Pharmaceutical Analysis and Quality of Complementary Medicines: *Sceletium* and Associated Products

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ABSTRACT

There has been an upsurge in the use of Complementary and Alternate Medicines (CAMs) in both developed and developing countries. Although herbal medicines have been in use for many centuries, their quality, safety and efficacy are still of major concern. Many countries are in the process of integrating CAMs into conventional health care systems based on the knowledge and use of traditional medicines. The quality control (QC) of herbal products usually presents a formidable analytical challenge in view of the complexity of the constituents in plant material and the commercial non-availability of appropriate qualified reference standards.

Sceletium, a genus belonging to the family Aizoaceae, has been reported to contain psychoactive alkaloids, specifically mesembrine, mesembrenone, mesembrenol and some other related alkaloids. *Sceletium* is marketed as dried plant powder and as phyto-pharmaceutical dosage forms. *Sceletium* products and plant material marketed through health shops and on the internet are associated with unjustified claims of specific therapeutic efficacy and may be of dubious quality.

Validated analytical methods to estimate *Sceletium* alkaloids have not previously been reported in the scientific literature and the available methods have focused only on qualitative estimation. Furthermore, since appropriate markers were not commercially available for use as reference standards, a primary objective of this study was to isolate relevant compounds, qualify them as reference standards which could be applied to develop appropriate validated qualitative and quantitative analytical methods for fingerprinting and assay of Sceletium plant material and dosage forms.

The alkaloidal markers mesembrine, mesembrenone and Δ^7mesembrenone were isolated by solvent extraction and chromatography from dried plant material. Mesembranol and epimesembranol were synthesised by hydrogenation of the isolated mesembrine using the catalyst platinum (IV) oxide and then further purified by semi-preparative column chromatography. All compounds were subjected to analysis by ^{1}H, ^{13}C, 2-D nuclear magnetic resonance and liquid chromatography-tandem mass spectroscopy. Mesembrine was converted to hydrochloride crystals and mesembranol was isolated as crystals from the hydrogenation reaction mass. These compounds were analysed and characterised by X-ray crystallography.

A relatively simple HPLC method for the separation and quantitative analysis of five relevant alkaloidal components in *Sceletium* was developed and validated. The method was applied to determine the alkaloids in plant material and dosage forms containing *Sceletium*.

An LCMS method developed during the study provided accurate identification of the five relevant *Sceletium* alkaloids. The method was applied for the quantitative analysis and QC of *Sceletium* plant material and its dosage forms. This LCMS method was found to efficiently ionize the relevant alkaloidal markers in order to facilitate their detection, identification and quantification in *Sceletium* plant material as well as for the assay and QC of dosage forms containing *Sceletium*.

The chemotaxonomy of some *Sceletium* species and commercially available *Sceletium* dosage forms were successfully studied by the LCMS method. The HPLC and LCMS methods were also used to monitor the bio-conversion of some of the alkaloids while processing the plant material as per traditional method of fermentation.

Additionally a high resolution CZE method was developed for the separation of several *Sceletium* alkaloids in relatively short analysis times. This analytical method was used successfully to fingerprint the alkaloids and quantify mesembrine in *Sceletium* and its products.

Sceletium species grown under varying conditions at different locations, when analyzed, showed major differences in their composition of alkaloids and an enormous difference was found to exist between the various species with respect to the presence and content of alkaloids. *Sceletium* and its products marketed through health shops and the internet may thus have problems with respect to the quality and related therapeutic efficacy.

The QC of *Sceletium* presents a formidable challenge as *Sceletium* plants and products contain a complex mixture of compounds. The work presented herein contributes to a growing body of scientific knowledge to improve the QC standards of herbal medicines and also to provide vital information regarding the selection of plant species and information on the specific alkaloidal constituents to the cultivators of *Sceletium* and the manufacturers of its products.

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ACD	advanced chemistry development
ACN	acetonitrile
ADRs	adverse drug reactions
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
Ar	aromatic
ARTG	Australian register of therapeutic goods
AOAC	association of official analytical chemists
ASTM	American society for testing and materials
ATM	African traditional medicine
atm	atmospheric
AUC	area under the curve
AUFS	absorbance units full scale
β	beta
BGE	background electrolyte
C	carbon
^{13}C	carbon 13 shift
C_{18}	octadecyl silane
CA	California
CAMs	complementary and alternative medicines
CDCl3	chloroform D1
$CHCl_3$	chloroform
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
cGMP	current good manufacturing practice
CHMP	committee of herbal medicine products
CID	collision induced dissociation
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CM	complementary medicines
CMC	complementary medicines committee
CMEC	complementary medicines evaluation committee
CMWG	complementary medicines working group
COSY	correlation spectroscopy
CTD	common technical document
CPMP	committee for proprietary medicinal products
CSFAN	center for food safety and nutrition
CZE	capillary zone electrophoresis
Δ	delta
δ	delta
°C	degree centigrade
DAD	diode array detector
DCM	dichloromethane
D_2O	deuterium oxide
DoH	Department of Health
DSC	differential scanning calorimetry
DSHEA	dietary supplement health and education act
ECCMHS	expert committee on complementary medicines in the health system
EMEA	European agency for the evaluation of medicinal products
EI	electron impact
EOF	electroosmotic flow
ERP	expedited registration procedure

