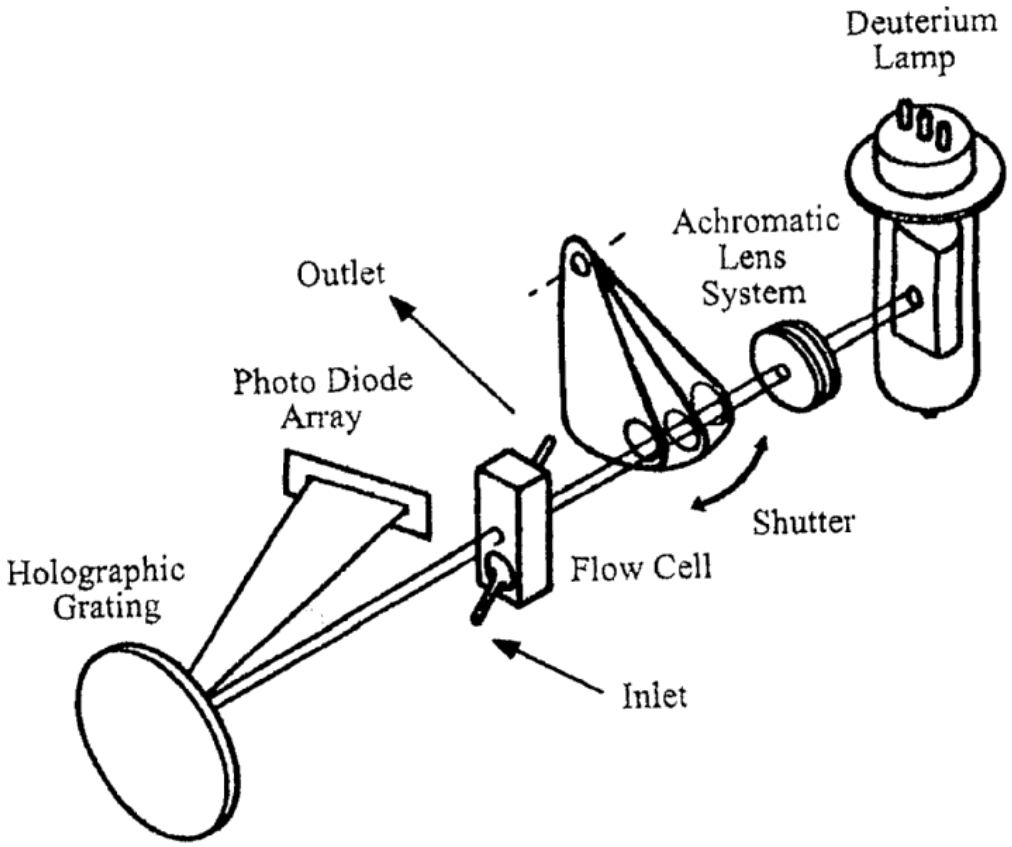


Figure 9: The diode array detector[17]

2.7. Thin layer chromatography

Thin layer chromatography is a subdivision of liquid chromatography, in which the mobile phase is a liquid and the stationary phase is a thin layer on the surface of a flat plate. [22,23] The mobile phase is the transport medium for the solutes to be separated as it migrates through the stationary phase by capillary forces. Substances that move slowly are attracted to the layer, whereas those that move quickly spend a smaller fraction of their time in the layer because of less affinity for it and more solubility in the mobile phase. At the end of development, each zone spreads owing to the

fluctuations in the movement of individual molecules in the zone due to factors such as particle size and uniformity in the layer[22].

2.8. Detection and Visualization

Following development, chromatograms are removed from the chamber and are air or oven dried to remove the mobile phase[22]. Coloured substances may be viewed in daylight without any treatment. Detection of colourless substances is simplest if compounds show absorption in the short-wave ultraviolet (UV) region (254 nm) or if they can be excited to produce fluorescence by short wave and/or long wave (366 nm) UV radiation. Otherwise the detection can be achieved by means of chromogenic reagents (producing coloured zones) or fluorogenic reagents (producing fluorescent zones), or by biological methods [22].

CHAPTER 3

3. MASS SPECTROMETRY

3.1. Introduction to Mass Spectrometry

Mass spectrometry (MS) is the most sensitive method of molecular analysis amongst the various spectrometric techniques[24]. It has the potential to yield information on the molecular weight as well as the structure of the analyte. The basis of MS is the production of ions, which are subsequently separated or filtered according to their mass to charge (m/z) ratio and detected.

It consists of five parts: sample introduction, ionization, ion separation, ion detection, and data handling. Sample introduction systems comprise controlled leaks, through which a sample vapour is introduced from a reservoir, various direct insertion probes for the introduction of solids (and low-volatility liquids), and combinations with various chromatographic techniques. The ionization of the analytes can be performed in a number of ways, e.g. electron ionization, chemical ionization, desorption ionization, and others[24].

In a magnetic deflection mass spectrometer, ions leaving the ion source are accelerated to a high velocity. The ions then pass through a magnetic sector in which the magnetic field is applied in a direction perpendicular to the direction of ion motion. When the acceleration is applied perpendicular to the direction of motion of an object, the object's speed remains constant, but the object travels in a circular path. Therefore, the magnetic sector is shaped in an arc; the radius and angle of the arc vary with different ion optical designs[24].

3.2. Introduction to ionization techniques

A wide variety of ionization techniques are available for organic mass spectrometry[8,25].The oldest and most frequently used is called electron impact ionization (EI). In electron impact ionization the analyte vapour is subjected to a bombardment by energetic electrons. Collision of the fast electron with a molecule can result in a weakly bonded electron being expelled from the molecule leaving a positively charged molecular ion. Some of these ions may have enough excess internal energy to fragment, producing ions of smaller mass. The numbers and masses of all these ions constitute the mass spectrum of a compound. EI is performed in a high vacuum ion source and EI spectra are highly reproducible as a result[25]. Chemical ionization (CI) is generally performed in relatively high-pressure ion sources, with pressure between 1 Pa and atmospheric pressure (10^5 Pa). In most cases the ionization is based on a chemical reaction between a reagent gas ion and the analyte. The reagent gas ion is produced by bombardment of a reagent gas by energetic electrons, i.e., by EI, followed by a series of ion molecule reactions[25].

3.3. Mass analysis

Four different principles are used in mass analysis. Two of those that will be discussed in greater detail are currently used routinely in LC - MS interfacing, sector and quadrupole[8,25]. In a single focusing instrument, for ions with mass m and z elementary charges, the kinetic energy of the ions is determined by the voltage V, with which the ions are accelerated towards the source exit slit[26]. Thus

$E_k = zev = 1/2\ mv^2$

where *e* is the elementary charge and *v* is the velocity of the ion. The magnetic field gives rise to a force perpendicular to the direction of movement, B*ve* that result in acceleration of the ion in a circular path:

$BzeV = mv^2/r$

where r is the radius of curvature of the path through the magnetic field. Combining these two equations leads to:

$m/z = B^2r^2e/2V$

This equation indicates that ions differing in m/z values are separated in space as each has a different radius of curvature. By variation of either B or V, ions of different m/z values can be detected by a detector at a fixed position behind a slit as being separated in time[25].

In a quadrupole shown in figure 10, only electric fields are used to separate ions according to their m/z values[27]. A quadrupole analyzer consists of four parallel rods or poles through which the ions are passed. Opposite rods are electrically connected and a constant voltage (DC) and alternate voltage (RF) are superimposed on the opposite pairs of rods. Depending on the produced electric field, only ions of a particular m/z will be focused on the detector, all the other ions will be deflected onto the rods. By varying the DC and RF voltages, different ions will sequentially be transmitted to the detector, resulting in the recording of a mass spectrum. The trajectory of an ion through a quadrupole is very complex. The four circular rods shown in the diagram in reality have a hyperbolic cross-section[27].

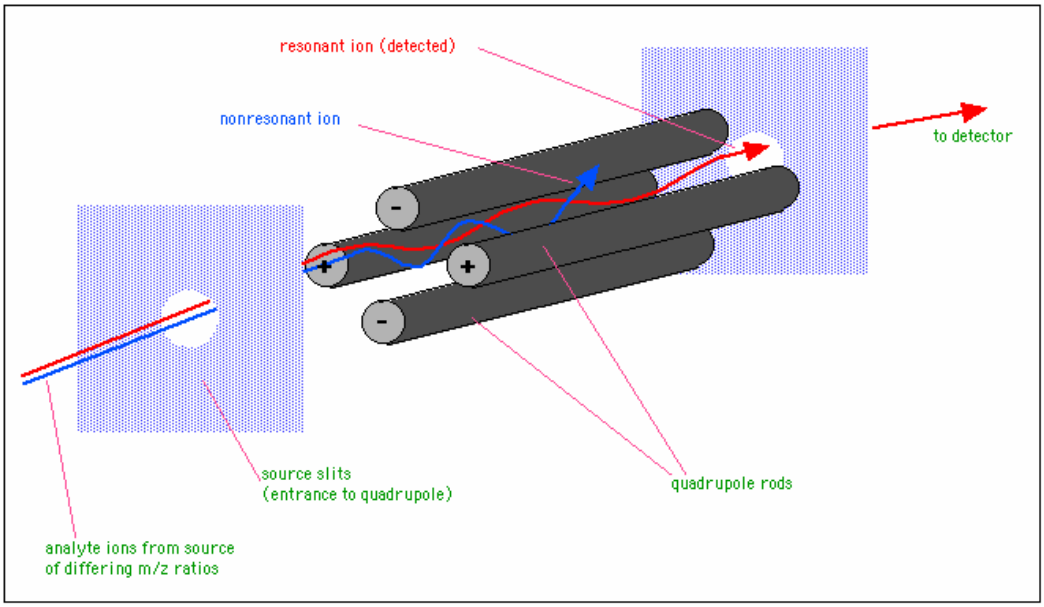


Figure 10: The quadrupole rods[27]

The opposite rods will have a potential of $+(U + Vcos(\omega t))$ and the other set of opposite rods a potential of $-(U + Vcos(\omega t))$ where U represents the fixed and $Vcos(\omega t)$ the radio frequency (RF) voltage respectively. When cos (ωt) cycles with time, t, the applied voltages on the opposed pairs of rods will vary in a sinusoidal manner but in opposite polarity. Along the central axis of the quadrupole assembly and also the axis between each adjoining rod the resultant electrical field is zero. In the transverse direction of the quadrupole, an ion will oscillate amongst the poles in a complex fashion depending on its m/z, the voltage U and V and the frequency, ω, of the alternating RF potential. By suitable choices of U, V and ω, only ions of a given m/z will oscillate stably through the quadrupole mass analyzer to the detector. All other ions will have greater amplitude of oscillation causing them to strike one of the rods. In practice, the frequency ω is fixed with typical values being 1 - 2 MHz. The length and diameter of the rods will determine an ultimate resolution that can be achieved by

the quadrupole assembly. The maximum mass range that is normally achieved is around 4000 Da with a resolution of around 2000[27].

3.4. Interfacing chromatography and mass spectrometry.

Combining chromatography with mass spectrometry offers the possibility of taking advantage of both chromatography as a separation technique and mass spectrometry as the identification method[24]. It plays an important role in environmental analysis, while LC - MS instrumentation is also heavily used in biochemical and biotechnological applications, as well as in many other fields of application[26,27,28]. The coupling of LC and MS is generally provided by means of thermo spray (TSP), particle beam (PB), atmospheric pressure chemical ionization (APCI), or electrospray Ionization (ESI) interfaces [29, 30].

3.5. Electrospray ionization

The electrospray interface is shown in figure 11[30,31,32]. Mobile phase from the LC column or infusion pump enters through the probe and is pneumatically converted to an electrostatically charged aerosol spray[33]. The solvent evaporates away reducing the droplets size and increasing the charge concentration at the droplet surface.

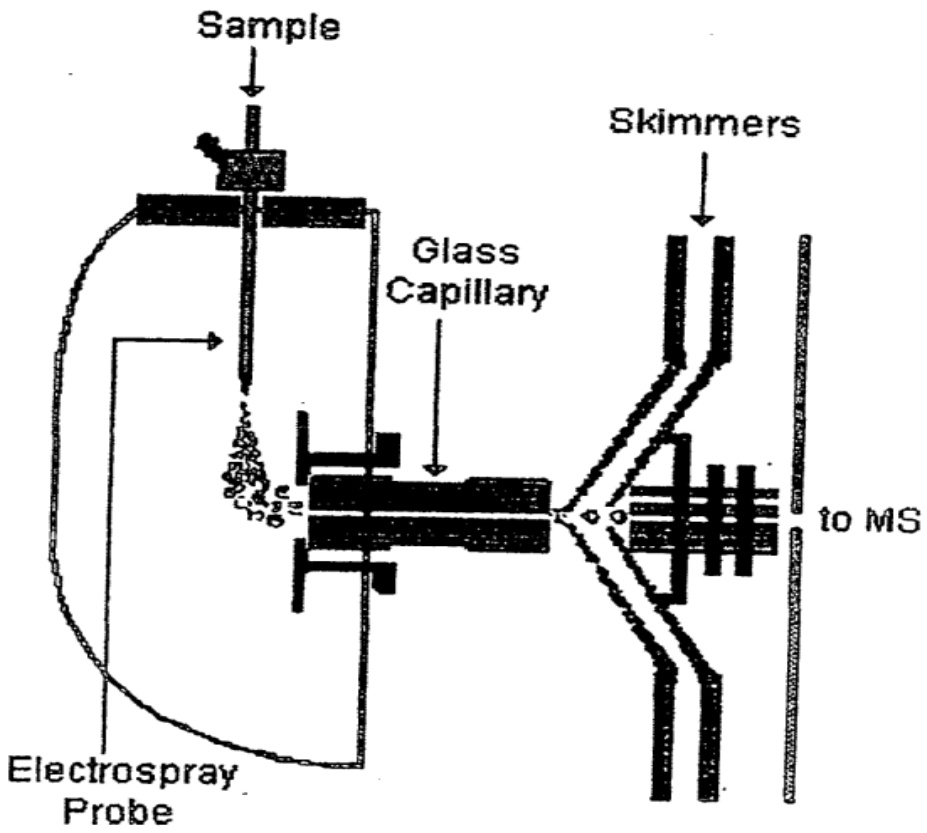


Figure 11: The general layout of the ESI[31]

The Coulombic repulsion overcomes the droplet's surface tension and the droplets explode. This 'Coulombic explosion' forms a series of smaller, lower charged droplets. The process of shrinking followed by explosion is repeated until individually charged 'naked' analyte ions are formed. The charges are statistically distributed amongst the analyte available charge sites, leading to the possible formation of multiply charged ions under the correct conditions. The process of droplet disintegration is shown in figure 12. Increasing the rate of solvent evaporation, by introducing a drying gas flow counter current to the sprayed ions, increases the extent of multiple charging[31].

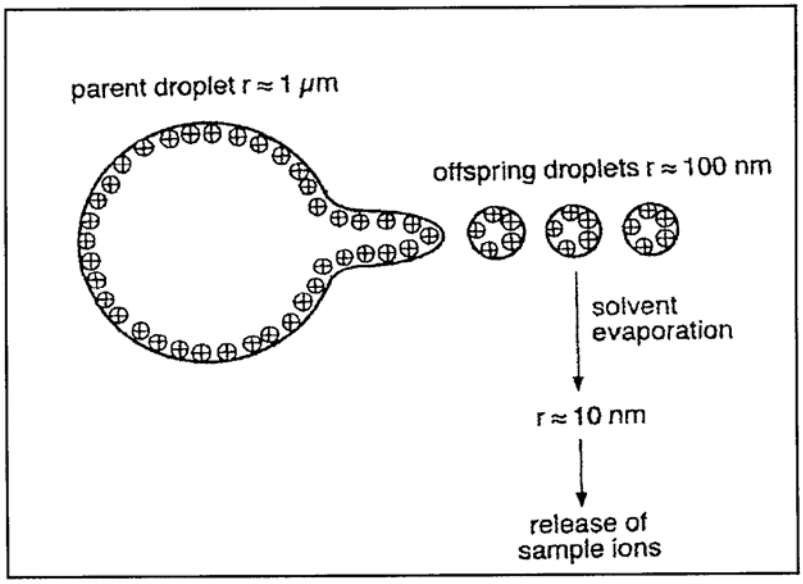


Figure 12: The process of droplet disintegration[31]

3.5.1. Nebulization

Nebulization in its simplest form is shown the figure 13. A sample is fed through the capillary tube and a high electric field at the tip of the tube pulls positive charge towards the electric front[34]. When the electrostatic repulsion becomes stronger than the surface tension, a smaller electrically charged droplet leaves the surface and travels through the surrounding gas to the counter electrode. In the figure 13(A) the capillary is at a positive potential compared to the counter electrode. In simple terms electrospray is the dispersion of a liquid into electrically charged droplets and combining two processes, droplet formation and droplet charging. Electric discharge is troublesome in the formation of negatively charged droplets. In the negative mode, the sprayer tip is at a high negative potential with respect to other parts of the source, and field emission of electrons from the sharp spray needle or from the sharp tip of the solvent front is a simple process[34]. The electrospray ionization allows rapid,

accurate and sensitive analysis of a wide range of analytes from low molecular mass (less than 2000 Da) polar compounds to biopolymers. Compounds less than 1000 Da produce singly charged protonated molecules ([M + H]+) in positive ion mode while low molecular weight analytes yield ([M - H]-) in the negative ion mode[31].

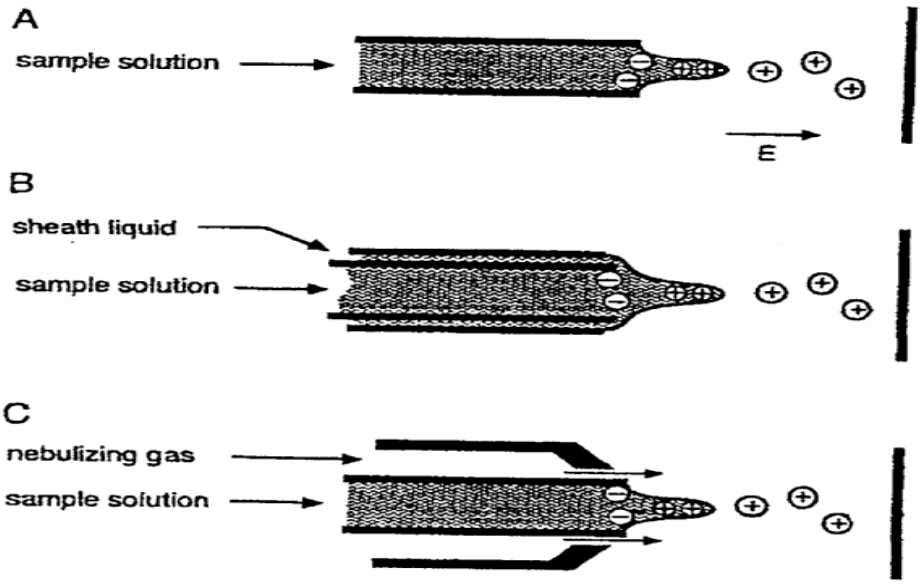


Figure 13: The nebulization process[34]

3.6. Atmospheric pressure chemical ionization (APCI)

APCI is a technique, which creates ions at atmospheric pressure. A sample solution flows through a heated tube where it is volatilized and sprayed into a corona discharge with the aid of nitrogen nebulization. Ions are produced in the discharge and extracted into the mass spectrometer. APCI is best suited to relatively polar, semi-volatile samples. An APCI mass spectrum usually contains the quasi-molecular ion, $[M+H]^+$. This technique is used as an LCMS interface because it can accommodate very high

(1 ml/min) liquid flow rates[35]. It can ionise less polar analyte that are not efficiently ionized by the electrospray process.

A general schematic diagram of an API source is shown in the figure 14. An API interface/source consists of five parts: the liquid introduction device, the actual API ion source region, where the ions are generated by means of electrospray ionization, APCI, or by other means, an ion sampling aperture, an atmospheric-pressure to vacuum interface, and an electrostatic focusing system, where the ions are transported into the mass analyzer [36,37,38,39,40].

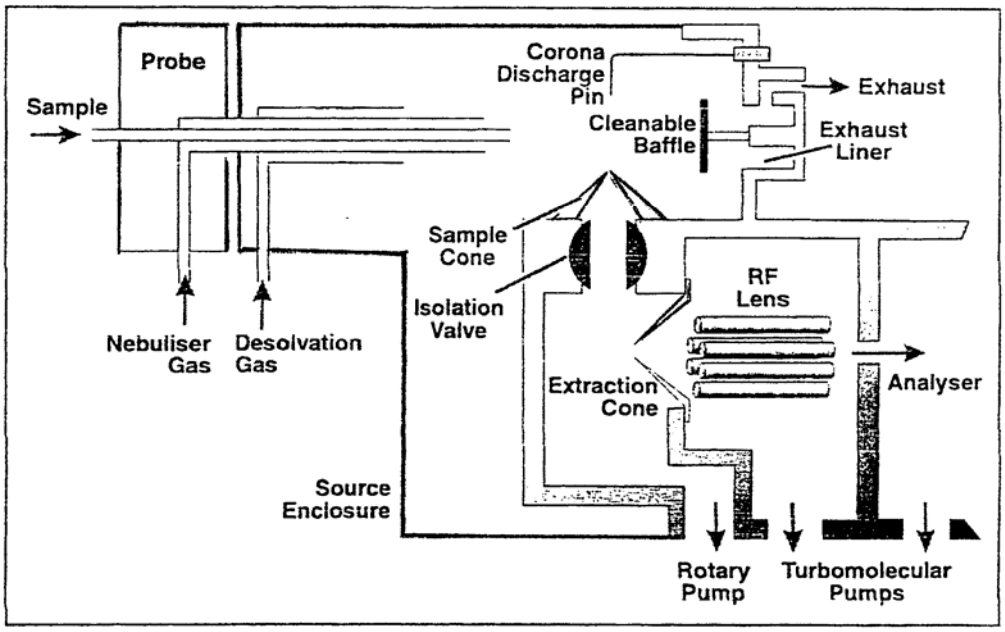


Figure 14: The APCI interface[27]

The column effluent from the LC is nebulized into an atmospheric pressure ion source region. Nebulization is performed pneumatically after which ions are produced from the evaporating droplets by gas-phase ion-molecule reactions initiated by electrons from a corona discharge. Analyte molecules introduced into the mobile phase react with the reagent ions at atmospheric pressure and become protonated in the positive

ion mode or deprotonated in the negative ion mode. The sample and the reagent ions pass through the sample cone prior to being extracted into the hexapole transfer lens through the extraction cone [27].

In a pneumatic nebulizer, a high-speed gas flow is used to mechanically disrupt the liquid surface and to form small droplets that are subsequently dispersed by the gas to avoid droplet coagulation[24]. Pneumatic nebulizers can be used to nebulize the LC column effluent either in an atmospheric-pressure region or in a reduced-pressure region, either directly into the ion source or into a reduced-pressure region separated from the ion source. The latter type is called a vacuum nebulizer[24].

In a vacuum nebulizer the column effluent is nebulized into an evacuated chamber that is connected to the ion source by means of a heated tube. The vacuum nebulizers are designed for microbore LC-MS, thus applying flow-rates in the 10-50 µl/min range. Higher flow rates cannot be introduced due to limitations in the heat transfer efficiency in the vacuum[24].

3.6.1. Sample inlet

The sample is introduced from a suitable liquid pumping system along with the nebulising gas to either the APCI probe or the electrospray probe. For nanoflow electrospray, metal-coated glass capillaries allow the lowest flow rates to be obtained while fused silica capillaries are used for flow injection analyses or for coupling to nano-HPLC[27].

3.7. MS operating modes

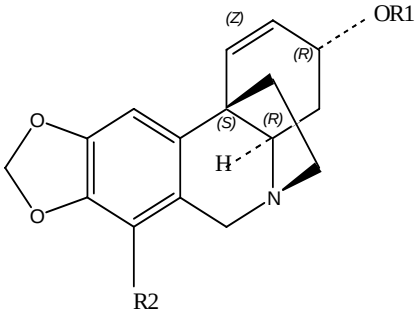
The mass spectrometer can be operated in a single or selected ion, full-scan or tandem MS (MS-MS) modes, and only the full scan mode (MS1) will be used in this study[27].

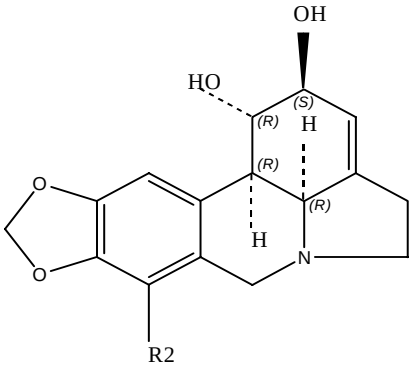
CHAPTER 4

4. PLANTS INTRODUCTION

4.1. *Boophane disticha* (*Amaryllidaceae* family)

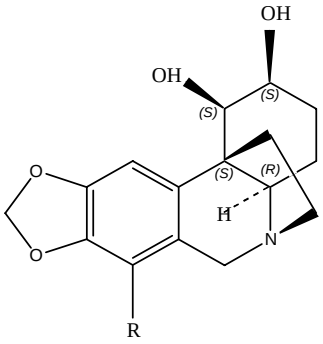
Boophane disticha (shown in illustration 1 on page 50), in addition to being poisonous, is used medicinally. The Xhosas use the dry outer scales of the bulb as an outer dressing after circumcision and as an application to boils[41,42,43]. In Europe the dry scales, moistened, were in the past applied as a dressing on boils, sores, whitlows (infection on the fingers by herpes virus, in children, this is often caused by thumb sucking or finger sucking while they have a cold sore) and septic cuts[42,44]. This is said to relieve pain and "draw out" pus. It is known to be used for arrow poisoning for shooting of smaller type of game by the Khoi and the San people[45,46]. The traditional way of extracting the compounds was with water. Thirteen alkaloids found in the bulbs of *Boophane disticha* are also known to exist in the other *Amaryllidaceae* family[47,48,49]. The principal alkaloid was isolated and identified as buphanidrine (3)[42]. Below is a list of compounds found in the *Amaryllidaceae* family [42,50].





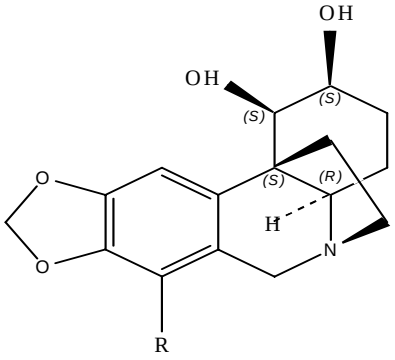
Crinine **(1)** *where R1 = R2 = H*
Krepowine **(2)** *where R1 = H, R2 = OH*
Buphanidrine **(3)** *where R1 = CH_3, R2 = OCH_3*
*Powelline***(4)** *where R1 = H, R2 = OCH_3*

Lycorine **(5)** *where R2 = H*



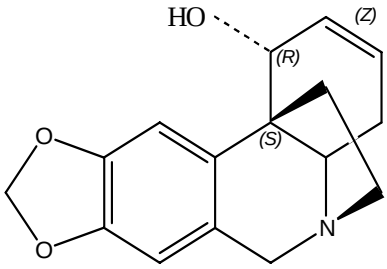
4a-Dehydroxycrinamabine **(6)** *where R = H*

1-Epideacetylbowdensine **(7)** *where R = OCH_3*

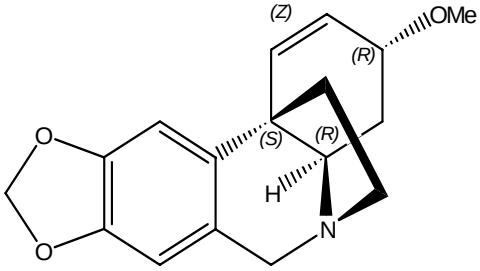


Crinanidine **(8)** *where R=H*

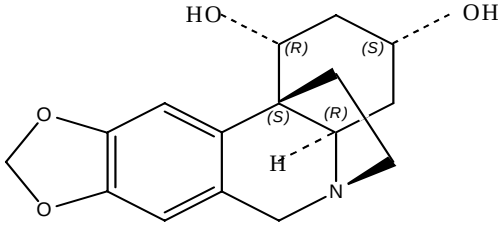
Undulatine **(9)** *where R = CH_3*



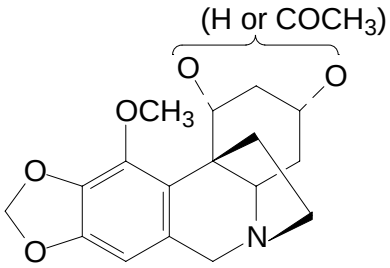
Buphanamine **(10)**



Buphanisine **(11)**



Buphanitine (Nerbowdine) **(12)**



Acetyl- Nerbowdine **(13)**

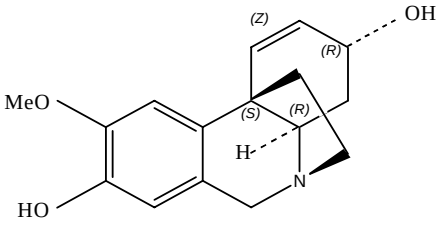
Illustration 1: *Boophane disticha* [51]

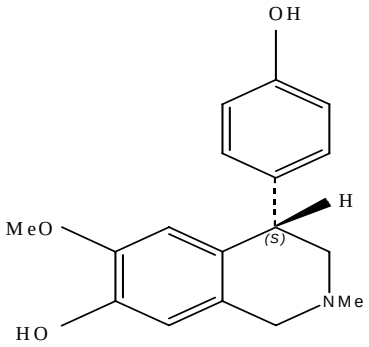
4.2. *Crinum macowanii* (*Amaryllidaceae* family)

This plant is the most widely distributed of all the Crinum[41] species of South Africa (see Illustration 2 on page 52). It is reported to be a Zulu remedy for various complaints, mainly for scrofula (TB of the neck), micturation (bladder complications) and rheumatic fever[52,53,54,55]. It is also used for blood cleansing, kidney and bladder diseases, glandular swelling, fever and skin problems such as sores, boils and acne (a skin condition which has plugged pores (black heads and white heads), inflamed pimples, and deeper lumps). Various *Crinum* species are used as arrow poison[41].

Both *Crinum macowanii* and *Boophane disticha* belong to the *Amaryllidaceae* family and have similar characteristics. There are many reports that describe the presence of alkaloids in this family and the two plants investigated have been reported to contain similar compounds[47,48,49,56,57,58].

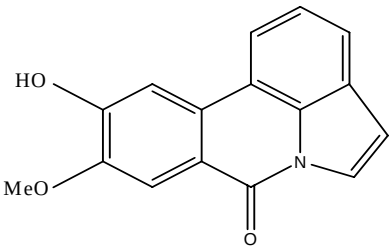
The active ingredient in *Crinum macowanii* was found to be Lycorine **(5)** while Crinamine **(17)** is also present in this species and is a respiratory depressant and a powerful transient hypotensive agent (causing low blood pressure or a lowering of blood pressure) in dogs[59]. Various effects have been ascribed to *Crinum* alkaloids, such as antitumor, hypotensive, and analgesic activity[41,60]. Other compounds found in *Crinum macowanii* in addition to alkaloids mentioned in the *Amaryllidaceae* family include Macowine **(14)**, Cherylline **(15)** and Pratorimine **(16)**[61,62,63,64,65].

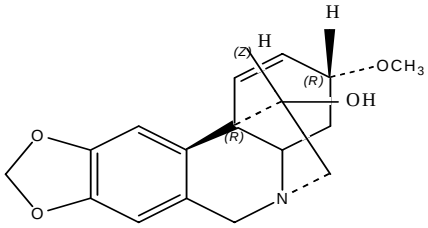




Macowine **(14)**

Cherylline **(15)**





Pratorimine **(16)**

Crinamine **(17)**

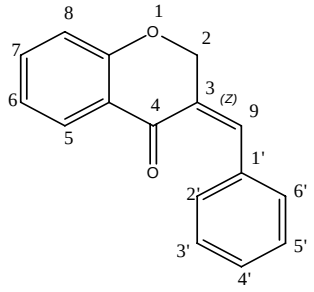
Illustration 2: *Crinum macowanii* in Mpumalanga province[66]

4.3. *Eucomis autumnalis*

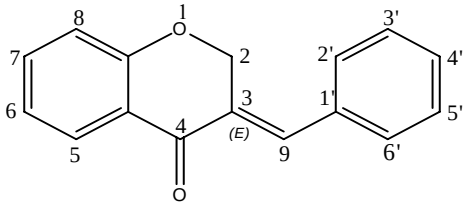
This bulbous plant has long, broad, soft-textured leaves with wavy margins (see Illustration 3 on page 56). Numerous small, yellowish-green flowers are borne on a thick central stalk[41]. The plant is used medicinally for low back pain, to assist in post-operative recovery and to aid in the healing of fractures. A decoction of the bulbs is also used for a variety of other ailments, including urinary disease, stomachache, fevers, colic (severe abdominal pain caused by spasm, obstruction, or distension of any of the hollow viscera, such as the intestines. Often a condition of early infancy, colic is marked by chronic irritability and crying.), flatulence (the presence of excessive gas in the digestive tract), hangovers, and syphilis (a chronic infectious disease caused by a spirochete *(Treponema pallidum),* either transmitted by direct contact, usually in sexual intercourse, or passed from mother to child in uterus, and progressing through three stages characterized respectively by local formation of chancres (An ulcer located at the initial point of entry of a pathogen) , ulcerous skin eruptions, and systemic infection leading to general paresis), and to facilitate childbirth[67,41]. The subspecies, *clavata* is also used for coughs and respiratory ailments, blood disorders, diarrhea and to prevent premature childbirth[41].

The genus *Eucomis* is indigenous to Southern and Eastern Africa[68]. Eucomin and eucomol are the main metabolites in one of the species called *Eucomis bicolor* (cf Table 1). Later, 3,9 dihyroeucomnalin, the 7-O-methyl as well as the 4-dimethyl derivatives were isolated (compound 21 – 27)[69]. The compounds showing a 4',5,7-oxygenation pattern are called "eucomins" and "punctins" bearing an additional methoxy group at C-6 and C-8 have been isolated from *Eucomis comosa* and *Eucomis autumnalis* together with some metabolites which belong to the eucomin

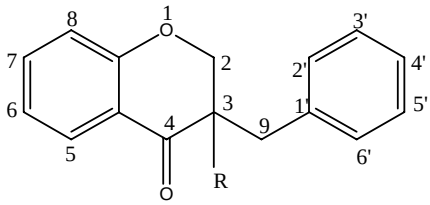
series[70,71,72,73,74,75]. The structures of the compounds found in *Eucomis* species are shown (**Structure type I – IV**) and table 1 shows various compounds derived from different substitution on these structural types. These type of compound that are found in *Eucomis* species are known to be homoisoflavanoids[69,70,71,72,73,74,75].



Structure type **I** (Z), Cis-



Structure type **II** (E), trans-



Structure type **III**, R = H

Structure type **IV**, R = OH

Table 1: Names of compounds of different structural type[42]

Compound	*Name*	*Substitution position*							*Structural type*
		3	***5***	***6***	***7***	***8***	***4'***	***5'***	
18	***Eucomin***		OH		OH		OH		II
19 &20			OH		OH		OCH_3		I, II
4			OH		OCH_3		OCH_3		II
21	***3,9-***		OH		OH		OH		III
22	***Dihydroeucomin***		OH		OH		OCH_3		III
23			OH		OCH_3		OCH_3		III
24			OCH_3		OCH_3		OH		III
25			OH		OH		OCH_3	OCH_3	III
26			OH		OCH_3		OCH_3	OH	III
27			OH	OCH_3	OH		OCH_3		III
28	***Eucomol***	OH	OH		OH		OH		IV
29		OH	OH		OH		OCH_3		IV
30		OH	OH		OCH_3		OCH_3		IV
31	***Eucomnalin***		OH	OCH_3	OH		OH		II
32	***3,9-*** ***Dihydroeucomin***		OH	OCH_3	OH		OH		III
33	***Punctatin***		OH		OH	OCH_3	OH		II
34,35			OH		OH	OCH_3	OCH_3		I,II
36	***3,9-Dihydro-***		OH		OH	OCH_3	OH		III
37	***punctatin***		OH		OH	OCH_3	OCH_3		III

Illustration 3: *Eucomis autumnalis*[66]

4.4. Methods for extraction and sample clean up.

Living organisms consists of complex mixtures of chemical compounds, usually held within cellular structures. In order for the compounds to be isolated for testing in bioassays, for determining their structure, or both, the initial step clearly involves separating them from cellular structural material (mostly protein, lipid and polysaccharides) and ideally from a large majority of unrelated substances co-existing in the organism. However in some cases those materials may be desired[1].

4.4.1. Factors to be considered in selecting an extraction method

a) Purpose of extraction

There may be four situations in sample extraction:

1. A chemical substance of known identity is to be extracted from an organism. In this case specific published procedures can be followed, and appropriate modifications made to improve the process[1].
2. Materials may be examined for the occurrence of particular chemical substances, such as alkaloids or flavanoids. In such situations, general extraction methods applicable to the chemical group of interest can be found in literature and are used. These would be followed by the appropriate chemical or chromatographic tests for the chemical group[1].
3. The organism (plant) may be used in traditional medicine, and is usually prepared in certain ways, e.g. traditional Chinese medicine frequently requires herbs to be boiled in water and aqueous mixture administered as medicine. This process must be mimicked as closely as possible if the extract is to be the subject of further biological or chemical studies, particularly if the purpose is to investigate traditional use[1].
4. The nature of the substance to be isolated is not predetermined in any way. This situation may arise where the aim is to test random organisms or those used traditionally for the presence of compounds with a specific biological activity[1].

b) Properties of compounds to be extracted

1) Polarity

Whether the compound to be isolated is predetermined or not, it is important to note the relationship between the method applied and the properties of the substance extracted. A general principle is 'like dissolves like'. Thus non-polar solvents will extract out non-polar substances, and polar solvents will extract polar materials [1].

2) Effect of varying pH

The ionizability of compounds is another important consideration, as the pH of the extracting solvent can be adjusted to ensure maximum extraction. For example, even non-polar alkaloids can be extracted into polar aqueous acid, as their basic nature ensures salt formation in acid. The salt dissociates into ions in aqueous solutions and the substance dissolves due to hydration of the positively charged, protonated alkaloids and the anion. Aqueous solvents at alkaline pH may similarly be used to extract acidic phytochemicals, e.g. fatty acids and phenols. It is important to ensure that the compounds will not break down at pH values employed [1].

3) Thermostability

The solubility of compounds in a solvent increases with increasing temperature and higher temperature facilitates penetration of the solvent into the cellular structure of the organism to be extracted. However, any advantage gained here will clearly be lost if the compound is unstable at higher temperatures. The formation of artefacts, i.e. new compound not initially present in the organism under study, is a possibility with many extraction methods [1.]

c) Properties of the solvents to be used

The principle of 'like dissolves like' is, again, applicable here, i.e. the nature of the solvent determines the type of chemicals it is likely to extract from the organism. Other properties of the solvent are the boiling point, flammability, toxicity, reactivity, presence of additives and cost [1].