ESCOP	European scientific cooperative on phototherapy
ESI	electro-spray ionization
EtOAc	ethyl acetate
EtOH	ethanol
E-MOH	epimesembranol
EU	European Union
FDA	food and drug administration
FL	Florida
FTC	federal trade commission
g	gram
GCMS	gas chromatography mass spectroscopy
GRAS	generally recognized as safe
^{1}H	proton
H_2	hydrogen gas
HCl	hydrochloric acid
H_2SO_4	sulphuric acid
H_3PO_4	orthophosphoric acid
HMBC	heteronuclear multiple bond correlation
HMP	herbal medicinal products
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HSQC	heteronuclear single quantum correlation
HV	high voltage
IS	internal standard
id	internal diameter
IL	Illinois
IPA	isopropyl alcohol
IT	ion trap
kV	kilovolt
LC	liquid chromatographic/ liquid chromatography
LCMS	liquid chromatography-mass spectrometry
LCMS/MS	liquid chromatography-tandem mass spectrometry
LoD	limit of detection
LoQ	limit of quantitation
LoD	loss on drying
Ltd	limited
M	molar
mg	milligram
µg	microgram
µA	current (microampere)
µl	microliter
µm	micrometer
ml	milliliter
MA	Massachusetts
MP	mobile phase
M-HCl	mesembrine hydrochloride
MCC	medicine control council
MRHA	medicines and health products regulatory agency
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MHz	mega hertz
MI	Michigan
MO	Missouri
mm	millimeter
mM	millimolar

mp	melting point
MRM	multiple reaction monitoring
MS	mass spectrometry
MT	migration time
MW	molecular weight
m/z	mass to charge ratio
N	normal
N_2	nitrogen gas
NaCl	sodium chloride
NaOH	sodium hydroxide
$NaBH_4$	sodium borohydride
NCCAM	national center for complementary and alternative medicines
ng	nanogram
NHP	natural health product
NHPD	natural health products directorate
NHPR	natural health products regulations
NHPs	natural health products
NIH	national institutes of health
NJ	New Jersey
NLEA	nutrition labeling and education act
nm	nanometer
NMR	nuclear magnetic resonance
2D-NMR	two dimensional NMR
NNFA	national nutritional foods association
NP	normal phase
NPD	nitrogen phosphorus detector
NY	New York
OD	outer diameter
ODS	office of dietary supplements
OH	hydroxyl
OTC	over the counter
PDA	photodiode array
PVDF	polyvinylidene difluoride hydrophilic filters
QA	quality assurance
QC	quality control
Q-HCl	Quinine hydrochloride
QSE	quality, safety and efficacy
®	registered trade mark
R	Registered
Rf	retention factor
RP	reversed phase
RSA	Republic of South Africa
RSD	relative standard deviation
RT	retention time
RMT	relative migration time
SS	stainless steel
SSRI	selective serotonin reuptake inhibitor
S/N	signal-to-noise
SD	standard deviation
SIM	single ion monitoring
SRM	single reaction monitoring
TGA	therapeutic goods administration
THMPD	traditional herbal medicinal products directive
TIC	total ion current
TLC	thin layer chromatography

TMD	traditional medicine
TMEC	traditional medicines evaluation committee
TRAMED	traditional medicines project
TRM	traditional medicines programme
UK	United Kingdom
UN	United Nations
US	United States
USA	United States of America
US FDA	United States federal drug act
USP	United States Pharmacopoeia
UV	ultraviolet
UV_{254}	UV short wavelength
UV_{366}	UV long wavelength
UV-Vis	ultraviolet-visible
WHO	World Health Organisation
www	world wide web

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Use of phytomedicines, natural products as well as complementary and alternate medicines (CAMs) in general, is now a global phenomenon which has gained tremendous popularity. Herbal medicines have been used as primary health care amongst the poor in many developing countries and have also gained much acceptance even in countries where conventional medicines (allopathic) are the predominant form of medical care. However, the use of herbal medicine varies depending on specific regions and culture around the world, which makes this form of treatment inconsistent. Safety and efficacy are major concerns due to poor documentation and a dearth of scientific research on this subject [1].

The World Health Organisation (WHO) estimates that currently up to 80% of the population in Africa use herbal medicines for some aspect of primary health care. Acceptance of herbal therapies in terms of CAMs by developed countries is reportedly 48% in Australia, 50% in Canada, 42% in the United States of America (USA), 75% in France, and about 90% in the United Kingdom (UK). The widespread use of herbal related therapies have provided a huge market for herbal products reaching $ 43,000 million in the year 2000 with an estimated increase of 5-15% growth per annum since 1991/92 [2].

Herbal medicines are considered an integral part of modern civilization and 25% of modern medicines reported to be derived from herbal origin are commonly used today [3]. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlate directly with their traditional use as herbal medicines [4]. Major pharmaceutical companies are currently conducting extensive research on various species of plants for their potential medicinal value.

The accumulated knowledge of herbal traditions are generally without modern scientific controls which are important to distinguish between the placebo effect, natural ability of the body to heal itself and the inherent benefits of the herbal medicine *per se* [5].

1.2 COMPLEMENTARY AND ALTERNATIVE MEDICINES

CAMs, as defined by the National Center for Complementary and Alternative Medicines (NCCAM), is a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine[a]. While some scientific evidence exists regarding some CAM[b] therapies, for most there are key questions that are yet to be answered through well-designed scientific studies. Questions such as whether these therapies are safe and whether they work for the diseases or medical conditions for which they are used are unanswered [6].

NCCAM, the USA government's lead agency for scientific research on CAM, distinguishes complementary medicines from alternative medicines. Complementary medicine is used together with conventional medicine. An example of a complementary therapy is using Aromatherapy to help lessen a patient's discomfort following surgery. Alternative medicine is used in place of conventional medicine. An example of an alternative therapy is using a special diet to treat cancer instead of undergoing surgery, radiation, or chemotherapy that has been recommended by a conventional doctor [6].

The terms 'complementary medicine' or 'alternative medicine' are used inter-changeably with traditional medicine[c] and herbal medicine in some countries. They refer to a broad set of health care practices that are not part of that country's own tradition and are not integrated into the dominant health care system. Due to the increasing popularity and use of CAM, agencies such as NCCAM are dedicated to exploring complementary and alternative healing practices in the context of rigorous science, training CAM researchers, and disseminating authoritative information to the public and professionals [6].

[a] Conventional medicine is medicine as practiced by holders of M.D. (medical doctor) or D.O. (doctor of osteopathy) degrees and by their allied health professionals, such as physical therapists, psychologists, and registered nurses. Other terms for conventional medicine include allopathic, Western, mainstream, orthodox, and regular medicine; and biomedicine. Some conventional medical practitioners are also practitioners of CAM [6].

[b] Other terms for complementary and alternative medicine include unconventional, non-conventional, unproven, and irregular medicine or health care [6].

[c] Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness [3].

According to the WHO, the definitions of herbal medicines, one of the categories under the CAM, include [1]**:**

- ***Herbs*** "include crude plant material such as leaves, flowers, fruits, seeds, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered".

- ***Herbal materials*** "include in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir-baking with honey, alcoholic beverages or other materials".

- ***Herbal preparations*** "are the basis for finished herbal products and may include comminuted or powdered herbal materials, or extracts, tinctures and fatty oils of herbal materials. They are produced by extraction, fractionation, purification, concentration, or other physical or biological processes. They also include preparations made by steeping or heating herbal materials in alcoholic beverages and/or honey, or in other materials".

- ***Finished herbal products*** "consist of herbal preparations made from one or more herbs. If more than one herb is used, the term mixture herbal product can also be used. Finished herbal products and mixture herbal products may contain excipients in addition to the active ingredients".

  However, finished products or mixture products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal.

  - ***Therapeutic activity*** refers to the successful prevention, diagnosis and treatment of physical and mental illnesses; improvement of symptoms of illnesses; as well as beneficial alteration or regulation of the physical and mental status of the body.

  - ***Active ingredients*** refer to ingredients of herbal medicines with therapeutic activity. In herbal medicines where the active ingredients have been identified, the preparation of these medicines should be standardized to contain a defined amount of the active ingredient, if adequate analytical methods are available. In cases where it is not possible to identify the active ingredients, the whole herbal medicine may be considered as one active ingredient.

1.3 TRADITIONAL USE OF HERBAL MEDICINES

Traditional use of herbal medicines refers to the long historical use of these medicines. Their use has been well-established and widely acknowledged to be safe and effective, and may be accepted by national regulatory/health authorities.

There are more than a hundred different therapies available as CAM treatments and herbal medicines that come under one of the five discrete clinical disciplines such as acupuncture, chiropractic, homeopathy, osteopathy and herbal medicine which are distinguished by established training and professional standards. Herbal medicines and acupuncture are the most widely-used CAM medicine therapies in the world [7].

1.3.1 Increased use of Herbal Medicines

Health care in the twentieth century has witnessed a revolution with a dramatic decline in mortality, consequently increasing life expectancy. Scientific innovations have led to the development of new medicines. However, these achievements have not changed much in terms of regular access to affordable essential medicines[d] to one–third of world population. It is observed that modern medicine is not likely to be a realistic treatment option. In contrast, herbal medicines are readily available, accessible and more affordable, even in remote areas [2]. Herbal medicines play an important role in health care in both developed and developing countries. The United Nations Conference on Trade and Development (UNCTAD) document states that many activities and products based on traditional knowledge are important sources of income, food, and healthcare for large parts of populations in various developing countries [2].