1) Toxicity

Another factor influencing the choice of the solvent is its toxicity. For example, inhalation of large amounts of chloroform or diethyl ether can cause respiratory depression and central anaesthesia. Many solvents cause defatting of the skin, leading to dermatological conditions [1].

2) Reactivity

It is important to be aware that the solvent itself may react chemically with the compounds to be extracted, resulting in the formation of artefacts. The potential chemical reactions occurring in acidic or basic environments have already been mentioned. In addition to this, solvents containing carbonyl groups may react with nucleophilic substances in the extract, and the use of methanol or ethanol may result in esterification [1].

CHAPTER 5

5. RESULTS AND DISCUSSION

5.1. Extraction of compounds from *Boophane disticha*.

5.1.1. Preparation of plant material for analysis

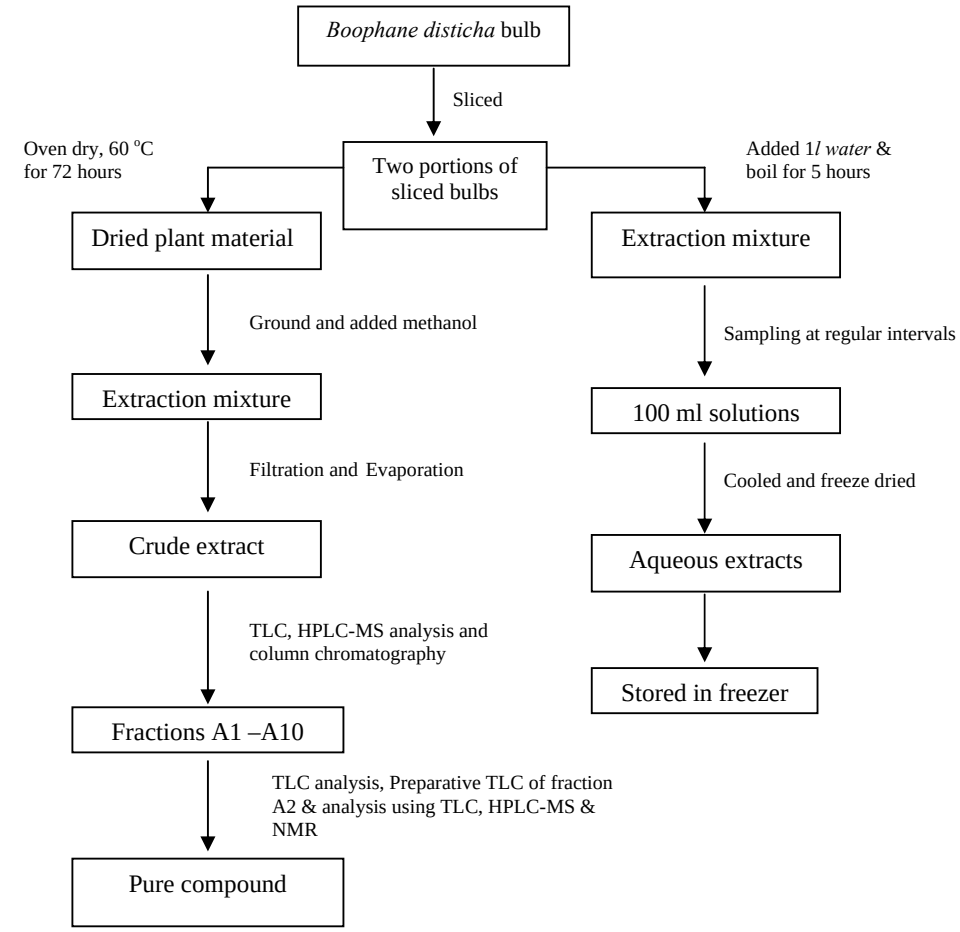
The fresh bulb of *Boophane disticha* was sliced into small pieces and separated into two portions. One portion was oven dried at 60 ^{o}C for 72 hours after which the dried plant material was extracted with methanol, plant material filtered off and the methanol evaporated to dryness. The dried methanol extract was analysed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) followed by further purification using column chromatography (silica gel as stationary phase) and preparative TLC as shown in scheme 1. The other portion of the sliced bulbs was boiled for five hours while taking samples for analysis at regular intervals.

5.1.2. Processing of a crude organic extract of *Boophane disticha*

The methanol extract was analyzed using silica gel thin layer chromatography (using two separate TLC plates) with chloroform: ethyl acetate: methanol (4:4:2, v/v/v) as the mobile phase. Several compounds were detected on the TLC plates using a UV lamp at a wavelength 360 nm. Spraying one plate with cerium sulphate and the other with Dragendorf reagent also indicated a complex mixture of compounds. Some of the compounds were visible under UV while others could only be detected after spraying with the reagents. An indication of the presence of alkaloids in the extract was established using Dragendorf reagent, which is specific for that class of compound[76]. An indication of the presence of the alkaloids was identified by compounds which stained orange with Dragendorf reagent.

In order to obtain more information on the chemical profile of the compounds in the extract, further analysis was conducted using high performance liquid chromatography - mass spectrometry (HPLC-MS). The analysis of the methanol extract using HPLC eluting with ammonium acetate/methanol (9:1, v/v) on a C-18 reverse phase column indicated that the extract contains a complex mixture of compounds. The UV max plot and total ion chromatograms of the extract are shown in figures 5.1 (A) and (B) respectively. Literature studies (see Chapter 3) reported that the alkaloid, Buphanidrine (3), with a molecular weight of 315 is present in *Boophane disticha*[41,43]. A selected ion search for m/z 316 (M+1) indicated a compound eluting at 41.73 minutes in the total ion chromatogram which corresponded to this molecular ion. The corresponding peak eluting at 41.67 minutes in the UV max plot showed UV absorption maxima at 214 nm and 286 nm, which were previously reported for Buphanidrine (3)[47]. Based on reported information and indications that the compound with molecular weight of 315 occurs in very low concentration (See total ion chromatogram, figure 5.1(B)), further purification of the extract aimed at the isolation of Buphanidrine (3) was warranted.

Scheme 1: Extraction and purification scheme of *Boophane disticha*



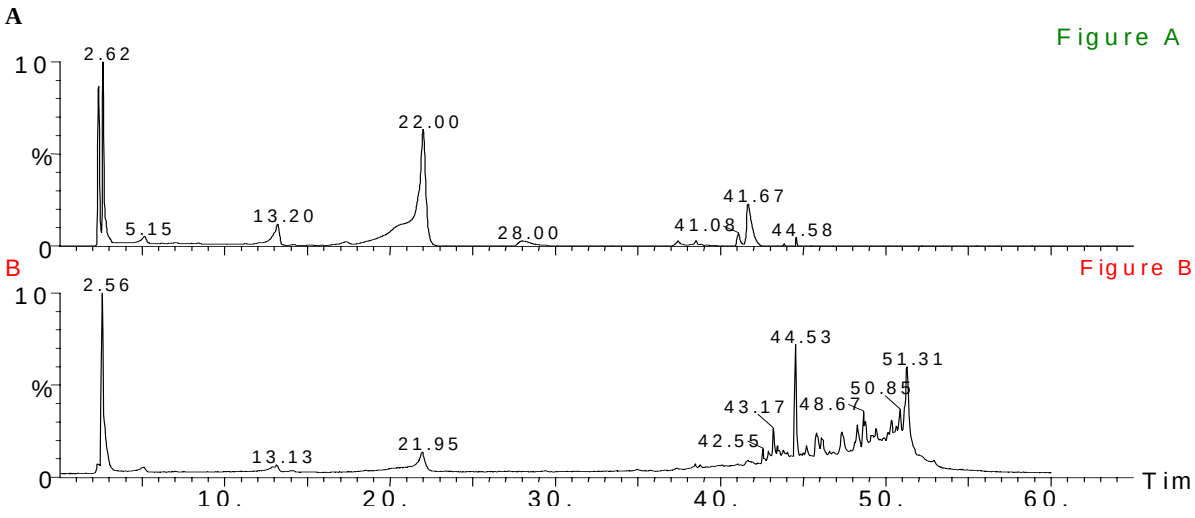


Figure 5.1 (A and B): The UV (Figure A) and total ion chromatograms (Figure B) of the methanol extract of *Boophane disticha*

5.1.3. Purification of *Boophane disticha* extracts

The crude methanol extract was purified using column chromatography with silica gel as the stationary phase and eluting with chloroform: ethyl acetate: ethanol (4:4:2, v/v/v). Seven fractions were collected and TLC analysis, with Dragendorf reagent to indicate the presence of alkaloids was used to identify which fractions contained alkaloid type compounds. One of the fractions (Fraction A_2), which showed the presence of alkaloids, was further chromatographed.

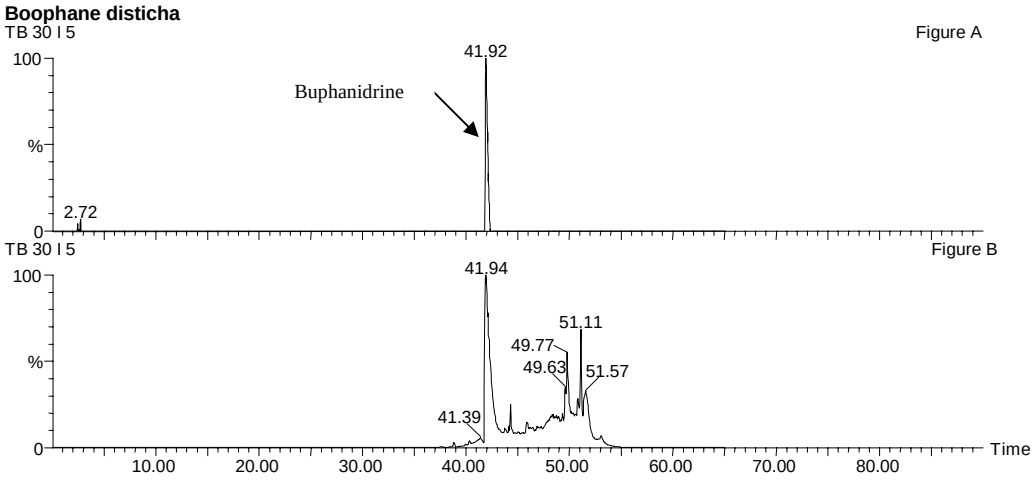
This fraction, A_2 (see Scheme 1) was further purified using silica gel preparative thin layer chromatography with chloroform: ethyl acetate: methanol (4:4:2, v/v/v) as eluant. After development of the chromatographic plates the compound staining with Dragendorf reagent was identified. This was done by cutting off a strip on the edge of the TLC plate, spraying this with the reagent and using the Rf value for the positively identified compound to mark out the alkaloid in the preparative TLC plate for further extraction. The bands, containing the alkaloids were separately scrapped off the plate, the silica gel ground to a powder, extracted with methanol, filtered and solvent evaporated. The process was repeated several of times to recover enough product. Only one band was extracted using this procedure while the remaining material was discarded. The fractions isolated were analyzed using TLC, HPLC-MS and NMR.

5.1.4 Analysis of the fractions by HPLC-MS and TLC.

TLC and HPLC-MS were used to analyze the fraction obtained from preparative thin layer chromatography. Two TLC plates were used to analyze the fraction and the samples were developed using chloroform/ethyl acetate/methanol (4:4:2, v/v/v) as the mobile phase. Visualizing under UV at a wavelength of 366 nm only one spot was

observed. When one plate was sprayed with cerium sulphate and the other with Dragendorf reagent only one compound was visible on both of the plates showing that the purification step was successful at this stage. The Dragendorf reagent showed an orange colour after spraying, which is indicative for alkaloids.

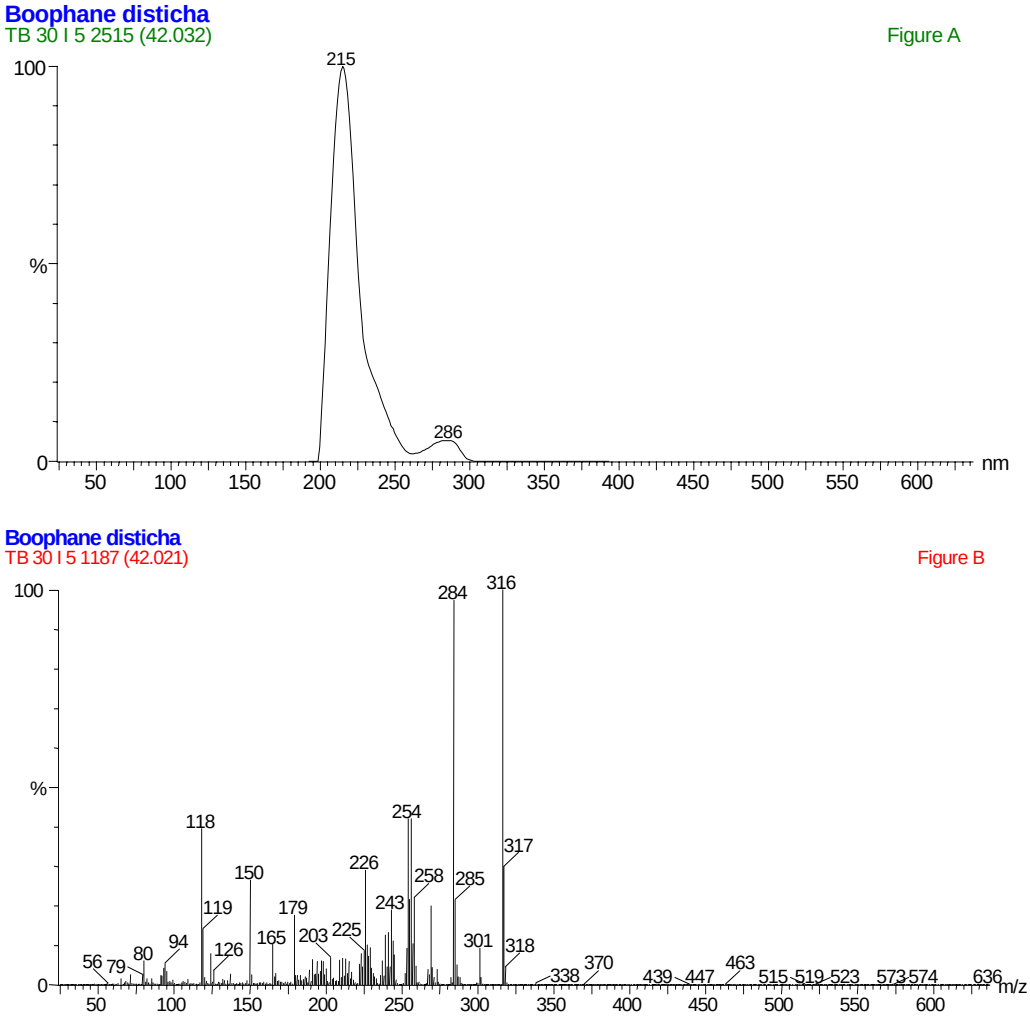
The HPLC-MS gradient system was developed with the initial conditions as ammonium acetate: methanol (9:1) on a C-18 reverse phase column while for the MS, a positive electrospray mode was used for the ionization. The UV max plot and the total ion chromatogram of the pure compound are shown in figure 5.2 A and B, respectively. The two figures show a compound with a retention time of 41.92 minute which had a UV maximum absorption at 214 nm and 286 nm, and a molecular ion at m/z 316 (M+1). The UV and MS spectra of this compound is shown in figure 5.3 (A and B) and results are given in details in Table 5.1.



Figures 5.2 (A and B): The UV max plot (Figure A) and the total ion chromatogram (Figure B) of the purified compound

Table 5.1: The UV and MS data from HPLC-MS of the purified compound

Retention Time (minutes)	**UV maximum absorption (nm)**	**m/z (M + 1)**
41.92(UV chromatogram)	214 and 286	
41.94 (Total ion chromatogram)		316

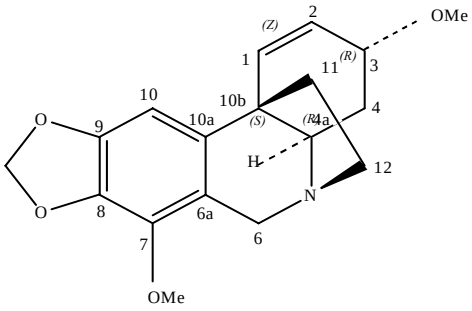


Figures 5.3(A and B): The UV and mass spectra of the purified compound

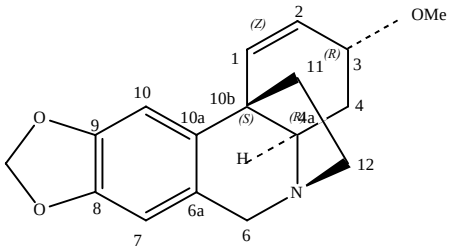
The MS chromatogram in figure 5.3 B shows the (M+1) ion at m/z 316, which correlates with the reported[49] molecular weight for the compound Buphanidrine (3).

The fragment (M+1-32) at m/z 284 corresponds to the loss of neutral methanol, indicative of the methoxy groups (–OCH_3) on the molecule. The quasi molecular ion at m/z 316 distinguishes Buphanidrine (**1)** from the closely related alkaloid Buphanisine (**3)** with molecular weight 285 (and only a single methoxy group) that was also reported to be isolated from *B. disticha.* (Pham et al., 1998).

The ^{1}H NMR and the ^{13}C NMR (in *deuterated*-$DMSO_3$) provided further evidence for the confirmation of the structure of the compound isolated. The NMR data was compared to that of the published data of the compound Buphanidrine (3) and Buphanisine (11)[49,77]. The comparison is given in Table 5.2 (^{1}H NMR) and Table 5.3 (^{13}C NMR).



Buphanidrine (3), m/z = 315



Buphanisine (11), m/z = 285

A closely related compound Buphanisine (11) [78], which was reported to be isolated from *Boophane disticha*, lacks a methoxy group at C-7 while Buphanidrine (3) has two methoxy groups at positions C-3 and C-7. This was used as the distinguishing factor between the two compounds as the isolated compound has a signal that corresponds to two methoxy groups at δ 3.32 and 3.94 in the proton NMR spectrum. Based on the literature[47], the signal at δ 3.32 was assigned to the C-3 methoxy group

and δ 3.94 to the C-7 methoxy group. Further evidence was obtained from the ^{13}C NMR spectra, as the chemical shift of the signal corresponding to C-7 of the isolated compound was δ 140.9 ppm while for the buphanisine (11) it is reported at 142 ppm. Because Buphanisine (11) doesn't have a 7-Ome susbstituent as compared to Buphanidrine (3), the there is no signal arising from that carbon in the ^{13}C NMR spectra for Buphanisine (11) while there is a signal for Buphanidrine (11) at δ 59.2 ppm arising from the same carbon.

The remaining NMR data for Buphanidrine (3) and that reported for Buphanisine (11)[77] are similar to each other (c.f. Table 5.2 and 5.3). Based on the mass spectral data and the differences observed in the comparative NMR between the isolated compound and the published data for the two alkaloids, it was concluded that the isolated compound is Buphanidrine (3). One compound (Lycorine) reported in other Amaryllidaceae species[79,80,48] was also confirmed to be available in *Boophane disticha* with the molecular weight 287 and giving the quasi-molecular ion (M+1) at 288 when analyzed with the positive electrospray ionization (ESI) mode of the mass spectrometer. This compound will be discussed in detail in the next section of the plant *Crinum macowanii.*

The discrepancy in chemical shifts between the NMR data and that reported in the literature (cf tables 5.2 and 5.3) for the 6β, 12 exo, 12 endo, and 12 exo protons could be attributed to the use of different solvents for the analysis. Deuterated chloroform was used as the solvent for the NMR analysis of the pure compounds as described in literature[47] while in our experiments deuterated DMSO was used. The other reason could be that the authors used a 300 MHz NMR instrument for the analysis as described in literature[47] while in our case a 400 MHz NMR instrument was used.

Table 5.2: ^{1}H NMR data for Buphanidrine (3)

Hydrogen Atom	Isolated Product δ_H /ppm (*J* (HH)/H_z) in *d* $DMSO_3$	Literature data of Buphandrine (3)[47] δ_H /ppm (*J* (HH)/H_z) in $CDCl_3$	Literature data of Buphanisine (11)[77] δ_H /ppm (*J* (HH)/H_z)in $CDCl_3$
1	6.49 (*d* , *J* = 10.4)	6.42 (*d*, J = 10.0)	6.61 (*d*, *J* = 10)
2	5.94 (*dd, J* = 4.5, 10.0)	5.95 (*dd, J* = 5.0,10.0)	5.96 (*dd, J* = 10.0,5.2)
3	3.78 *m*	3.77 *m*	3.82 *m*
4α	2.28 (*broad, J* = 13.3)	2.46 (*brd, J* = 13.5)	2.08 (*ddd, J* =13.8,13.4, 4.1)
4β	1.59 (*ddd, J* = 13.6,13.6, 4)	1.60 (*ddd, J* = 4.0, 13.5, 13.4)	1.59 (*ddd, J* = 13.8,13.4, 4.1)
4a	3.40 (*dd, J* = 3.2, 12.6)	3.5 (*dd, J* = 3.5, 13.5)	3.34 (*dd, J* = 3.9, 13.4)
6α	4.30 (*d, J* = 17.2)	4.35 (*d, J* = 17.0)	4.40 (*d, J* = 16.7)
6β	3.32 (*d, J* = 17.2)	3.95 (*d, J* = 17.0)	3.80 (*d, J* = 16.7)
7	None	None	6.47 *s*
10	6.42	6.50 s	6.83 s
11endo	1.95 (*ddd, J* =5.4, 10.9, 12.1)	1.99 (*ddd, J*=6.0, 10.5, 12,5)	1.91(*ddd, J* = 12.8, 12.2, 5.90)
11exo	2.92 (*ddd, J* = 5.7,8.8,13.2)	2.20 (*ddd, J* = 4.0, 9.0, 12.6	2.16 (*ddd, J* = 12.8, 9.1, 5.9)
12endo	3.50 (*ddd, J* = 3.2,8.5, 12.8)	2.99 (*ddd, J* = 6.0, 9.0, 13.0)	3.38 (*ddd, J* = 4.3, 11.2, 13.2)
12exo	3.50 *m*	3.64 *m*	2.89 (*ddd, J* = 5.9, 9.1, 13.2)
-O-CH_2-O-	5.82 and 5.84 (*2d, J* = 1.6)	5.81 and 5.82 (*2d, J* = 1.5)	5.87 and 5. 86 (*2d, J* = 1.70)
3-OMe	3.32 *s*	3.29 s	3.45 *s*
7-OMe	3.94 *s*	3.94 *s*	

Table 5.3: The ^{13}C NMR Chemical shift assignments of Buphanidrine (3) in *deuterated*-$DMSO_3$

Carbon Atom	Isolated Product (Buphanidine (3)) δ_c /ppm in deuterated- $DMSO_3$	Literature data for Buphanidrine(3)[47] δ_c /ppm in $CDCl_3$	Literature data for Buphanisine (11)[77] δ_c /ppm in $CDCl_3$
1	131.8 D	132.1 *D*	132.9 *D*
2	125.7 D	125.5 *D*	125.3 *D*
3	72.13 D	72.2 *D*	72.7 *D*
4	27.9 *T*	28.0*T*	28.9 *T*
4_a	63.04 *D*	62.8 *D*	63.1 *D*
6	58.1 *T*	58.1 *T*	62.4 T
6_a	115.8 *S*	115.8 *S*	126.4 *S*
7	140.9 *S*	140.8 *S*	142 *S*
8	133.6 *S*	133.4 *S*	145.6 *S*
9	148.5 *S*	148.2 *S*	146 *S*
10_a	96.94 *D*	103.2 *D*	102.9 *D*
10_b	43.2 *S*	44.3 *S*	44.32 *D*
11	43.5 *T*	43.4 *T*	44.28 *T*
12	53.3 *T*	53.3 *T*	53.6 *T*
-O-CH_2-O-	100.7 *T*	100.6 *T*	100.9 *T*
3-OMe	56.6 *Q*	56.5 *Q*	56.4 *T*
7-OMe	59.2 *Q*	59.1 *Q*	

5.1.5. Aqueous extraction of compounds from the bulbs of *Boophane disticha*

The traditional preparation of the plant is by boiling the bulb in water for several hours. The purpose of the aqueous extraction in this study was to establish any differences in the concentration of the active compound Buphanidrine, at different times while boiling. The bulb of *Boophane disticha* is known to have toxic compounds[41] and after several hours of boiling the extract is ready for drinking as a medicine. It is possible that the extended boiling is done for extraction of the compounds at safe concentrations.

The aqueous extract of *Boophane disticha* was prepared by the addition of water to the wet plant material that was previously cut into smaller portions. The mixture of

the plant material with water was placed on a heating plate and boiled while a portion of the boiling mixture was withdrawn at hourly intervals for analysis.

The samples collected at regular intervals were cooled and freeze-dried and then analyzed using HPLC MS. Approximately 10 mg of the sample was dissolved in 2 ml of water (previously degassed) and the sample solution was filtered with the 0.45 µm nylon filter and injected (25 µl) on the HPLC-MS system, using a gradient mobile method. The compound Buphanidrine (3) was detected in the region of 41.98 minutes in the UV chromatogram and a general decrease in the peak area at this retention time for the compound was observed in the samples taken as the boiling continued (See Table 5.4 for the peak area for Buphanidrine (3) obtained from the UV chromatogram). This decrease in the peak area could be attributed to a decrease in concentration of Buphanidrine (3), as the peak area of the compound in the UV chromatogram is proportional to concentration. The UV chromatograms that show a decrease in peak area on this compound with retention time 41.98 minutes are shown in figures 5.4 to 5.9. The compound Lycorine (5) that was shown to be present in extracts of the plant was detected at retention time of 17.42 minutes (in UV Chromatogram) also showed a decrease in the peak area as the boiling continued until the compound could not be detected in the extract after 3 hours of boiling.

Table 5.4: Results from the analysis of aqueous extracts of *Boophane disticha* using HPLC-MS

Sample labels	Sampling time (in hours)	Retention time (in minutes) from UV chromatogram	Peak area of Buphanidrine from UV chromatogram
TB 13 AA	1	41.98	8478399
TB 13 BB	2	42.08	4874898
TB 13 CC	3	42.12	3349362
TB 13 DD	3.5	42.16	3145711
TB 13 EE	4	42.16	3057704
TB 13FF	5	42.20	2606112

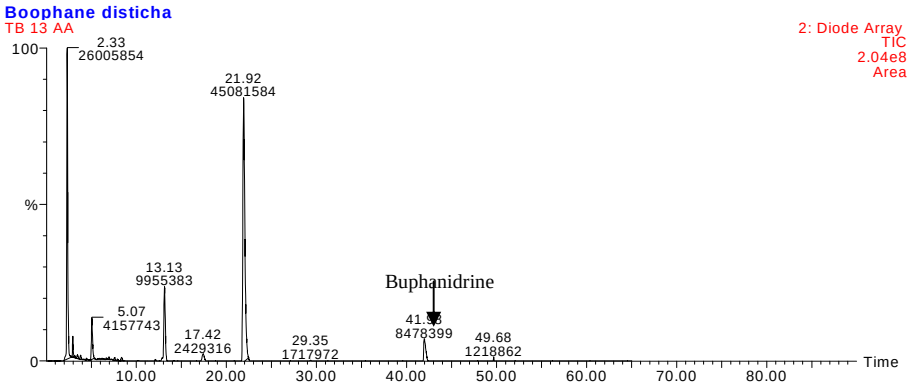


Figure 5.4: UV chromatogram of the water extract after 1 hour of boiling, retention time of Buphandrine (3) at 41.98 minutes

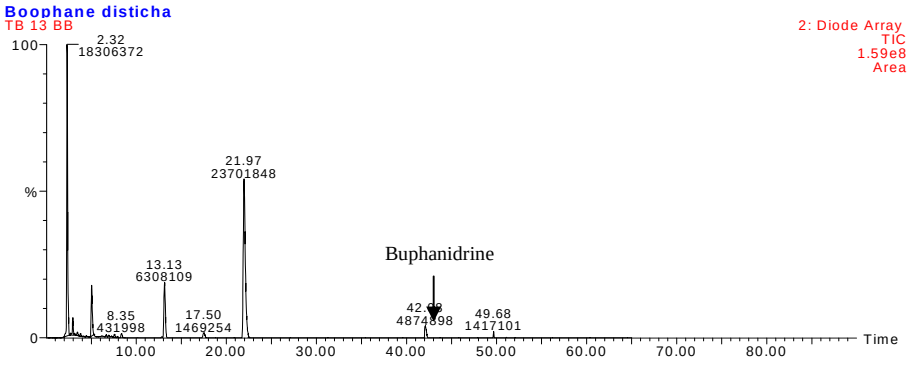


Figure 5.5: UV chromatogram of the water extract after 2 hour of boiling, retention time of Buphandrine (3) at 42.08 minutes

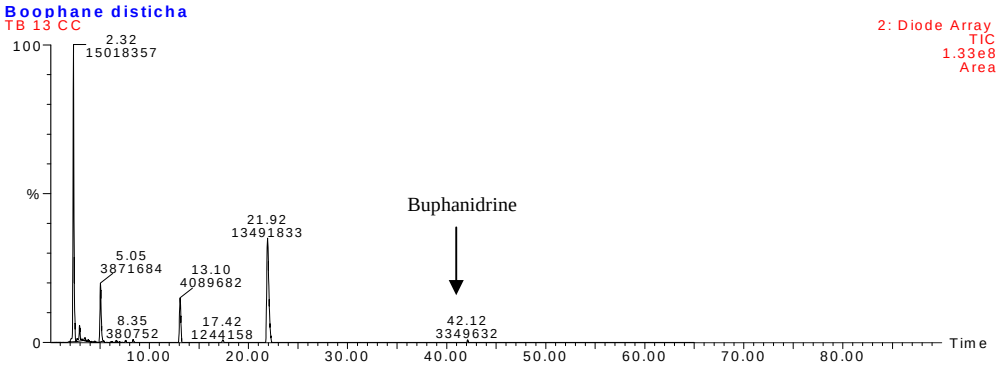


Figure 5.6: UV chromatogram of the water extract after 3 hour of boiling, retention time of Buphandrine (3) at 42.12 minutes

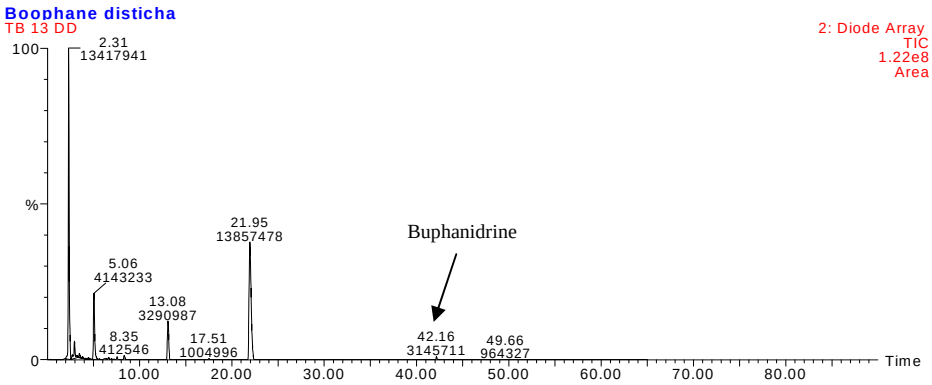


Figure 5.7: UV chromatogram of the water extract after 3.5 hour of boiling, retention time of Buphandrine (3) at 42.16 minutes

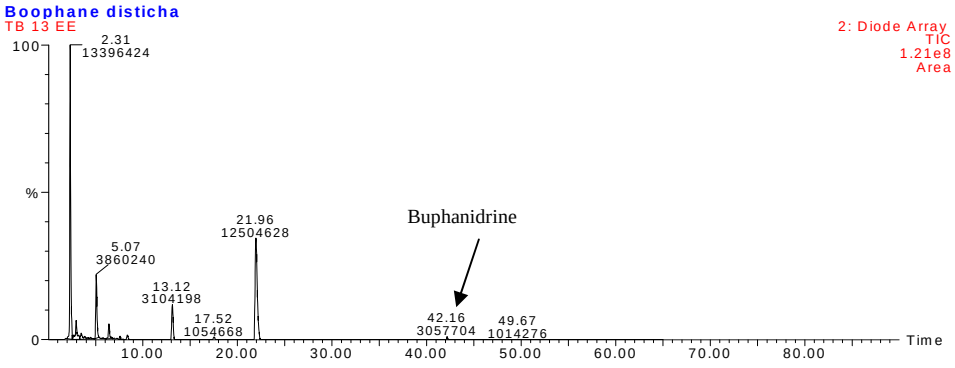


Figure 5.8: UV chromatogram of the water extract after 4 hour of boiling, retention time of Buphandrine (3) at 42.16 minutes

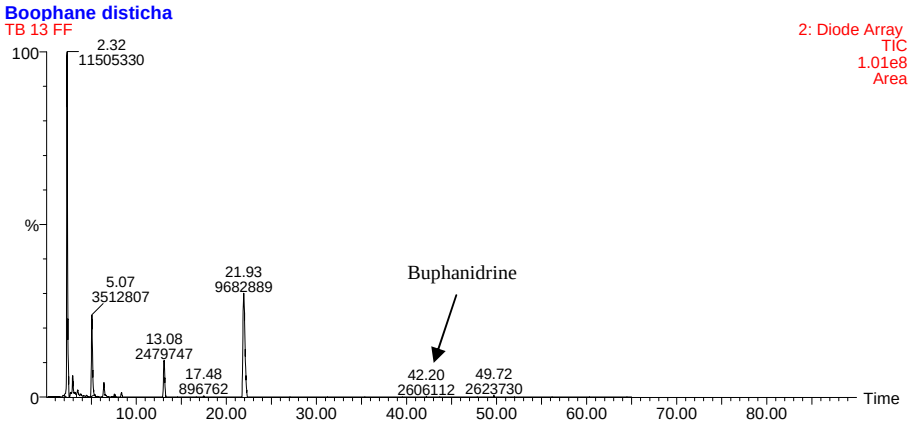


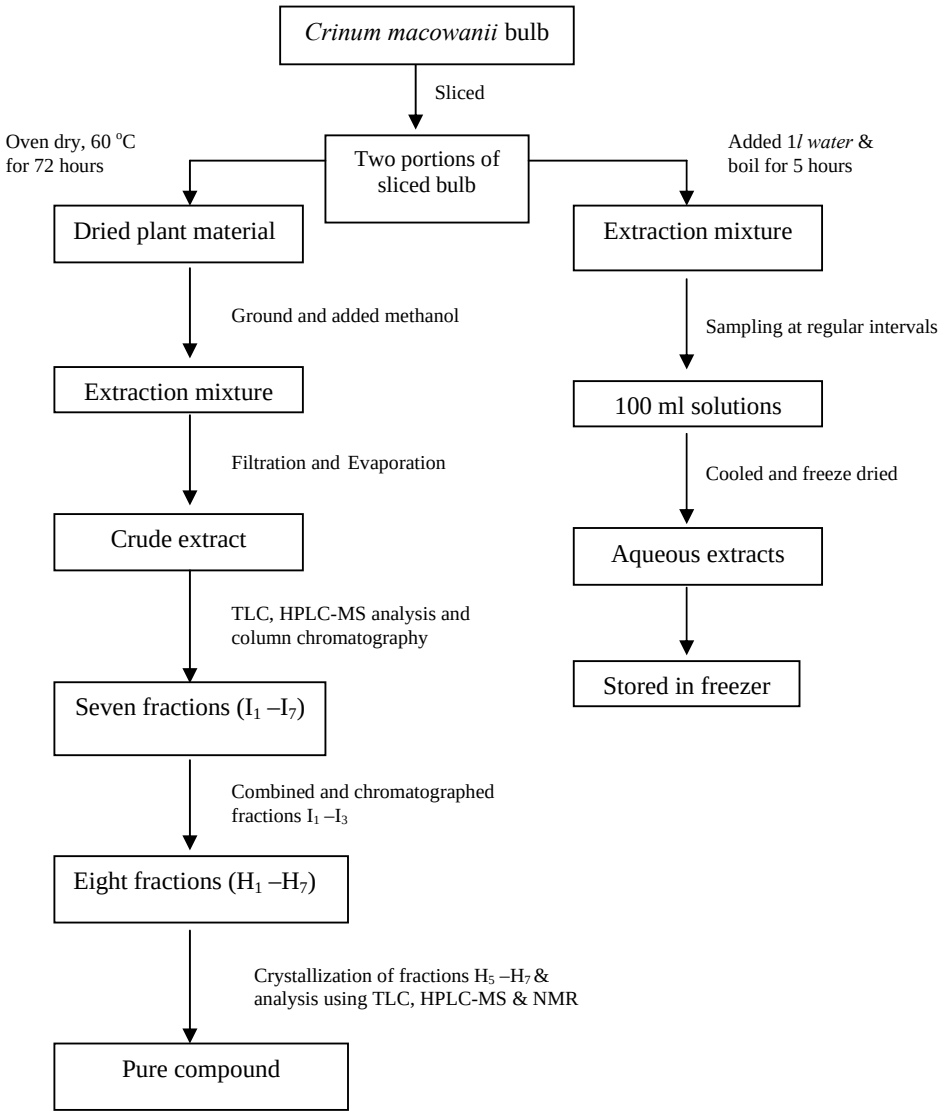
Figure 5.9: UV chromatogram of the water extract after 5 hour of boiling, retention time of Buphandrine (3) at 42.20 minutes

5.2. Extraction of compounds from *Crinum macowanii*

5.2.1. Preparation of the bulb of *Crinum macowanii* for analysis

The fresh bulbs of *Crinum macowanii* were sliced, separated into two portions and one portion oven-dried at 60 ^{o}C for 72 hours. The dried material was then ground and the fine powdered material was extracted with methanol. After filtration, the methanol was evaporated under vacuum and the crude extract was stored for analysis and further processing. Another portion of the sliced bulbs was boiled for five hours while taking samples at regular intervals.

Scheme 2: Extraction and purification scheme of *Crinum macowanii*



5.2.2. Analysis of the crude extract of *Crinum macowanii.*

The crude extract obtained from the extraction of the processed plants with methanol was analyzed using thin layer chromatography (TLC) to establish the presence of alkaloids and other compounds. The sample was applied to two TLC plates with chloroform: ethyl acetate: methanol (4:4:2, v/v/v) as the mobile phase. The two TLC plates were visualized under a UV at 366 nm, which indicated several compounds that were visible at this wavelength. One TLC plate was sprayed with Dragendorf reagent, which showed orange spots indicative as a positive test for alkaloids[76]. The crude extract was also analyzed using HPLC-MS and the figures 5.10 (A and B) shows the UV absorption spectrum and the total ion chromatogram, respectively. From these chromatograms a complex mixture of compounds was observed and no conclusive results could be obtained and it was decided to purify the extract.