In the last decade, there has been a global upsurge in the use of CAMs in both developed and developing countries. Among the various reasons quoted for the increasing popularity of herbal medicines are assumptions that orthodox medicines are synthetic and can cause side-effects, dissatisfaction with the treatment received in modern medicine and chronic diseases and other diseases, that cannot be cured by modern medicines [7]. For about 2,000 varieties of minor illnesses and serious diseases it is reported that only 40% may be cured using orthodox medicines [8]. This leaves a wide gap that herbal medicines can fill along with other therapies in CAM and hence accounts for the growing popularity.

[d] Essential medicines are those medicines that should be available, affordable and accessible to treat the majority of health problems in the population.

Although by the 1950s, Pharmacognosy, the study of plants affecting health, was thought to be a dying science, revival of interest in herbalism as a part of the mainstream remained a global phenomenon. New recognition of the value of traditional medicines and indigenous pharmacopeias and the need to make health care affordable for all caused this new movement, which was driven by consumers. The drive of "return to nature" was led by the consumers' desire to control their health to the extent of preventing and treating diseases, including chronic and incurable conditions. One of the major reasons for the increased interest in herbalism is the perception of consumers that pharmaceuticals agents are expensive and high-risk in contrast to herbal remedies that are perceived to be natural and safe. Other factors that contributed to an exponential increase in herbal agents are, increase in public knowledge, access to information through a variety of sources such as the media, journals and internet. Among the major types of herbal medicines that have evolved, Asian, European, Indigenous and Neo-Western have topped the list. The first two types have been used for thousands of years and appear in pharmacopeias and are better understood [9].

As a consequence of increased popularity of herbal medicines, the role of traditionally trained practitioners who identified ingredients, harvested plants at specific times, prepared remedies under strict rules and prescribed appropriately, have been replaced by mass production of herbal medicines providing increased access of herbal medicines to consumers in the market [10]. The herbal industry expanded from a small market segment to a major industry globally, with total access to health food stores as well as drug stores and supermarkets as channels of distribution [10].

1.3.2 Safety and Efficacy of Herbal Medicines

Even though herbal medicines have been in use according to long-standing indications, their safety and efficacy are a major concern. There are many reports on adverse drug reactions (ADRs) which could be attributed to intrinsic and extrinsic factors, by the nature of the phyto-chemicals in the plant itself and others due to lack of good manufacturing practices (GMPs), adulteration, wrong species identification and improper quality control (QC) analytical methods [11].

In the USA, manufacturers are expected to accept responsibility for the safety of herbal products but are not expected to provide safety information to regulatory bodies, before marketing. Thus there is limited safety information available prior to marketing the product. Post marketing safety reports are also not required to be submitted or maintained. Lack of appropriate medical supervision may result in increased interactions with food and other medicines being used concomitantly [12]. Interactions with patients' physiology and/or with medication regimens are possible with concomitant use of herbal medicines along with orthodox medicines. Hence, people with chronic illness such as renal and liver diseases, the elderly, pregnant/nursing women, children, those with impending surgery and

patients on prescription medicines such as anticoagulants, hypoglycemics, antidepressants, sedatives, are under greater risk when using herbal medicines [13]. Even though herbal medicines are used extensively, limited evidence is available to support efficacy in the form of randomized controlled trials (RCTs). For example, in 2001, the USA had recorded systematic reviews with statistically significant evidence of efficacy only for four herbs- Garlic, *Gingko biloba*, St. John's wort and saw palmetto [11]. Potential drug interactions can be dangerous, especially since an estimated 75% patients do not inform their health care providers regarding their herbal supplements [12]. At the same time, it is equally important to consider that life-threatening effects of herbal remedies are extremely rare in comparison to those associated with pharmaceutical products. These events are more likely due to adulterants in the formulations, unknown interactions in complex mixtures as a result of undisclosed pharmaceutical interactions, inappropriate dosage or use, or due to underlying factors with the individual patient [14].

The growing number of herbal medicines along with pharmaceuticals results in endless possibilities of interactions. When words like 'alternative' 'unorthodox' medicines are used by health care professionals, they are perceived as judgmental by the patients [13]. Without adequate efficacy and toxicity profiles of herbal medicines, the evidence is lacking for the outcomes. Alternative therapies are reported to be rarely discussed by patients with their physicians, due to fear of criticism [13]. Perceptions that herbal drugs being natural are equated to safe and efficacious due to their origin, is a cause of concern [15].

Herbal post marketing surveillances to detect serious adverse drug reactions (ADRs) are not in place [9]. ADRs may be caused due to intrinsic and extrinsic factors inherent in phytochemicals of products. Reviews show that pre-operative period use of herbal medicines leads to interactions with anesthetics and other medications commonly used during surgery e.g. Garlic, *Ginko biloba* and Ginseng can increase risk of bleeding during surgery. *Kava-Kava* and Valerian result in increased sedative effect of anesthetics [11].