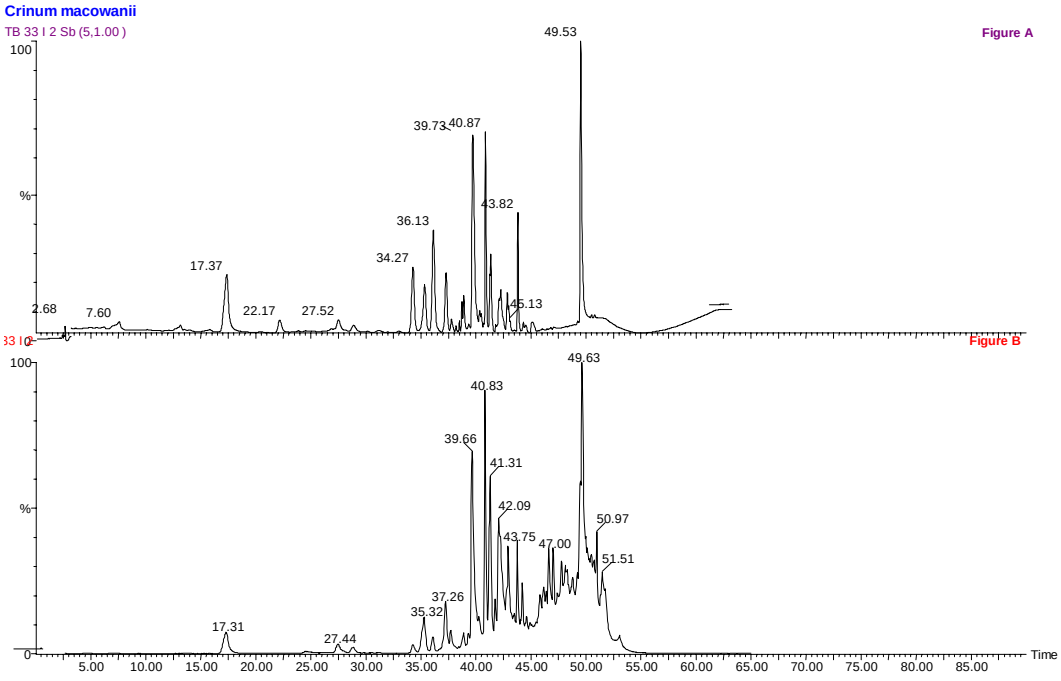
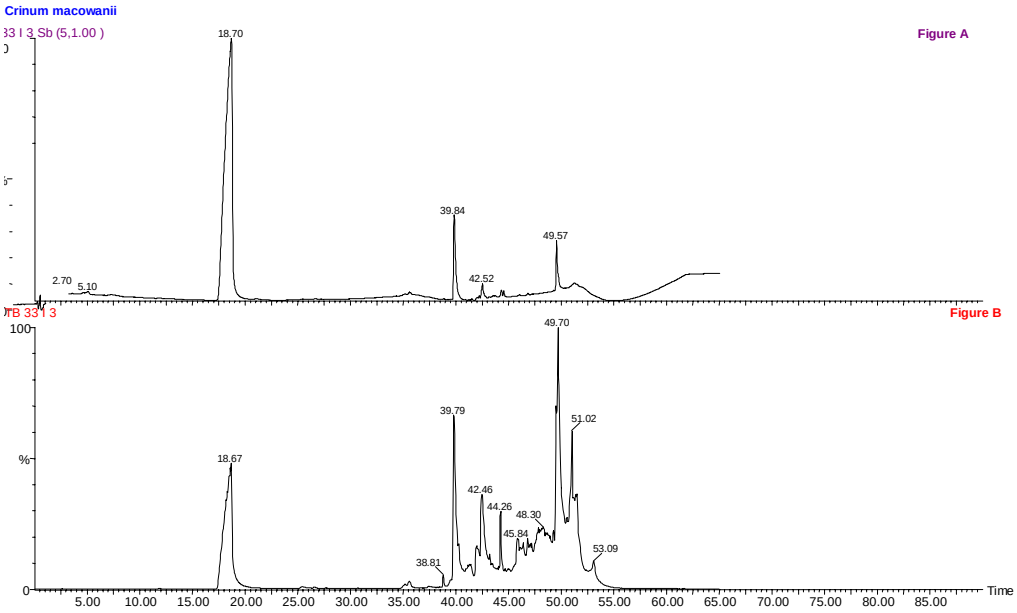


Figure 5.10 (A and B): The UV maximum plot (Figure A) and total ion chromatogram (Figure B) of the crude *Crinum macowanii* extract

5.2.3. Separation and analysis of compounds from the methanol extract

The methanol extract was chromatographed on silica gel with chloroform/ethyl acetate/methanol (4:4:2, v/v/v) as the eluant and seven fractions were collected separately based on their polarity. These fractions were analyzed using two TLC plates with chloroform/ethyl acetate/methanol (4:4:2) as the eluant. The two TLC plates were initially visualized under UV at 366 nm and were sprayed separately with cerium sulphate and Dragendorf reagents. Three fractions displayed compounds with an orange color when sprayed with Dragendorf reagent indicative of alkaloids. These three fractions were then combined and re-chromatographed on silica gel with chloroform/ethyl acetate/methanol (4:4:2, v/v/v) as the eluant. TLC was used to monitor the efficiency of the purification. A white compound crystallized from one of the fractions and was filtered off.

The crystals were dissolved in methanol and the resulting solution was analyzed using TLC and only one compound was visualized under UV at 366 nm and when sprayed with Dragendorf reagent, an orange colour was observed indicating the presence of an alkaloid. The sample was also analyzed using HPLC-MS with a gradient mode starting with ammonium acetate/methanol (9:1) on a C18 reverse phase silica column and the mass detector operating on the electrospray positive mode. The UV maximum plot and the total ion chromatogram of the sample are shown in figures 5.11 (A and B), respectively. The major compound was found to have the retention time of 18.70 minutes in the UV chromatogram and the equivalent in the total ion chromatogram at 18.76 minutes. The UV maximum absorption and molecular ion for this compound are given in Table 5.5.



Figures 5.11 (A and B): The UV maximum plot (Figure A) and the total ion chromatogram (Figure B) of the fraction isolated.

Table 5.5: The UV and MS data obtained from HPLC-MS analysis of purified compound in 5.2.3

Retention Time (in minutes)	UV maximum absorption(nm)	m/z (M +1)
18.70 (UV chromatogram)	204 and 289	
18.67 (total ion chromatogram)	-	288

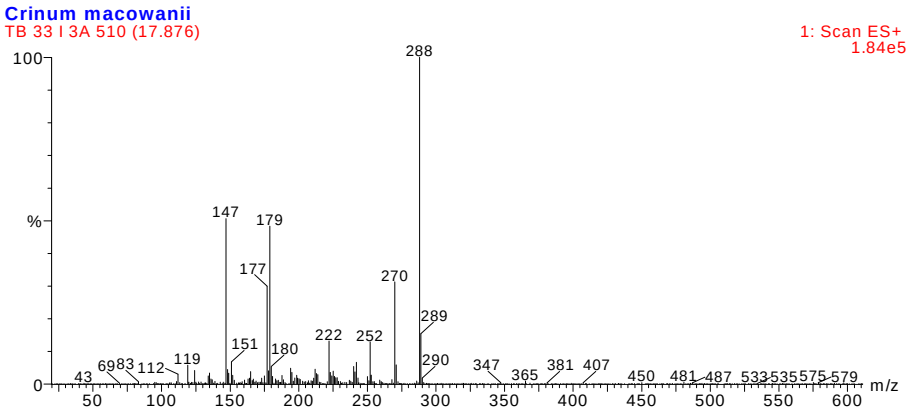
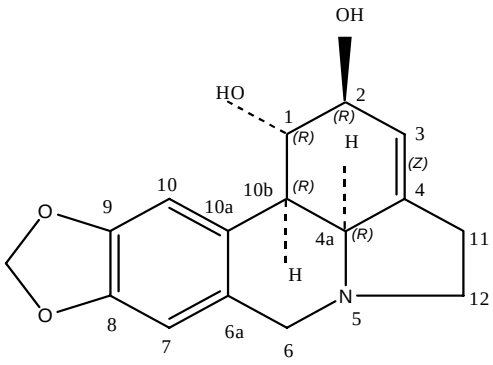


Figure 5.12: The positive ion electrospray mass spectrum of Lycorine

The UV maximum absorption for the Buphanidrine (3) (214 nm and 286 nm) are close to that of the isolated compound (204 nm and 289 nm) suggesting that the two compounds might be related and or might belong to the same class of compounds. Based on the mass spectrum of the compound indications are that this compound isolated was Lycorine (5), an alkaloid which has been reported to be isolated from other *Amaryllidaceae* family species[47,48,78,79,81].

The mass spectrum of the compound isolated is shown in figure 5.12 and has a molecular ion (M+1) at m/z 288, the same as that of Lycorine[49]. The mass spectrum indicates the fragment at m/z 270 (M+1-18) is associated with a loss of a water molecule indicative of the presence of a hydroxy group. A further fragment at m/z 252 (M+1-36) represents the loss of a second water molecule. These indicate the presence of two hydroxyl groups in the compound providing further evidence that the compound is Lycorine (5).



Lycorine (5) m/z = 287

The ^{1}H NMR and ^{13}C NMR of the purified compound were obtained to confirm its structure and the spectra were compared to that in literature[49,80,82,83,84] c.f. Tables 5.6 and 5.7. The presence of methylenedioxy group at C-8 and C-9 was indicated by the methylene proton signal at δ 5.90 and δ 5.89 (1 H, each singlet) and the presence of the aromatic protons (H-7 and H-10) occurred at δ 6.62 and δ 6.975 (both singlets) in

the ^{1}H NMR spectrum. The ^{13}C NMR spectrum revealed that the sixteen resonances are due to the presence of the four methylene, the seven methine and five quaternary atoms. Tables 5.6 and 5.7 below show the chemical shifts of the respective protons and carbons atoms from the NMR analysis.

The discrepancy in chemical shifts between the NMR data and that reported in the literature (cf tables 5.6 and 5.7) could be attributed to the use of different solvents for the analysis. Methanol was used as the solvent for the NMR analysis of the pure compounds as described in literature[81] while in our experiments chloroform was used. The other reason could be that the authors used a 270 MHz NMR instrument for the analysis as described in literature[81] while in our case a 400 MHz NMR instrument was used.

Table 5.6: ^{1}H NMR chemical shifts assignment of Lycorine

Hydrogen Atom	**Isolated Product, NMR in $CDCl_3$ δ_H /ppm (*J* (HH)/H_z)**	**Literature data of Lycorine C[81] in CD_3OD δ_H /ppm (*J* (HH)/H_z)**
1	4.22 (*dd*, *J* = 1.6, 1.3)	4.58 (*dd*, *J* = 2.2, 1.1)
2	3.98 *m*	4.26 *m*
3	5.32 (*br s*)	5.77 (*br s*)
4_a	3.29 (*d*, *J* = 11.8)	3.95 (*d*, *J* = 11.8)
6	3.97 *m*	4.48 *d* & 4.19 *d* (*J* = 2.2, 1.1)
6α	-	-
7	6,62 *s*	6.80 *s*
10	6,97 *s*	6.98 *s*
10_b	2,45 (*dd*, *J* = 11.5, 2.7)	2.99 (*dd*, *J* = 11.8, 2.2)
11	2,37 *m*	2.88 *m*
12	3.16 *m* & 2.92 *m*	3.75 *m* & 3.49 *m*
-O-CH_2-O-	5,90 *s* & 5,89 *s*	5.95 *s* & 5.95 *s*

Table 5.7: The ^{13}C NMR chemical shifts assignment of Lycorine

Carbon Atom	Isolated Product (Lycorine) in $CDCl_3$ δ_c /ppm	Literature data for Lycorine [81] in CD_3OD δ_c /ppm
1	70,2 D	70.1 *D*
2	71,7 *D*	71.9 *D*
3	118,5 *D*	122.9 *D*
4	141,7 *S*	137.9 *S*
4_a	60,7 *T*	61.8 *T*
6	56,7 *T*	54.2 *T*
6_a	129,7 *S*	130.6 *S*
7	106,9 *D*	108.8 *D*
8	145,1 *S*	149.7 *S*
9	145,6 *S*	148.1 *S*
10	105,0 *D*	106.4 *D*
10_a	129,7 *S*	125.7 *S*
10_b	40,1 *D*	38.2 *D*
11	28,1 *T*	30.3 *T*
12	53,2 *T*	55.1 *T*
-O-CH_2-O-	101.7 *T*	102.8 *T*

The NMR and mass spectral data provide sufficient evidence that the compound isolated is Lycorine (5). The compound Buphanidrine (3) with the molecular mass of 315 and the retention time of 42 minutes was also detected in this plant using the HPLC-MS through a selected ion scan. However this compound has not been reported to be isolated from the plant. This compound was previously discussed in the section of *Boophane disticha*.

5.2.4. Aqueous extraction of compounds from *Crinum macowanii*

Traditional medicines are often prepared by boiling plant material in water for different time periods as determined by the traditional practitioner. The purpose of this study was to attempt to mimic the traditional preparation in the laboratory and to chemically analyse the resulting extracts. The extraction was done by boiling the wet bulbs cut into small pieces in a litre of water over five hours while samples were taken for analysis at regular intervals. The aqueous samples were freeze-dried and stored in a freezer.

Each of the freeze dried samples (results shown in table 5.8) was dissolved in distilled water and analysed using HPLC-MS. The compound Lycorine (5) was identified in the corresponding extracts at retention time 17.88 minutes in the UV chromatogram. The molecular mass of 287 for this compound was confirmed earlier; with a pseudo molecular ion (M+1) indicated at 288 in the positive electrospray mass spectrum using the mass detector and is shown in figure 5.12. The area under the peak in the UV chromatogram that corresponded to Lycorine (5) in each of the extracts taken at the different time points indicated that there was a decrease in this area. This decrease points to a reduction in the concentration of Lycorine during the extraction with increased boiling times, however as an internal standard was not used for the analysis these results are non conclusive but only indicative of this trend. The decrease in the peak area of Lycorine in the UV chromatogram may also be due to dilution of the compound as more of water-soluble compounds or materials are extracted as the boiling continued.

Table 5.8: HPLC results for aqueous samples of *Crinum macowanii*

Sample labels	Sampling time (in hours)	Retention time (in minutes)	Peak area of Lycorine from UV chromatogram
TB 33 A	1	17.88	6720613
TB 33 B	2	17.79	5522394
TB 33 C	3	17.82	5185160
TB 33D	3.5	17.88	4715225
TB 33 E	4	17.88	4496630
TB 33 F	5	17.85	4079423

The UV maximum plots showing the peaks and Lycorine with retention time in the region of 17.88 minutes and their corresponding peak areas are shown in figures 5.13 – 5.18. Some of the other peaks in the UV chromatogram of the extracts of plant also showed a decrease in the peak area as the boiling continued indicating that these also decrease in concentration as boiling continues, however here again as an internal standard was not used, this trend in only indicative.

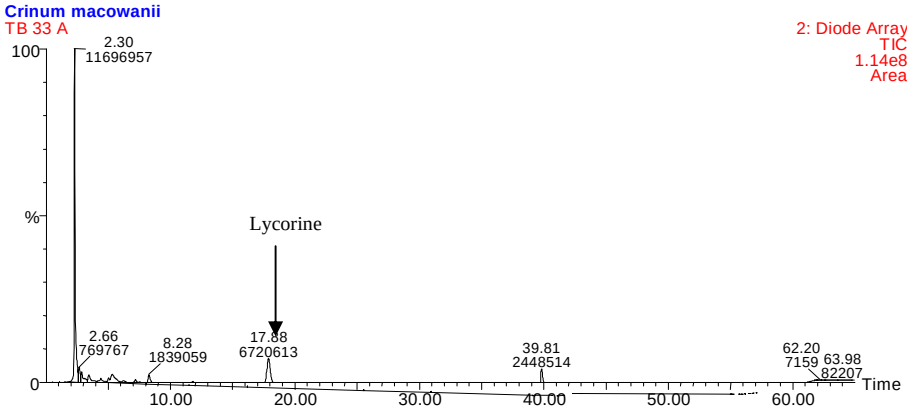


Figure 5.13: UV chromatogram from water extract after 1 hour of boiling, retention time of Lycorine at 17.88 minutes

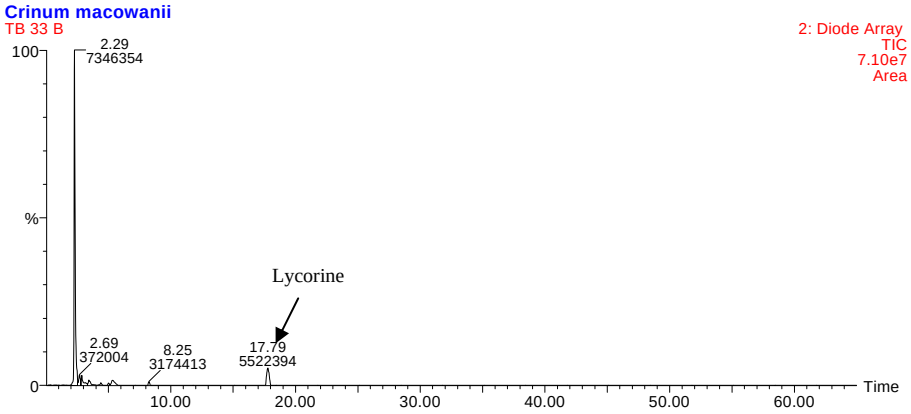


Figure 5.14: UV chromatogram from water extract after 2 hours of boiling, retention time of Lycorine at 17.79 minutes

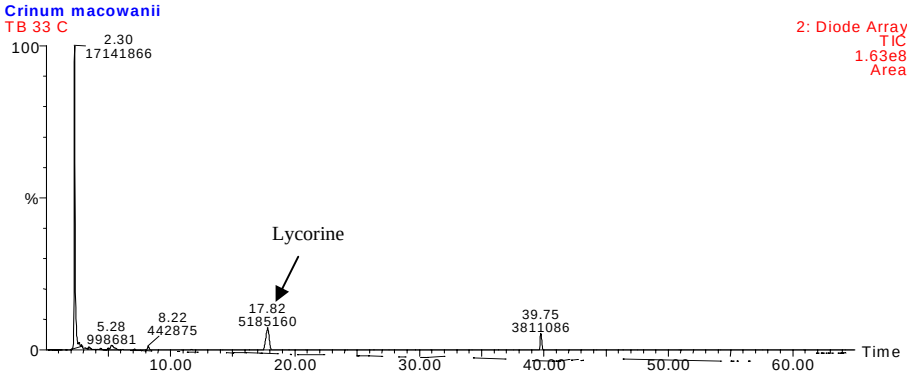


Figure 5.15: UV chromatogram from water extract after 3 hours of boiling, retention time of Lycorine at 17.82 minutes

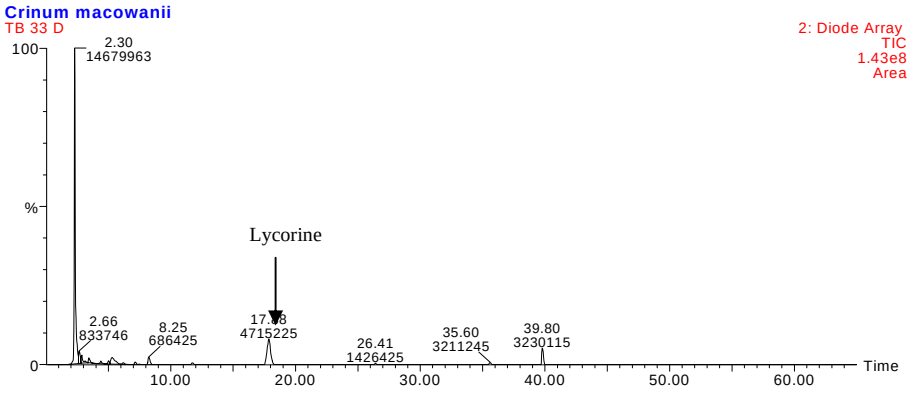


Figure 5.16: UV chromatogram from water extract after 3.5 hours of boiling, retention time of Lycorine at 17.88 minutes

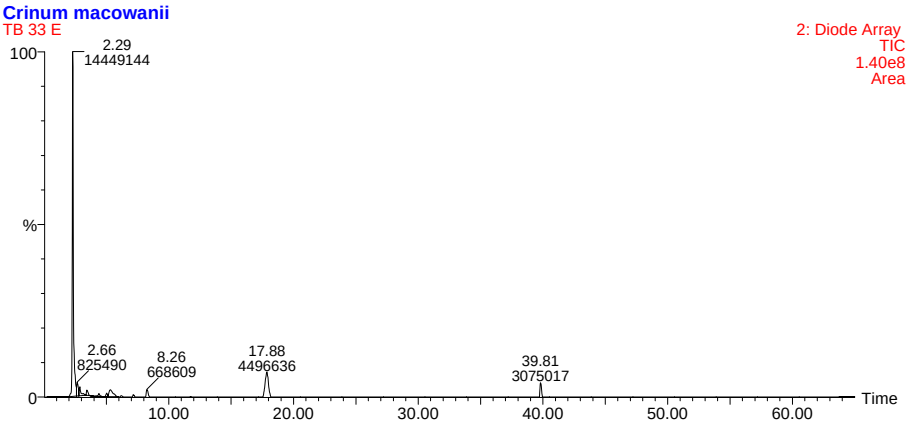


Figure 5.17: UV chromatogram from water extract after 4 hours of boiling, retention time of Lycorine at 17.88 minutes

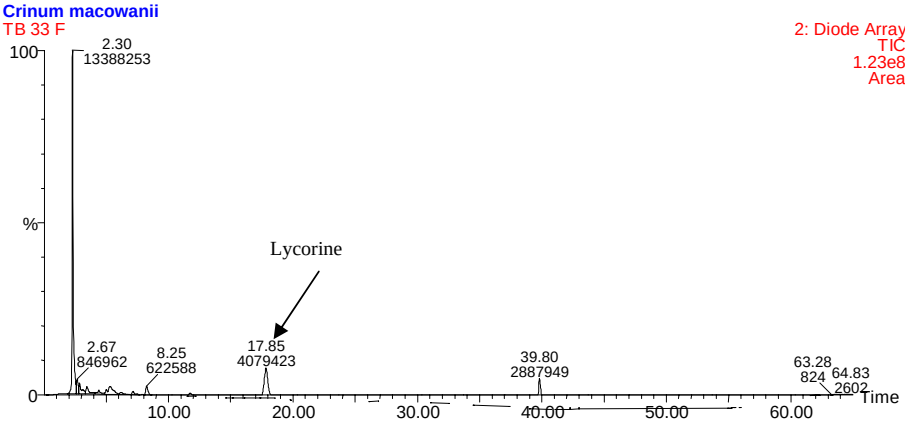


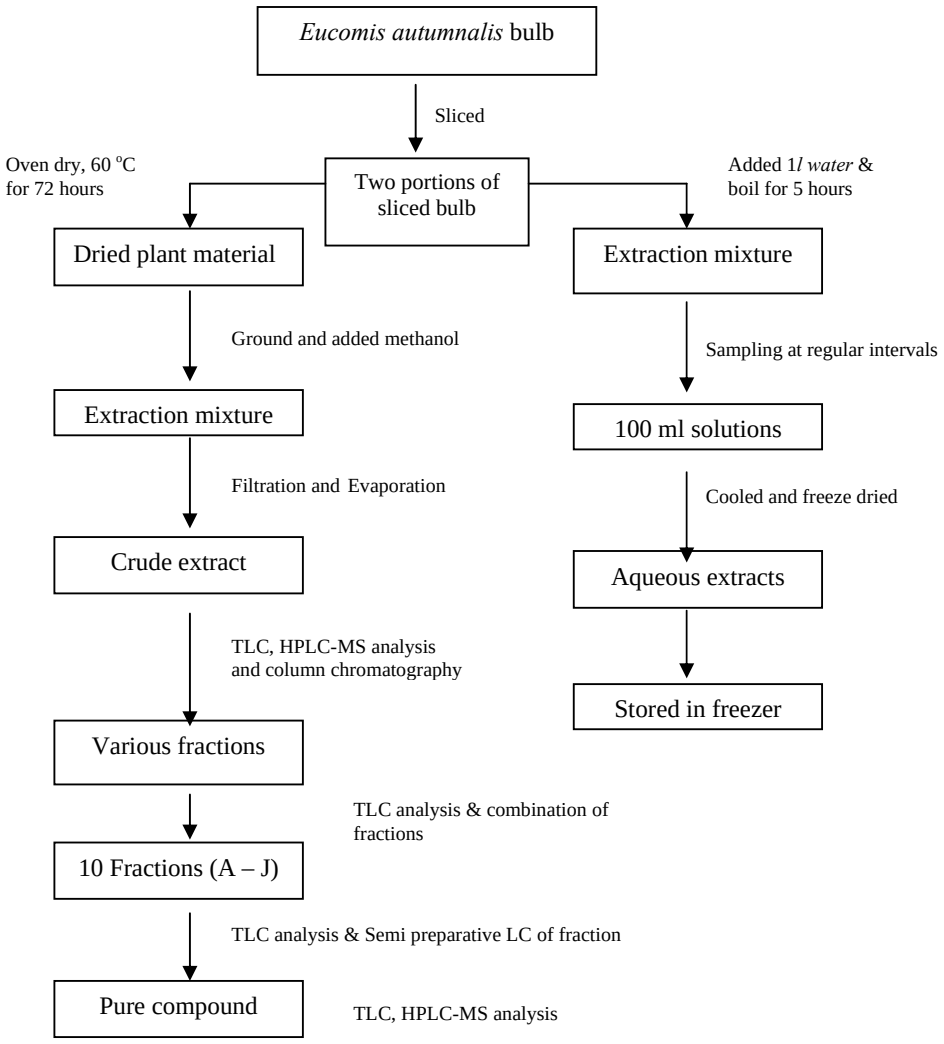
Figure 5.18: UV chromatogram from water extract after 5 hours of boiling, retention time of Lycorine after 17.85 minutes

5.3. Extraction of compounds from *Eucomis autumnalis*

5.3.1. Preparation of extracts from the bulbs of *Eucomis autumnalis*

A portion of sliced bulbs of *Eucomis autumnalis* was oven dried at 60^{o}C for 72 hours. The dried material was ground and the resulting fine powder was extracted with methanol for 48 hours. Scheme 3 illustrates the plant preparation and extraction process. Another portion of the sliced bulbs were boiled for five hours while taking samples at regular intervals for analysis.

Scheme 3: Extraction and purification scheme of *Eucomis autumnalis*



5.3.2. Purification and analysis of the crude methanol extract

The methanol extract was chromatographed by column chromatography using silica gel as the stationary phase and hexane/ethyl acetate/methanol (4:4:2, v/v/v) as the eluant. The fractions obtained were analysed by TLC using the same eluant used for column chromatography and the developed TLC plates were visualised under UV (at 366 nm), sprayed with a solution of cerium sulphate in sulphuric acid. Compounds showed a yellow colour when the plate was sprayed with this solution and baked in an oven. The methanol extract was also analysed using HPLC-MS, with the HPLC operating on a gradient mode starting with water/acetonitrile (9:1, v/v) and ending with water/acetonitrle (0:10, v/v) over 90 minutes. The MS was changed from positive ESI mode to negative ESI mode as no peaks were detected in the positive ESI mode. The resulting UV max and total ion chromatograms are given in figures 5.19 (A and B) and illustrate that the crude extract contained several compounds.

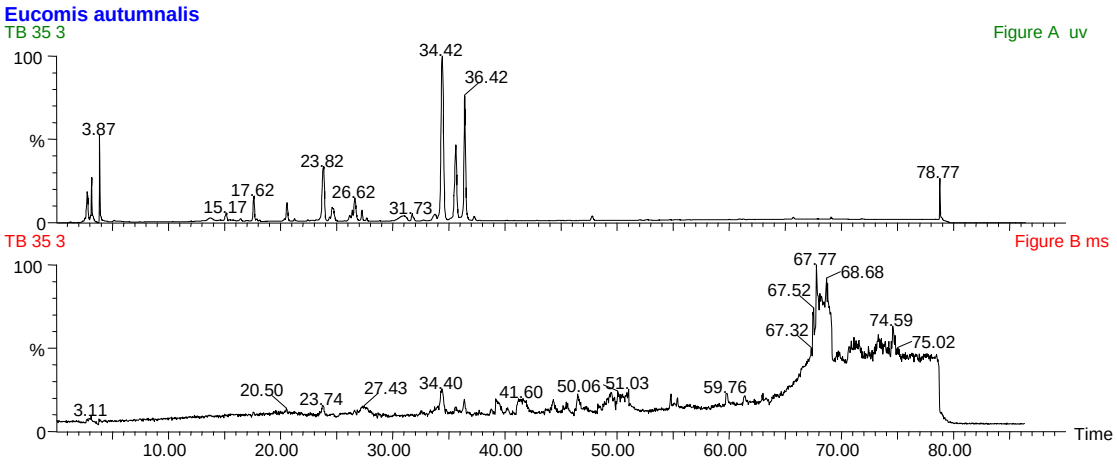


Figure 5.19 (A and B): The UV (Figure A) and total ion chromatograms (Figure B) of crude *Eucomis autumnalis* extract (negative electrospray mode)

The fractions obtained from column chromatography were impure and were further chromatographed on silica gel using the same solvent system as described earlier. A number of fractions were collected, analysed using TLC and combined into 10

fractions (labelled A-J). Fraction B was further chromatographed on silica gel using the same mobile phase as the one above. The third fraction that was obtained from the second purification was analysed using TLC, which showed one yellow spot after spraying cerium sulphate and baking in the oven at 60^{o}C. HPLC-MS analysis of this fraction using reverse phase as the stationary phase indicated four major peaks in the UV max and TIC chromatograms (figure 5.20 (A and B)).

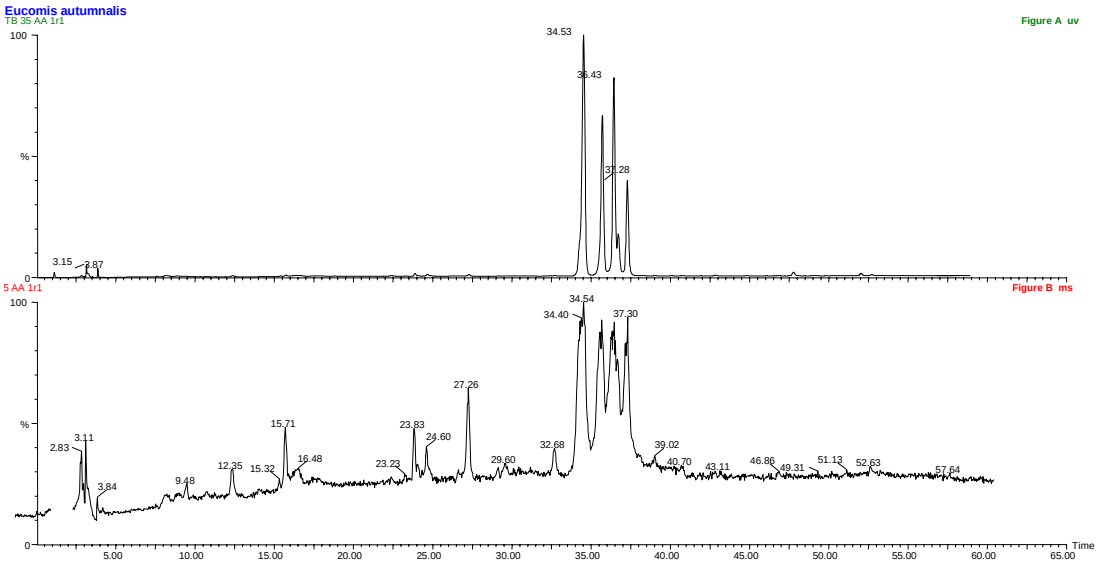
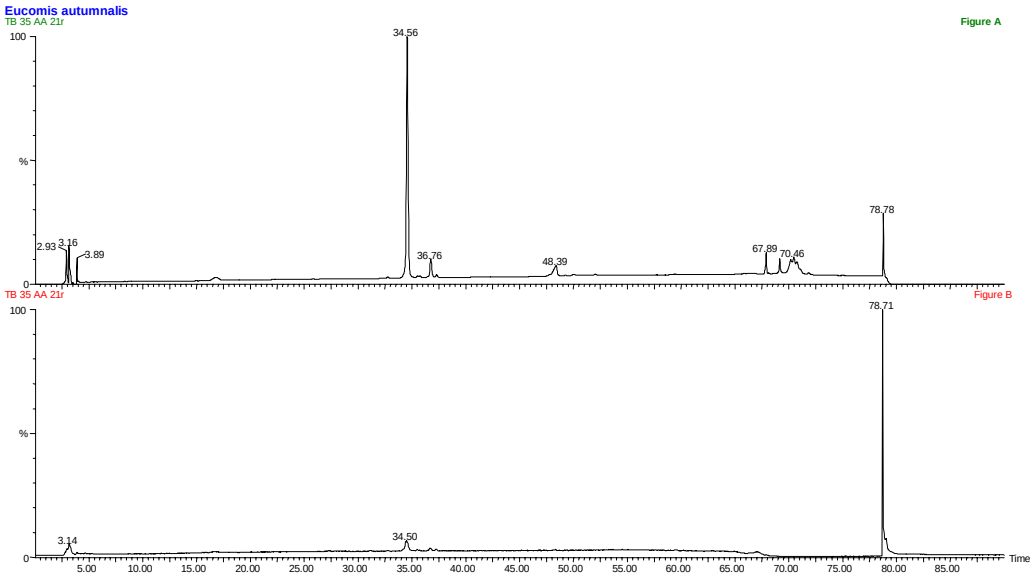


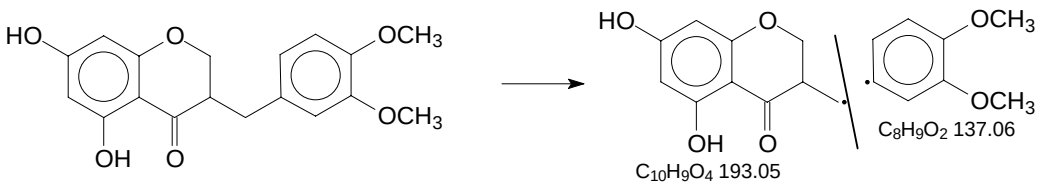
Figure 5.20 (A and B): The UV max plot (Figure A) and total ion chromatograms (Figure B, negative ESI mode) of the rechromatographed fraction

A preparative HPLC with C-18 reverse phase silica as the stationary phase and methanol: water (9:1, v/v) as an isocratic eluant was used for the purification of the fraction containing the four compounds. This resulted in the isolation of a pure compound with retention time of 34.56 minutes. This compound was analysed using TLC which showed a single compound. Further HPLC-MS analysis of this compound showed one major peak as can be seen on the UV maximum plot and the total ion chromatogram in figures 5.21 (A and B).

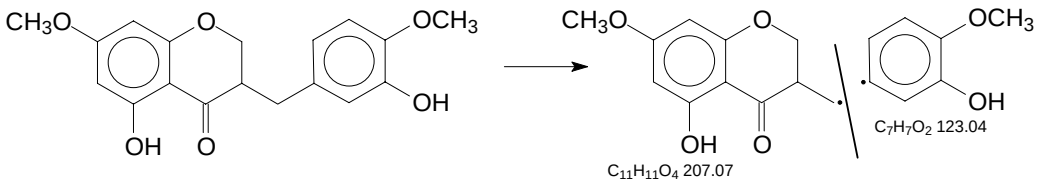


Figures 5.21 (A and B): The UV (Figure A) and total ion chromatograms (Figure B) negative ESI mode) for the pure compound (at 35 minutes retention time)

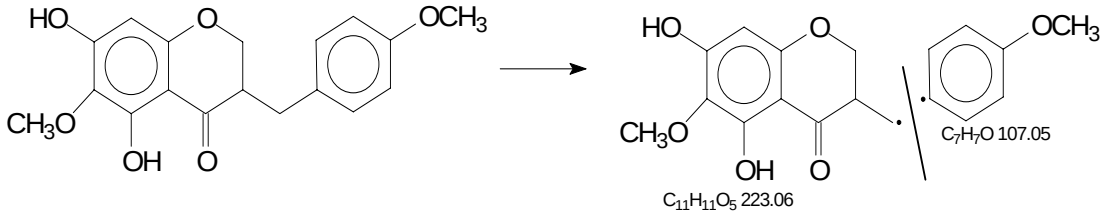
The negative ESI–MS of the compound showed the quasi molecular ion peak with m/z 329 (M-1). This could be related to compound 25 as described in table 1 of chapter 4, 3-(3,4-Dimethoxybenzyl)-5,7-dihydroxychroman-4-one with molecular mass 330 that was previously isolated from *Eucomis autumnalis* (*Hyacinthaceae*)[69]. However from literature[72] there are three compounds in this plant with the molecular mass of 330. The structures of these compounds with molecular weight 330 are shown as Compounds 25-27. The mass spectrum was used to provide information on the molecular weight of the isolated compound and the UV (figure 5.22(A)) of this compound shows the main broad absorption at 292 nm.



Compound 25: 3-(3,4-Dimethoxybenzyl)-5,7-dihydroxychroman-4-one, m/z = 330



Compound 26: 3-(4-Hydroxy-5-methoxybenzyl) - 5-hydroxy -7-methoxychroman-4-one, m/z = 330



Compound 27: 5,7-Dihydroxy-6-methoxy-3-(4-methoxybenzene) chroman-4-one, m/z = 330

The MS spectrum given in figure 5.22 (B) of the purified compound shows the loss of the fragment 137 at m/z =193 which corresponds to the loss of the 3,4-dimethoxybenzyl fragment equivalent to compound 25. The presence of the peak at m/z = 193 points to the presence of the chromanone unit of the molecule (cf Figure compound number 25) and the presence of the peak at m/z = 314 represents the loss of a CH_3 group (from the methoxy group) from the quasi-molecular ion. The absence of peaks at m/z = 207 and m/z = 223 (cf compound number 26 and 27 respectively) exclude these two compounds and point to the fact that the isolated compound has the structure of compound 25. Compounds with the structures similar to those found in table 1 in chapter 4 are known be homoisoflavanoids and have been reported to be present in the bulbs of *Eucomis autumnalis*[71]. Similar compounds were also isolated from the bulbs of *Scilla* spicies[72,74].