Despite all these issues with herbal medicines, there is lack of stringent requirements for safety and efficacy information for herbal medicines because one of the justifications is that herbs have been used over centuries. Consequently, evidence based studies are not seen as a requirement. On the other hand, manufacturers cannot patent herbs, incentives to conduct clinical trials are low. The high cost of funding research and regulatory requirements are avoided in phyto-pharmaceuticals. This situation results in the lack of availability of data regarding adverse effects, drug interactions and dependence issues [13].

1.3.3 Quality of Herbal Medicines

The quality of herbal medicines is a major concern worldwide. Lucrative markets for herbal medicines are fuelled by heightened consumer interest. Herbal preparations are not governed by the same safety criteria as prescription and over the counter (OTC) products. Absence of QC requirements for potency and purity as well as lenient labeling standards results in standards and quality of herbal medicines not being maintained. Product integrity is not established because of lack of safety testing and purity between batches and manufacturers. The amount of active ingredient can vary greatly between brands and some may not even contain the assumed active ingredients [7].

The factors that affect active constituents of herbal plants include, age of the plant, temperature, daylight length, atmosphere, sampling, toxic residues, manufacture and preparation, rainfall, altitude, soil, microbial contamination, deterioration and heavy metals contamination, amongst others [13]. Variation in activity of phyto-pharmaceuticals is probably due to the presence of various constituents. Illegal contamination or spiking with prescription ingredients and impurities are also possible because potency and purity are not monitored by regulatory agencies. When the raw materials for herbal medicines are collected from the wild or cultivated fields, toxic contaminants can arise from conditions where the plants are grown or collected, conditions where they are dried, processed, stored and transported before and during manufacture [7].

Other problems specific to the quality of herbal medicines are that they are mixtures of many constituents and all the active principles may be unknown. Selective analytical methods and monographs may not yet exist. If several relevant constituents have to be considered, standardization appears to be more complicated because of the variation of the ratios of the constituents in different batches [12].

Different growth, harvest, drying and storage conditions results in variation in quality of raw materials [12]. Hence it is important to cultivate plants under standardized conditions. The polarity of the extraction solvent, mode of extraction and instability of constituents can influence composition and the quality of extracts. Phyto-pharmaceuticals intended for use as medicines must be standardized and the pharmaceutical quality approved, since reproducible efficacy and safety is based on reproducible quality [12].

1.3.4 Adulteration and Contamination of Herbal Medicines

Quality, safety and efficacy (QSE) issues of herbal medicines are not only due to the lack of testing procedures. It is generally assumed that herbal medicines are safe and can be consumed without proper medical advice. There are many reports regarding herbal products being contaminated or spiked with synthetic drugs and also with heavy metals such as lead, mercury, cadmium, thallium and arsenic. The other contaminants could be due to contamination with pesticides and/or microbial growth due to improper storage and processing [7]. It is also reported that mis-identification of certain plants, such as *Digitalis purpurea*, *Atropa belladonna* and *Scutellaria laterflora*, have had serious medical implications [9]. Substitution of herbal medicines with cheaper plants of unknown potency can also lead to substandard products [11].

1.3.5 Responding to the Challenges of Herbal Medicines

Herbal remedies are used as prophylactics to maintain or enhance good health or prevent certain conditions from occurring. They are popular and are promoted as safe and efficacious [9]. For example, in the USA, during the early 1900s, herbal therapy was a major aspect of the pharmacopoeia. It has been reported that, nearly one quarter of pharmaceutical agents originate in whole or in part from naturally occurring chemicals in plants [13]. It is a well known fact that digitalis, reserpine and aspirin continue to be used as standard therapy for cardiovascular indications. The continued use and increasing popularity of phyto-pharmaceuticals was highlighted by a study in the USA that showed a marked increase in the use of herbal agents during 1990 to 1997. The suggested increase of 47% in total visits to alternative medicine practitioners with an estimated increase of 45% expenditure accounts for the sale of herbal medicines exceeding US$ 1.5 billion a year with an estimated 60-70% of the American population using them [16]. It is reported that less that one-third of these people inform their conventional health care providers of their current medicinal therapy [16, 17].

Currently, studies are being conducted to establish herbal-drug transmission *in-utero* or through mother's milk, pharmacokinetic behaviour of plant derived medicines, allergic reactions, problems associated with long term use, effects on internal organs, effects under predisposing conditions and interactions with prescribed medicines. Their effect to potentiate or diminish activity when used with prescribed medicines is also being increasingly studied [9, 14].

Much emphasis is placed on reviews such as Cochrane systematic reviews, which focuses not only on efficacy but also on expanded safety and quality. Methods for the standardization of active ingredients and methods for minimizing risks of contamination are related to quality. Safety issues involve occurrence of adverse effects, contraindications for use, e.g. herb-drug interactions or allergies to these herbal products. There is a need for Cochrane reviews because herbal products do not have package inserts like conventional pharmaceuticals and hence the much-needed information on expanded safety and quality is not available. It is essential that the information provided meet the needs of consumers, clinicians and researchers [18].