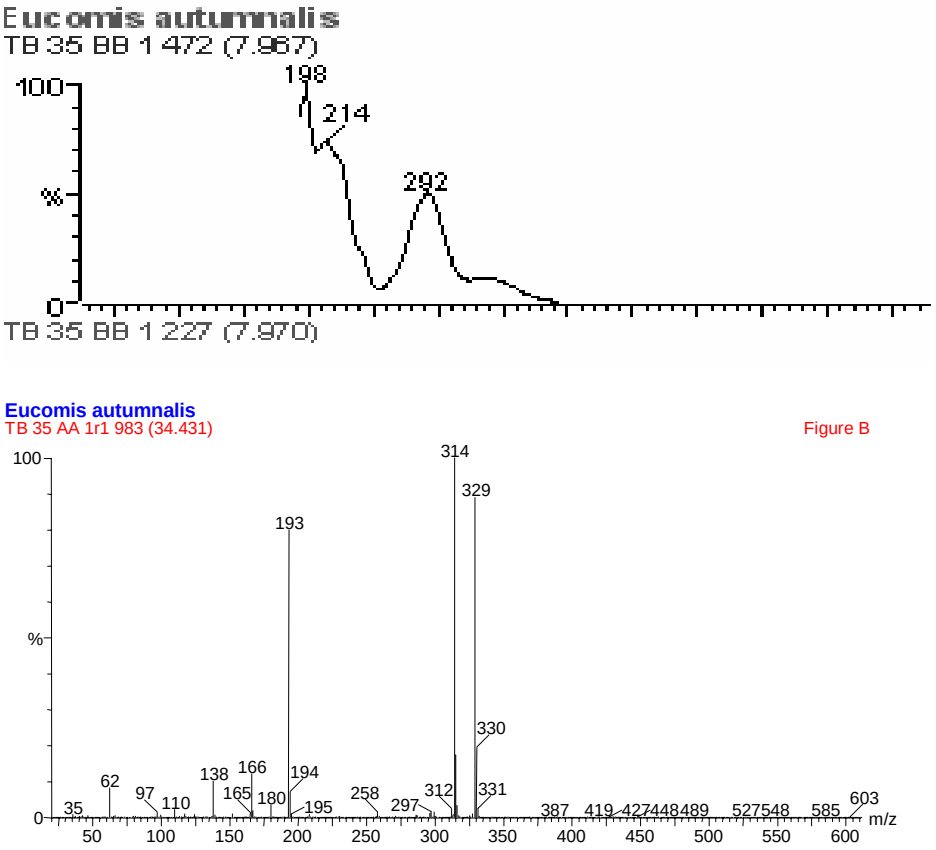


Figure 5.22: The UV spectrum and negative ion ESI mass spectrum of peak at retention time 34.50 minutes

5.3.3. Aqueous extraction of compounds from *Eucomis autumnalis*

The traditional preparation of plants is more often done by adding water to plant material and boiling for several hours. An aqueous extraction was prepared to try and mimic this preparation in the laboratory and it involved boiling the bulbs over a period of time to determine the levels of the Compound 25: 3-(3,4-Dimethoxybenzyl)-5,7-dihydroxychroman-4-one at different time intervals. The samples taken at different time intervals were freeze-dried and analysed using HPLC-MS with the HPLC operating on a gradient mode starting with water/acetonitrile (9:1, v/v) and ending with water/acetonitrile (0:10, v/v) over 90 minutes. Results from the analysis of the samples using HPLC MS are shown in Table 5.9. The results show that a general decrease in the peak area corresponding to compound 25 was observed as the

boiling continued, however as a internal standard was not used these results can be regarded and indicative of the trend. The figures 5.23 to 5.28 shows the UV maximum chromatogram as the boiling proceeded and illustrated the decrease in concentration of compound 25 at the retention time of approximately 34 minutes. As the traditional preparation requires a boiling period of up to three hours, this could mean that the levels of the compounds in the aqueous extract reaches concentration levels which are deemed safe and effective for the traditional use at that stage. This could be one way of traditionally getting the right dosage for medicinal usage.

Table 5.9: HPLC analysis results for aqueous samples of *Eucomis autumnalis*

Sample no	Sampling time (hours)	Retention time (in minutes)	Peak area of Compound 25 from UV chromatogram
TB 37 A	1	34.62	7282679
TB 37 B	2	34.62	4047709
TB 37 C	3	34.66	3029114
TB 37 D	3.5	34.66	2742688
TB 37 E	4	34.63	2117969
TB 37 F	5	34.67	844650

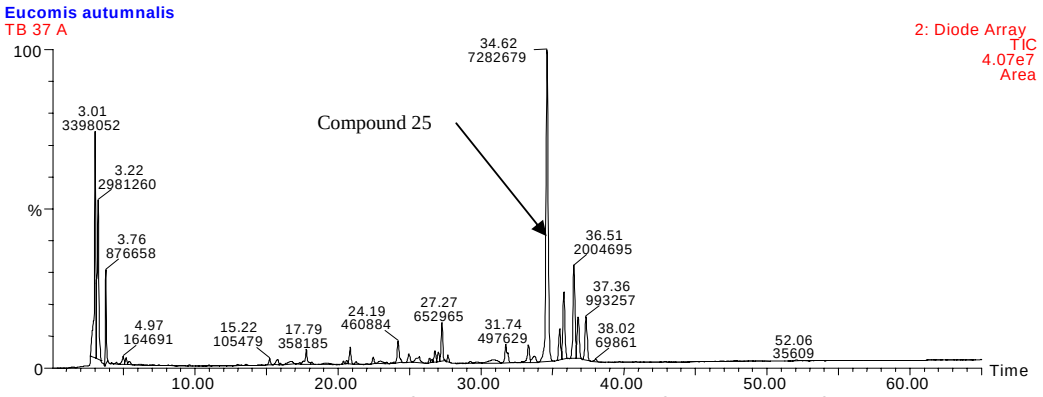


Figure 5.23: UV chromatogram from water extract after 1 hour of boiling, retention time of Compound 25 at 34.62 minutes

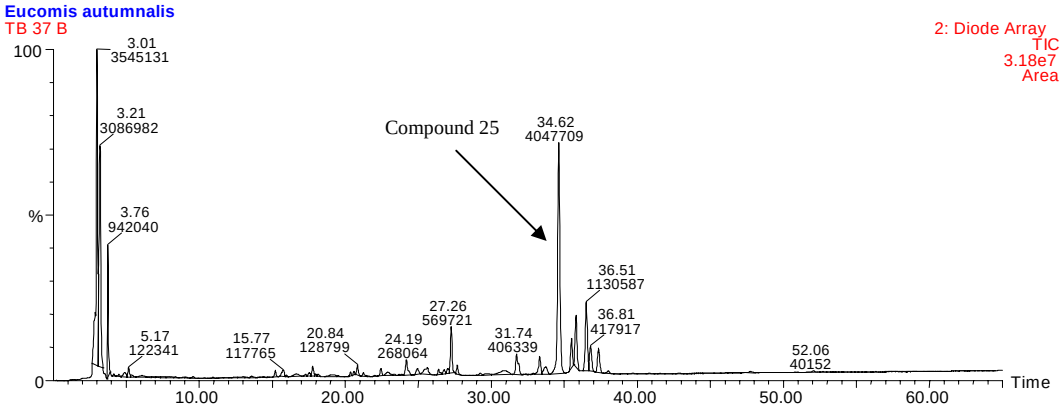


Figure 5.24: UV chromatogram from water extract after 2 hour of boiling, retention time of Compound 25 at 34.62 minutes

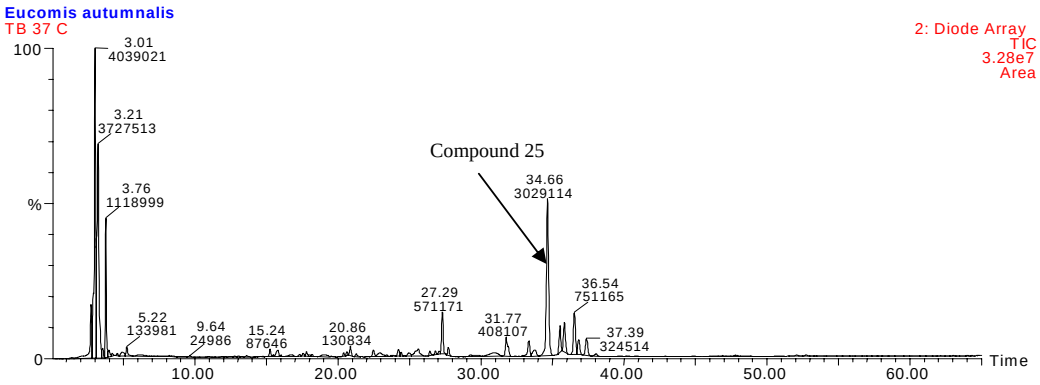


Figure 5.25: UV chromatogram from water extract after 3 hour of boiling, retention time of compound 25 at 34.66 minutes

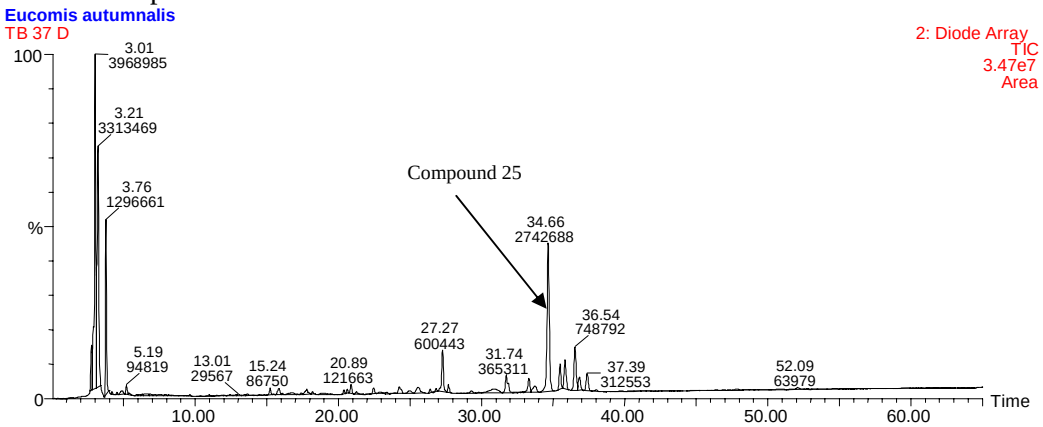


Figure 3.26: UV chromatogram from water extract after 3.5 hour of boiling, retention time of Compound 25 at 34.62 minutes

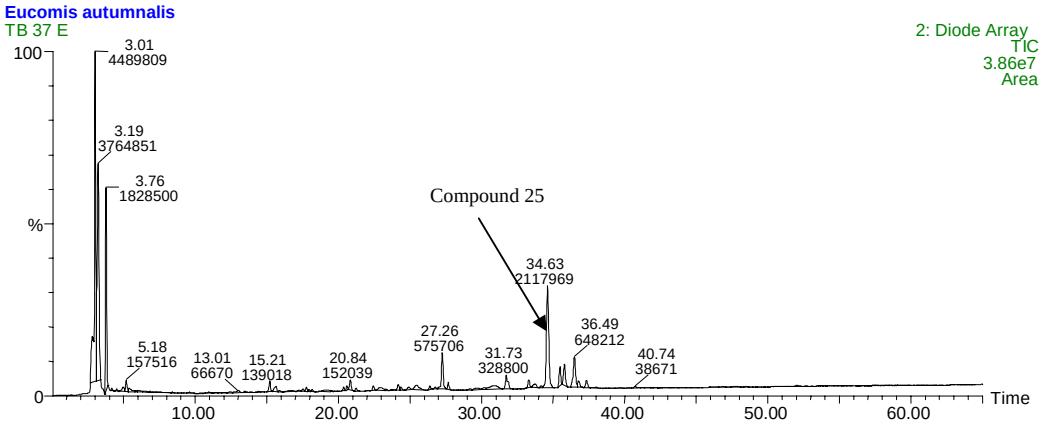


Figure 5.27: UV chromatogram from water extract after 4 hour of boiling, retention time of Compound 25 at 34.63 minutes

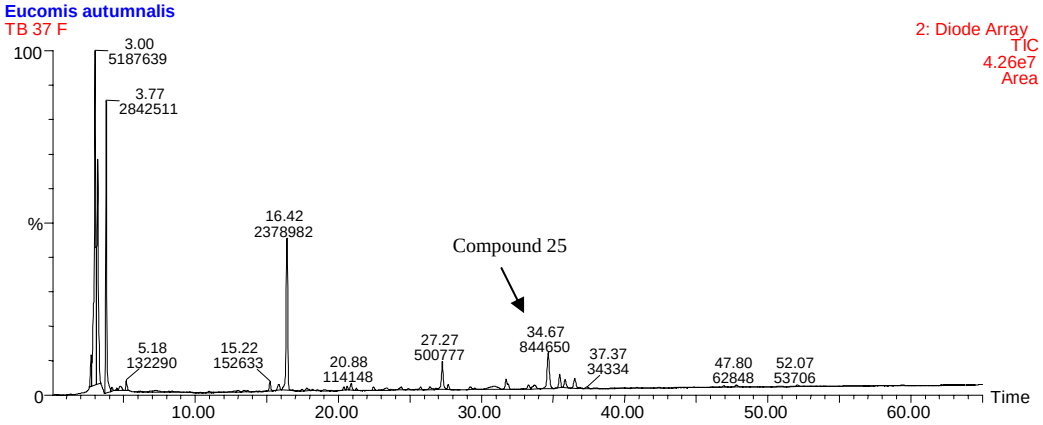


Figure 5.28: UV chromatogram from water extract after 5 hour of boiling, retention time of Compound 25 at 34.67 minutes

Chapter 6

6. CONCLUSION

Methods used for extract preparation of the bulbs from three plants involved slicing, drying the sliced plants in the oven, extraction of dried materials with solvents and boiling of fresh bulbs in water for five hours while collecting samples at different time intervals. These two methods can affect the chemical composition in the resulting extracts as the extraction of the compounds is dependant on the polarities of the solvents. Harvesting is also important because any species may display a variation within its chemical constituents due to the environmental conditions under which it is grown and the period in its life history when collection took place. In our study all compounds reported in previous studies to be present in the plants were identified and confirmed. This meant that the choice of methods for extracts preparation was found to be effective and stable to the compounds.

Chromatography played an important role by providing information on different fractions during the different steps of extract processing and purification. Column chromatography, which is one of the oldest separation methods, was used for the purification of the compounds from the crude extracts and thin layer chromatography provided information on the purity of the fractions and effectiveness of the separation. The use of the modern chromatographic techniques *viz*. HPLC-MS provided additional information for monitoring of the separation. The UV photodiode array detector was able to guide the separation process by detecting compounds which possess a UV chromophore while the MS was able to give molecular mass

information for the compounds of interest even if these did not possess a UV chromophore. This demonstrates the added advantage of MS linked to a purification system. For the analysis of *Boophane disticha* and *Crinum macowanii*, ammonium acetate was used as a buffer in the mobile phase. This was necessary in reverse phase chromatography using the HPLC, as the retention of analytes is related to their hydrophobicity and the active compounds in these two bulbs are known to be alkaloids that are weak bases. The more hydrophobic the analyte, the longer it is retained and hence a buffer is needed to control the degree of ionisation (protonation) of the alkaloids. Acids lose a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases. Therefore, when separating mixtures containing acids and/or bases by reversed phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible results. A gradient method with a mobile phase consisting of acetonitrile and water was used for the analysis of compounds in *Eucomis autumnalis.* The active compounds found in this bulb were known to be very weak acids (homoisoflavanoids) and there was no need to use buffer.

The MS chromatogram obtained from both the negative and positive modes of the electrospray ionization gave information on the molecular mass of the compounds in the plants. Both the negative and positive electrospray modes of ionization do not provide a consistent fragmentation pattern similar to that given by the electron impact ionization. ESI is preferred for compounds which are ionic or very polar or thermo labile, or with masses higher than 1000 and APCI is preferred for compounds which are not very polar. The choice of the ionisation technique used depended on the type of compounds investigated. Polar compounds are easily analyzed with ESI, less polar

with APCI, volatiles with APCI, non-polar better done with GC/EI. For the analysis of the compounds in *Boophane disticha* and *Crinum macowanii* a positive mode electrospray was used and for *Eucomis autumnalis,* a negative mode. The reason for this is based entirely on the type of compounds found in each bulb. Electrospray ion sources are soft ionization sources that produce mostly protonated molecular ions, MH^+ and the basic alkaloids found in both *Boophane disticha* and *Crinum macowanii* were identified using their MH^+. The molecular weight of a compound is easily determined by this technique. For small molecules, positive electrospray produces one peak, the MH^+ peak at M+1 amu. Acidic analytes are normally detected using the negative ion electrospray and the detected ion is the M-H anion, at M-1 amu. In negative-ion electrospray ionization, analytes typically deprotonate to become negatively charged and for the analysis of compounds found in *Eucomis autumnalis,* an $(M-H)^-$ peak at M-H amu was found in the mass spectrum. Peaks of lower mass were also observed when the negative mode was applied but far fewer than normally found in election impact ionisation mode.

Eleven alkaloids were previously reported[41,46-50] to be present in the bulbs of *Boophane disticha* and *Crinum macowanii*. In this study the use of HPLC-MS has for the first time been shown to be successful in identifying some of these compounds in these plants. In the case of *Boophane disticha* the presence of Buphanidrine (3) in the extracts of the bulbs was identified using HPLC-MS. In order to confirm that the technique was accurate, the compound was purified by column chromatography and its structure confirmed using NMR thereby confirming the results from the HPLC-MS. The compound was also identified in the extracts of *Crinum macowanii* mainly through the mass spectrum and retention time obtained from the HPLC-MS. This is

the first report of this compound being identified in this plant species using HPLC-MS. In the case of *Crinum macowanii* the compound Lycorine (5) was also identified in the extracts of the plant bulbs using HPLC-MS. NMR was used to confirm the presence of the compound after it was purified through column chromatography. In this case Buphanidrine (3) was identified to be present in the extract of the plant using the retention time and MS spectrum obtained from the HPLC-MS. The occurrence of this compound in *Crinum macowanii* is reported for the first time using HPLC-MS demonstrating the usefulness of this technique in identifying compounds in complex extracts without purification. The data has demonstrated that HPLC-MS is a very powerful technique in identifying compounds of interest in complex mixtures without having to go through the process of tedious purification which in this case was only used for confirmatory purposes and to prove the concept. The technique could also be useful for future standardization of these bulbs for as part of its scientific validation of their medicinal uses.

The compound, 3-(3,4-Dimethoxybenzyl)-5-hydroxy-7-methoxychroman-4-one (25) with molecular mass 330 was reportedly isolated in the bulbs of *Eucomis* species[69,71,73,74] and was shown to be present in *Eucomis autumnalis* (*Hyacinthaceae*) through HPLC-MS. The usefulness of the technique was demonstrated as two other similar compounds with molecular masses of 330 also occur in the *Eucomis* species. The compound, 3-(3,4-Dimethoxybenzyl)-5-hydroxy-7-methoxychroman-4-one (25) was identified and confirmed based on its fragmentation pattern in the mass spectrum which did not match that for the other two compounds of similar molecular mass. The HPLC-MS method developed for the analysis of extracts of *Eucomis autumnalis* can

be used for future standardization of products derived during the scientific validation of the medicinal properties of the plant.

The use of hot water as an extraction solvent at temperatures at approximately 100°C was explored in this study. In this case it was done to determine the effect of boiling on the chemical profile of the chemical constituents in the aqueous extracts of the plants. Although inert internal standards were not used for the quantitative analysis of the targeted compounds, Buphanidrine (3) from *Boophane disticha*, Lycorine (5) from *Crinum macowanii*, 3-(3,4-Dimethoxybenzyl)-5-hydroxy-7-methoxychroman-4-one (25) from *Eucomis autumnalis,* the concentrations of these compounds using the HPLC-MS appeared to decrease as the boiling was continued. This suggested that the extracts were suitable for medicinal use after some hours of boiling indicating that the traditional preparation of long boiling times is required to reach correct concentration of the compounds in the dosage forms. The technique provided scientific evidence, that the time used for the traditional preparation influences the chemical profile of the extract. In analytical chemistry terms it would be desirable to add an inert internal standard to study accurate concentration profiles of the active substances as the boiling process continues.

CHAPTER 7

7. Experimental

The Agilent High Performance Liquid Chromatograph (HPLC) 2690 coupled to the Photodiode Array Detector 996 (variable wavelength detector) and the Micromass Quattro LC Mass Spectrometer was used for all the HPLC and MS analyses. The solvents used for HPLC analysis were of HPLC grade and were degassed before use and the stationary phase used for all the HPLC experiments was the Agilent Hypersil LC Column. Nuclear Magnetic Resonance Spectra were recorded on a Bruker WM – 400 instrument operating at 399.9 MHz for ^{1}H and 100.6 MHz for ^{13}C nuclei. The abbreviations s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad are use in connection with *^{1}H NMR* data and in case of the ^{13}C NMR data, capital letters to refer to the patterns resulting from directly bonded (^{0}C,^{0}H) couplings [^{1}J (CH)].