The trend in bridging the gap between CAMs and allopathy is being promoted by creating awareness of potential interactions of herbal remedies with allopathic treatments by eliciting information on admission questionnaires, increasing sensitivity amongst future practitioners by developing courses in CAMs and complementing this effort by offering continuing education courses for health care professionals [9]. The integration process is being guided by proactive approaches such as publishing research on efficacy of CAMs in major medical journals, continuing medical education for physicians on CAMs and hospitals with CAM services [19]. NCCAM measures disease burden criteria such as prevalence, mortality, years of life lost and cost of illness before allocating funds for further research on CAMs, thus setting research priorities [20]. Adopting herbal use patterns as part of a patient's documented history and treatment plan is another important way in which measures are being put into place in developed countries [12]. Increase in scientific research to provide evidence in integrating CAMs into mainstream medical practice is expensive and time-consuming. The policy of integration is met with several obstacles due to lack of evidence and limited funding available for CAMs compared to conventional medicines [21].

1.4 SITUATION OF HERBAL MEDICINES IN AFRICA

In August 2000, the resolution on 'Promoting the Role of Traditional Medicine in Health Systems: A Strategy for the African Region', acknowledged that about 80% of the population living in the African region depend on traditional medicine for their health care needs [2]. There is evidence in all African communities that the use of traditional medicines is historic. For centuries, Africa's people have experienced free and open access to a diverse and rich biological resource. Africans have exchanged and traded these resources among themselves for food, fuel, medicine, shelter, and economic security. The globalization of trade and economies exerts intense pressure to open up Africa's natural resources and markets to conform to global trade rules by trans-national corporations [22]. Africa's biodiversity is threatened due to increasing privatization of the biological resource base. The livelihoods and rights of many local communities depend upon these resources and the knowledge and technologies they have developed have contributed to the biodiversity,

conservation and use. Global trade has increased corporate control of Africa's biological resources and healthcare systems. The collective rights of many African communities are undermined due to increased trade of its natural wealth, especially its medicinal plants, and without compensation [22].

Traditional medicine is cheaper and more readily available than Western medicine. In Ghana, there is a traditional doctor for every 224 people; the ratio of modern Western-style doctors is typically a ratio of 1:21,000. In Swaziland this ratio is reported to be at 1: 110 for traditional doctors compared to 1: 10,000 for western doctors [23].

1.5 REGULATORY CHALLENGES OF HERBAL MEDICINES

Differences in quality, variation between batches and current flexible regulatory control on herbal medicines exerts pressure on consumers to take onus regarding the purchase and use of herbal medicines [13]. Among the most common problems with the use of herbal medicines are adulteration, substitution, contamination, misidentification, lack of standardization, non-adherence to GMPs and inappropriate labeling [7, 11]. Substitution or adulteration with other herbs or synthetic medicines done either intentionally or by mistake is also known [7]. A direct consequence of the lack of an enforceable regulatory system on the practitioners and formulators of herbal medicines, results in use of remedies not bound by labeling requirements. Hence the regulatory challenges of requiring claims of content, QSE of herbal remedies are often not satisfied.

In 1991, the WHO developed a policy of traditional medicine in collaboration with its member states in the review of national policies, legislation and decisions on the nature and extent on the use of traditional medicine in their health systems. It also notes that there are differences that exist between its member states, in defining and categorizing herbal medicines. Depending upon the regulations that apply to foods and medicines in each specific country , a medicinal plant may be defined as a food, a dietary supplement or a herbal medicine. This situation makes it difficult to define the concept of herbal medicines for the purposes of national drug regulations, which also confuses patients and consumers [24].

The main objective of the WHO was to facilitate the integration of traditional medicine into national health care systems, to promote the rational use of traditional medicine through the development of technical guidelines for international standards of herbal medicines and related therapies and provide information on various forms of traditional medicine. The guidelines developed in this regard define basic criteria for the evaluation of safety, efficacy and quality of herbal medicines to assist in developing documentation for national regulatory authorities and manufacturers. Accordingly, a resolution was adopted by the World Health Assembly in May 2003 (WHA56.31) on

traditional medicine. The resolution requested WHO to support member states by providing internationally acceptable guidelines and technical standards and also evidence-based information to assist member states in formulating policy and regulations to control the QSE of traditional medicines [24].

1.5.1 Regulatory Situation of CAMs in Different Countries

1.5.1.1 Republic of South Africa Regulations

In the RSA, efforts are being made to regulate CAMs by the Department of Health (DoH) through an officially appointed government committee, the Complementary Medicines Committee (CMC) and a separate committee, Committee for African Traditional Medicines (ATM's) under the auspices of the Medicines Control Council (MCC). The mandate of the CMC is to attend to issues relating to QSE of CAMs and to develop specific regulations for their control and registration. The current Medicines and Related Substances Control Act (Act 101 of 1965) is more appropriate only for the regulation of allopathic medicines and most complementary medicines do not readily meet the requirements of the current medicine registration system as prescribed by Act 101 [25] .

The DoH, in a media statement dated 26th September 2006, makes the South African public aware that the committee has called for producers of CAMs to submit their products. In response to this, the statement purports that about 20,000 products were received, of which 14,000 products have been assessed to establish their contents. Meanwhile, the CMC is apparently in the process of developing guidelines and establishing a regulatory framework for the evaluation and registration of CAMs. Draft regulations which have been published for public comment are being reviewed for the comments that have been received. Upon consideration by the legal unit of the DoH, the recommendations are to be submitted to the Minister of Health (Figure 1.1).

The regulations which follow after this process are expected to regulate and ensure that all CAMs marketed or approved for marketing in the RSA, should meet the required quality, safety and efficacy standards and would be of benefit to the public [26].

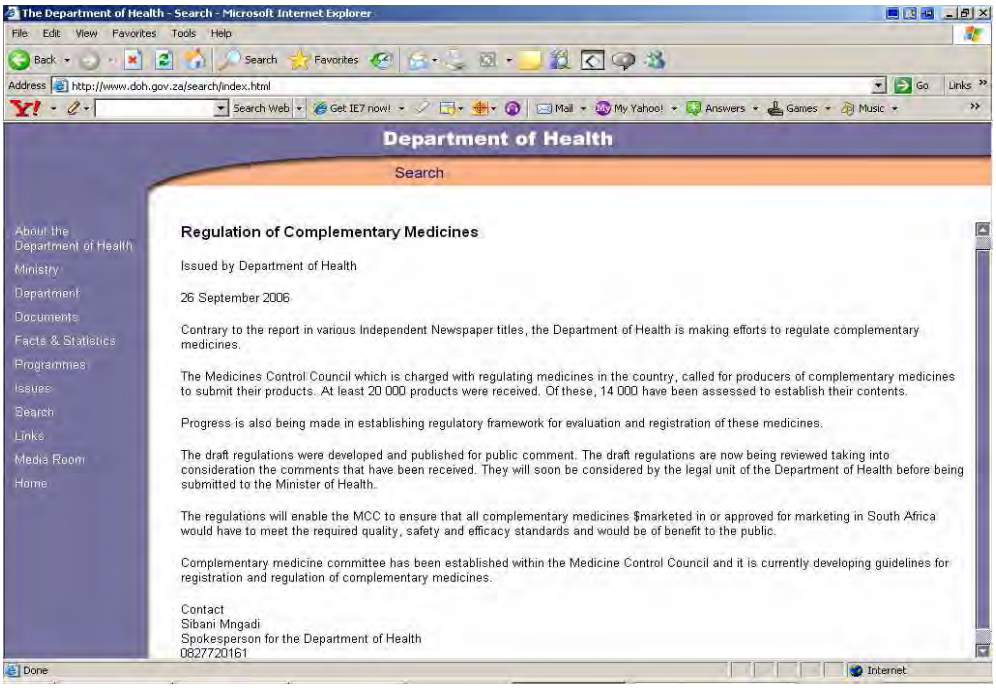


Figure 1.1 Communication from South African Department of Health on complementary medicines. (http://www.doh.gov.za/search/index.html date accessed 15-11-2006)

The CAMs categories [26] in the RSA include the following:

- Herbal medicines
- Ayurvedic medicines
- Chinese medicines
- Sogwa Rigpa medicines
- Unani-Tibb medicines
- Aromatherapeutic Essential oils
- Nutraceuticals
- Homeopathic medicines
- Biochemical tissue salts
- Anthroposophical medicines
- Homotoxicologic medicine
- Gemmotherepeutic medicines
- Energy substances
- CAM Combination medicines (made up from any of the above sub-categories)
- Combination medicines (made up from any of the above sub-categories AND medicines classified as “orthodox” medicines)
- Sports Performance preparations
- Weight Management products

1.5.1.2 The United States of America Regulations

Since the Food Drug and Cosmetic Act (FD & C Act) was passed in the late 1930s, the United States Food and Drug Administration (FDA) has regulated dietary supplements, nutraceuticals and other related herbal products in terms of QSE, with herbal medicines receiving more attention. Natural products have been considered under "generally recognized as safe" (GRAS) status when confirmed by qualified experts and without any further contradictions by other experts [10].

The Dietary Supplement Health and Education Act (DSHEA) was signed into law in October 1994, and it amended the FD & C Act by prescribing GMPs for dietary supplements. Since these regulations do not impose standards for non-availability of analytical methods, unless included in the regulations, this effectively protects dietary supplement manufacturers from proving the efficacy of the product prior to marketing for public consumption. The manufacturer is only expected to assure the FDA that the product is expected to be reasonably safe and they may advertise functional claims for supplements and also health claims as described by the FDA. This situation has prompted the US Congress to revise the DSHEA to exert more control by the government authority to remove substandard products and require manufacturers to file adverse event reports (AERs) with the FDA. The FDA's Center for Food Safety and Nutrition (CFSAN) is in the process of drawing up guidelines for reporting AERs [27].