Thin layer chromatography (TLC) was carried out on precoated silica gel plates (thickness 0.25mm) with eluants used as specified in the analysis sections number 7.1.3, 7.1.6, 7.1.7, 7.1.8, 7.2.3, 7.2.4, 7.2.5, 7.3.3, 7.3.4 and 7.3.6. Column chromatography was performed with Merck 60 silica gel (0.0630 – 0.2000 mm). The solvents used for TLC, column chromatography and extractions were of technical grade and were distilled before use. Preparative high performance liquid chromatography was carried out on a Waters Preparative LC 500 instrument.

7.1. *Boophane disticha*

7.1.1. Aqueous extraction of compounds from *Boophane disticha*

A bulb of *Boophane disticha* (838.78 g) was sliced and separated into two portions. One portion (428.08 g) was dried in an oven at 60 ^{o}C. The other portion (410.08 g) was further sliced and added to hot water (1000 ml). The mixture was boiled for five hours on a hotplate, during which portions of the extract (100 ml) was removed at regular hour intervals. After each sampling time the extract was filtered, cooled and freeze-dried. The freeze-dried samples were weighed [A (1st hour) = 1.24 g, B (2nd hours) = 2.66 g, C (3rd hour) = 5.54 g, D (3.5 hours) = 4.85 g, E (4th hour) = 3.53 g, F (5th hour) = 3.65 g] and stored in a freezer until further use.

7.1.2. Preparation of samples from *Boophane disticha* using methanol.

The other portion of *Boophane disticha* (428.08 g) from section 7.1.1 was further sliced and dried in the oven at 60 ^{o}C. Methanol (300 ml) was added to the dried material (200.01 g) and extracted by stirring for 48 hours. The mixture was filtered and methanol was evaporated to give a dry extract (6.89 g). The extract was kept in the freezer until further use. Thin layer chromatography was used to analyse the extract. The eluent used was chloroform: ethyl acetate: methanol (4:4:2, v/v/v) and the spray used colour development after visualization under UV (λ = 366 nm) was Dragendorf reagent.

7.1.3. Thin layer chromatography

Thin layer chromatography (TLC) was done to test for the solvent system (eluent) that will be most compatible for further processing of the extracts obtained. The following eluants were tested on the various extracts generated in 7.1.2:

1. Chloroform: ethyl acetate: methanol (4: 4: 2, v/v/v)
2. Chloroform: methanol (95: 5, v/v)
3. Benzene: methanol (95: 5, v/v)
4. Ethyl acetate: hexane: HOAc (10: 10: 1, v/v/v)
5. Chloroform: methanol: 25% NH_3 (85: 14: 1, v/v/v)

Two TLC plates were spotted with the same samples and developed in a tank with chloroform: ethyl acetate: methanol (4: 4: 2, v/v/v). After the plates were fully developed, they were air dried and viewed under UV (λ=366 nm). One TLC plate was sprayed with cerium sulphate solution and the other with Dragendorf reagent. This process for testing the compatible eluent was repeated for all the eluents stated above.

7.1.4. Preparation of Dragendorf reagent

i. $BiNO_3$ (1.7 g) was added to Acetic acid (20 ml): Water (80 ml).

ii. KI (40 g) was added to water (100 ml)

A stock solution was prepared by adding 1:1, v/v of the solutions prepared above. Stock solution (1 ml) was mixed with acetic acid (2 ml): water (10 ml) to prepare the final solution, which is known as the Dragendorf Reagent.

7.1.5. Preparation of the cerium sulphate spray

A 6N H_2SO_4 solution was prepared by diluting concentrated sulphuric acid with water. About 20 g $CeSO_4$ was added to 100 ml of 6N H_2SO_4 and the solution was stirred until it changes the colour to orange.

7.1.6. Isolation of compounds using column chromatography

The dry methanol extract (0.45 g) from 7.1.2 was chromatographed on silica gel (1000.00 g) using chloroform: ethyl acetate: methanol (4:4:2, v/v/v) as the eluent. A number of fractions (8-10 ml) were collected. The fractions obtained were spotted on a TLC plate and developed in a tank with chloroform: ethyl acetate: methanol (4:4:2, v/v/v). The fractions were combined to give four fractions and evaporated to form dry samples (A_1–A_4), which were further analysed with TLC. Two TLC plates were spotted with the samples (A_1–A_4) and developed in chloroform: ethyl acetate: methanol (4:4:2, v/v/v). After the plates were developed, they were air dried and viewed under UV (λ = 366 nm). One TLC plate was sprayed with cerium sulphate spray and the other with Dragendorf reagent. These samples (A_1 – A_4) were also analysed with HPLC-MS, with the HPLC running on a gradient mode to be discussed in Section 7.4 and the MS running on a positive Electrospray mode with the conditions shown in Section 7.5.

7.1.7. Preparative thin layer chromatography of sample A_2 from 7.1.6

Sample A_2 (22.10 mg) was dissolved in chloroform for further purification. Two preparative thin layer chromatography plates were spotted and developed in chloroform: ethyl acetate: methanol (4:4:2, v/v/v). After the TLC plates were fully developed, they were dried and visualized under UV (λ = 366 nm). The spots visualized under UV were marked with a pencil. A small portion of the edge of each plate was cut off and sprayed with cerium sulphate while the other was sprayed with dragendorf reagent. One spot that was marked with a pencil was still visible after spraying with Dragendorf reagent. The band was then scraped off the plate and ground. The ground material was then added to chloroform (20 ml) and the silica gel

was filtered off the chloroform layer, which was evaporated to give a pure compound (10.00 mg). The preparative TLC procedure was repeated and a similar compound was obtained (7.00 mg)

7.1.8. HPLC-MS analysis of the extracts and fractions of *Boophane disticha* obtained from silica gel column chromatograhy

A. Aqueous samples

The HPLC analysis of the aqueous extracts from 7.1.1 was done using the masses shown in table 7.1:

Table 7.1: Sample masses taken for HPLC analysis (*Boophane disticha*)

Extract no	Time of boiling (hours)	Mass taken (mg)
A	1	10.50
B	2	10.20
C	3	9.80
D	3.5	10.00
E	4	10.10
F	5	10.10

All extracts given in Table 7.1 were dissolved in 2 ml of water (previously degassed). The water was degassed by filtering with filter type 0.45 µm. After the samples were thoroughly mixed, they were filtered with 0.45 µm nylon filter and injected (25 µl) on the HPLC-MS, which was programmed to run on a gradient method to be outlined in section 7.4 table 7.5.

B. Organic samples

1. The analysis of the methanol extract as well as other fractions obtained from sections 7.1.2, 7.16 and 7.17 was done by taking the samples (5.00 mg) and dissolved in degassed methanol (2ml), the sample solution filtered with the 0.45 µm nylon filter and injected (25 µl) on the HPLC system, which runs on a gradient method.
2. Pure samples (0.30 mg) from sections 7.1.7 (which only showed one compound when analysed with TLC) were dissolved in degassed methanol (2 ml) and the solution filtered with the 0.45 µm nylon filter. The sample (25 µl) was injected on the HPLC-MS system, which runs on a gradient method outlined in Table 4.5 and the MS tuned to the positive electrospray ionisation (+ESI) mode.

7.2. Crinum macowanii

7.2.1. Aqueous extraction of compounds from *Crinum macowanii*

A bulb (847.38 g) of *Crinum macowanii* was sliced and separated into two portions. One portion (419.16 g) was dried in an oven at 60 ^{o}C. The other portion (423.81 g) was further sliced and added to hot water (1000 ml). The mixture was boiled for five hours on a hotplate, during which a portion of the extract (100 ml) was removed at regular intervals. After each sampling time the extract was filtered, cooled and freeze-dried. The freeze dried samples were weighed [A (1st hour) = 2.86 g, B (2nd hours) = 3.24 g, C (3rd hour) = 4.56 g, D (3.5 hours) = 3.89 g, E (4th hour) = 3.08, F (5th hour) = 3.21 g] and stored in a freezer until further use.

7.2.2. Extraction of dried material of *Crinum macowanii* using methanol.

The other portion of *Crinum macowanii* (419.16 g) was further sliced and dried in the oven at 60 ^{o}C. Methanol (300 ml) was added to the dried material (387.59 g) and extracted by stirring for 48 hours. The mixture was filtered and methanol was evaporated to give a dry extract (8.04 g). The extract was kept in the freezer until further use.

A portion of the extract (10 mg) was dissolved in degassed methanol (2 ml) and the solution was filtered through the 0.45 µm nylon filter. The sample (25 µl) was then injected into the HPLC-MS system, running on a gradient method (see section 7.4) and the mass spectrometer tuned to the positive electrospray ionisation (+ESI) mode.

7.2.3. Thin layer chromatography

Thin layer chromatography (TLC) was done to test the solvent system (eluant) to be used for further processing of the extracts. The following eluants were tested on the samples obtained in 7.2.2:

1. chloroform : ethyl acetate : methanol (4 : 4 : 2,v/v/v)
2. chloroform: methanol (9: 1,v/v)
3. chloroform: methanol: water (67 : 32.5 : 0.5,v/v/v)
4. ethyl acetate: hexane (6 : 2,v/v)

Two TLC plates were spotted with the same samples and developed in a tank with chloroform: ethyl acetate: methanol (4:4:2,v/v). After the plates were fully developed, they were air dried and viewed under UV (λ = 366 nm). One TLC plate was sprayed with cerium sulphate spray and the other with Dragendorf reagent. This process for testing the compatible eluent was repeated for all the eluants stated above.

7.2.4. Separation of compounds from the extract of *Crinum macowanii.*

The methanol crude extract (3.741 g) from 7.2.2, was chromatographed on silica gel (800.00 g) with chloroform: ethyl acetate: methanol (4:4:2, v/v/v) as the eluant and seven fractions (I_1 - I_7) were collected. These fractions were analysed with thin layer chromatography using the same eluant as for column chromatography. After visualization of the spots shown on the TLC plate under UV, it was further sprayed with Dragendorf reagent. The first three samples (I_1 - I_3) were combined and the remaining ones (I_4 - I_7) were analysed with HPLC-MS using the gradient method (LC) for alkaloids and the positive mode of the electrospray ionisation.

7.2.5. Further chromatography on the sample from 7.2.4.

A column was packed with silica gel (300.00 g) and the combined sample (I_1 - I_3) from 7.2.4 was chromatographed with chloroform: ethyl acetate: methanol (4:4:2). A number of fractions were obtained and TLC was used to analyse these for the presence of alkaloids. The samples were combined into eight fractions (H_1 - H_8) and fractions H_5-H_7 was found to be containing the same compound, which crystallized immediately after the evaporation of the solvent to a small amount (20 ml). The mother liquor was then removed with a pipette and methanol was added to the crystallizing compound to further wash the white solid formed. After evaporation of methanol a dry white powder (27.10 mg) was obtained. The sample was then ready for analysis with HPLC-MS, ^{1}H NMR and ^{13}C NMR. For NMR the samples were dissolved in deuterated DMSO.

7.2.6 HPLC-MS analysis of the fractions of *Crinum macowanii*

A. Aqueous samples

The HPLC analysis, of the aqueous extract from section 7.2.1 was done by using masses shown in table 7.2:

Table 7.2: Sample masses taken for HPLC analysis (*Crinum macowanii*)

Sample no	Time of boiling (hours)	Mass taken (mg)
A	1	10.10
B	2	10.00
C	3	9.90
D	3.5	10.00
E	4	10.30
F	5	10.10

All extracts given in Table 7.2 were dissolved in 2 ml water (previously degassed). Filtering with filter type 0.45 µm degassed the water. After the samples were thoroughly mixed, they were filtered with 0.45 µm filter and injected (25 µl) into the HPLC system, which was programmed to run on a gradient method outlined in section 7.4, table 7.5.

B. *Organic samples*

1. Each of the fractions I_4 - I_6 (5.00 mg) prepared from 7.2.4, were dissolved in degassed methanol (2 ml) and the solutions were filtered through with 0.45 µm filter. The samples (25 µl) were injected into HPLC-MS system, running on a gradient method.
2. Samples H_4 - H_7 (0.30 mg) from 7.2.5, were dissolved in degassed methanol (2 ml) and the solutions were filtered with 0.45 µm filter. The samples (25 µl) were injected on the HPLC-MS system, which operates on a gradient method and the MS tuned to the positive electrospray ionisation (+ESI) mode.

7.3. Eucomis autumnalis

7.3.1. Aqueous extraction of compounds from *Eucomis autumnalis*

The bulb of *Eucomis autumnalis* (431.46 g) was sliced into two smaller portions. One portion (236.31 g) was dried in an oven at 60 ^{o}C. The other portion (195.15 g) was further sliced and added to hot water (1000 ml). The mixture was boiled for five hours, after which a portion of the extract (100 ml) was removed at one-hour intervals. After each sampling the extract was filtered, cooled and freeze-dried. The freeze dried samples were weighed [A (1st hour) = 3.12 g, B (2nd hours) = 3.79 g, C (3rd hour) = 4.64 g, D (3.5 hours) = 4.98 g, E (4th hour) = 3.21 g, F (5th hour) = 2.76 g] and stored in a freezer until further use.

7.3.2. Extraction of compounds from *Eucomis autumnalis* using methanol.

The bulb of *Eucomis autumnalis* (236.31g) was sliced and oven dried for 48 hours. Methanol (350 ml) was added to the dried material (211.12 g) and extracted for 48 hours. The solid material was filtered and methanol was evaporated to obtain a dry extract (20.58 g). The extract was kept in the freezer until further use.

A portion of the sample (10.00 mg) was dissolved in degassed methanol (2 ml) and the solution was filtered with 0.45 µm filter. The sample (25 µl) was then injected into the LC-MS system, which runs on a gradient method and the mass spectrometer tuned to the negative electrospray ionization (-ESI) mode.

7.3.3. Thin layer chromatography

In order to further process the extract obtained from above, thin layer chromatography was done, and the following solvent systems (eluents) were tested on the methanol extract from 7.3.2 to find the best one that can be used for the column chromatography.

1. hexane: ethyl acetate (3: 2, v/v))
2. chloroform: methanol (9: 1, v/v)
3. chloroform: methanol: mater (67 : 32.5 : 0.5, v/v/v)

A TLC plate was spotted with the extract from 4.3.2 and developed in a tank with hexane: ethyl acetate (6:4, v/v). After the plates were fully developed, they were air dried and viewed under UV (λ=366nm). The plate was sprayed with cerium sulphate spray for colour development. This process for testing the compatible eluent was repeated for all the eluents stated above.

7.3.4. Separation of compounds from the extract of *Eucomis autumnalis*.

A crude extract (4.456 g) from 7.3.2, was chromatographed on silica gel (800.00 g) with hexane: ethyl acetate (3:2, v/v). A number of fractions were collected and analysed with thin layer chromatography (hexane: ethyl acetate (3:2, v/v)). After visualization of the spots shown on the TLC plate under UV (λ = 366 nm), it was further sprayed with cerium sulphate. Fractions showing yellow spots on the TLC plate were combined and analysed with LC-MS using the gradient method (LC) to be discussed in section 7.4 (Table 7.7) and the negative electrospray ionisation mode.

7.2.5. Further chromatography on the sample from 7.3.4.

A column was packed with silica gel (400.00 g) and the combined fractions with yellow spots in section 7.3.4 were chromatographed with hexane: ethyl acetate (6:4, v/v) as the mobile phase. A number of fractions were obtained and analysed with thin layer chromatography for the presence of homoisoflavanoids, which possess a UV chromophore. The samples were then combined into ten fractions (A–J) and then analysed with LC-MS.

7.3.6. Preparative liquid chromatography on the sample from 7.3.5.

The samples from 7.2.5, which were not easily separated by column chromatography and thin layer chromatography, were further purified using reverse phase semi preparative liquid chromatography. The preparative liquid chromatograph was set up with methanol: water (9:1, v/v) as the mobile phase and a C-18 reverse phase column. Sample A (10 mg) from 7.3.5 was dissolved in methanol (2 ml) and the mixture was injected (20 µl) on the semi preparative LC system. Fractions were collected separately based on the UV absorption of the separated compounds as they eluted from the column. In this way pure compounds with different retention times were obtained. The process was repeated until a reasonable quantity (30.00 mg of a pure compound) was obtained. The samples obtained were then analysed by HPLC-MS.

7.3.7. HPLC-MS analysis of the fractions of *Eucomis autumnalis*

A. Aqueous samples

The HPLC analysis of the aqueous samples from 4.3.1 was done by taking masses shown in table 7.3:

Table 7.3: Sample masses taken for HPLC analysis (*Eucomis autumnalis*)

Sample no	Time of boiling (hours)	Mass taken (mg)
A	1	10.00
B	2	10.10
C	3	9.90
D	3.5	9.98
E	4	10.20
F	5	10.10

All extracts given in Table 4.3 were dissolved in degassed distilled water (2 ml). Degassed water was obtained by filtering with filter type 0.45 µm filter. After the samples were properly mixed, they were filtered with 0.45 µm filter and injected (25 µl) into the HPLC system, which was programmed to run on a gradient method outlined in table 7.5.

7.4. The HPLC gradient methods used.

The liquid chromatography method used for the analysis of *Boophane disticha* and *Crinum macowanii* consisted of a buffer, is 0.01 M ammonium acetate in water, acetonitrile and methanol (HPLC grade). The gradient was designed such that the mixture is suitable to the column specifications. This means that the pH of the mixture should be within the range of the column pH specifications. The mobile phase used for *Crinum macowanii* and *Boophane disticha* is given in Table 7.4 and 7.5.

Table7.4: Initial conditions of the gradient method

Solvents	Solvent Line	Initial Solvent Ratio (%, v/v)
Ammonium acetate in water	A	90
Acetonitrile	B	0
Methanol	C	10

The flow rate was 1 ml/min and the column temperature was 40^{o}C. The Waters 996 PDA conditions (wavelength range) were 193 nm to 400 nm. The gradient time table contained the following entries:

Table 7.5: The gradient time table for HPLC method used for alkaloids.

Time	Solvent line A (ml)	Solvent line B (ml)	Solvent line C (ml)	Flow rate (ml/min)
0	90	0	10	1
33	60	0	40	1
40	10	0	90	1
48	0	0	100	1
58	90	0	10	1
65	90	0	10	1

The liquid chromatographic method and parameters used for *Eucomis autumnalis* are given in table 7.6 and 7.7.

Table 7.6: Initial conditions of the gradient method homoisoflavanoids analysis

Solvents	**Solvent Line**	**Initial Solvent Ratio (%, v/v)**
Water	A	90
Acetonitrile	B	10

The flow rate was 1ml/min and the column temperature was 40^{o}C. The Waters 996 PDA conditions (wavelength ranges) were 193 m to 400nm. The gradient time table contained the following entries:

Table 7.7: The gradient time table for LC method used for homoisoflavanoids

Time	**Solvent line A (ml)**	**Solvent line B (ml)**	**Flow rate (ml/min)**
0	90	10	1
61	20	80	1
64	0	100	1
75	0	100	1
75.1	90	10	1
90	90	10	1

7.4. The MS methods used.

For the two plants *Crinum macowanii* and *Boophane disticha* the following MS conditions used:

Tuning Parameters: ES+ (positive electrospray mode)

Capillary: 4 kV

Cone: 60 V

Extractor: 5 V

RF Lens: 0.5 V

Source Block Temperature: 140 ^{o}C

Desolvation Temperature: 300 ^{o}C

For *Eucomis autumnalis* the following MS conditions used:

Tuning Parameters: ES- (negative electrospray mode)

Capillary: 2 kV

Cone: 35 V

Extractor: 5 V

RF Lens: 0.5 V

Source Block Temperature: 140 ^{o}C

Desolvation Temperature: 350 ^{o}C

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