FDA regulations require that stipulated information appears on dietary supplement labels. Information regarding the dietary supplement label should include a descriptive name of the product stating "supplement"; the name and place of business of the manufacturer, packer, or distributor, a complete list of ingredients and the net contents of the product [28].

It is suggested that the FDA should modify quality inspections such that the manufacturers participate in voluntary GMP quality audit programmes, which are conducted by organisations like the National Nutritional Foods Association (NNFA). In addition, updating of United States Pharmacopoeia (USP) monographs is reported to be underway along with analysis of 28 botanical monographs and more than 100 supplements, conducted with the use of modern instruments to ensure product quality. In this respect, National Institutes of Health (NIH) Office of Dietary Supplements (ODS) has contracted the Association of Official Analytical Chemists (AOAC) dietary supplement task group to provide accurate analytical methods [27].

The advertising of dietary supplements is regulated by the Federal Trade Commission (FTC) in close association with the FDA, which includes informercials for dietary supplements and related products. The advertising and promotional materials which are received by postal mail are regulated by the U.S. Postal Inspection Service [27].

Traditional Medicinals Inc., a manufacturer of herbal medicines commented to the FDA and highlighted that the herbal products that are regulated as “Dietary Supplement Products” by the US FDA are further regulated in Canada as “Natural Health Products” by the Health Canada Natural Health Products Directorate (NHPD) and also by “Traditional Herbal Medicinal Products” in European Union member states. Many US companies which market herbal products internationally must therefore internally harmonize various regulatory requirements. The manufacturer strongly urges FDA to review the existing guidelines in this regard with respect to the other regulatory counterparts, in particular Health Canada NHPD, the UK Medicines and Healthcare products Regulatory Agency (MHRA) and the Australian Therapeutic Goods Administration (TGA), and to make a concerted effort to harmonize certain relevant parts of the claim substantiation guidance. Such an effort will help US companies to streamline their activities to substantiate the product quality under various regulatory authorities [29].

1.5.1.3 Australian Regulations

The Australian Department of Health and Ageing, Therapeutic Goods Administration, in March 2006, released Australian Regulatory Guidelines for Complementary Medicines (ARGCM) Part II for Listed Complementary Medicines.

The Therapeutic Goods Act (1989) laid the initial framework for the regulatory aspects for all therapeutic goods in Australia to ensure the QSE. CAMs imported or manufactured in Australia must be listed or registered in the Australian Register of Therapeutic Goods (ARTG). The general criteria for listed complementary medicines is that the products must contain only ingredients that have been approved for use in Listed Complementary Medicines that make only general and/or medium level indications as defined in the guidelines. The guidelines clearly define an herbal substance with the intention that only those herbal ingredients that comply with that definition are currently permitted to be included in the Medicines List. The product after being listed and approved for marketing has to undergo a random post market review process to confirm that certifications at the time of listing are correct [30].

The quality standards for listed complementary medicines have to be met for active ingredient and the finished product. The British Pharmacopoeia (BP) and Therapeutic Goods Orders (TGOs) are the officially recommended specification standards for active ingredients. In the case of finished products, comprehensive information on composition, product development, formulation, overages and manufacturing process have to be submitted [30].

The listed medicines are considered as low-risk medicines as they are assessed by the TGA. These products are assessed for quality and safety and not for efficacy. The listed products which claim therapeutic benefits may pose health risks to consumers through long term administration or inappropriate self-medication. These products require pre-market assessment for QSE [30].

Post marketing vigilance is mostly related to monitoring of the product by random audits and analytical testing for the advertised ingredients. TGA has an integrated system in place for reporting of ADR monitoring associated with complementary medicines and is in a position to expedite recall procedures if removal of such products is warranted. The labelling and presentation of complementary medicines should follow the Therapeutic Goods Act 1989 [30].

Presently a joint Australia-New Zealand Therapeutic products agency is being established for the regulation of herbal substances, which is known as - The Agency, wherein the complementary medicines comprising of herbal medicines and medicinal products containing herbal substances are to be regulated as medicines. The medicines are to be regulated on a risk-based approach and classified as Class I or Class II, which dictates the level of regulatory control and the manner of licensing. Class I medicines will be evaluated after the product license has been issued whereas Class II medicines are considered as higher risk medicines and a license will only be issued after stringent evaluation [31].

1.5.1.4 Canadian Regulations

Natural Health Products (NHP) in Canada is regulated by the Natural Health Products Directorate (NHPD) under a Canada Gazette for natural health products regulations as per the Food and Drugs Act. As per the gazette, the regulations apply to the sale, manufacture, packaging, labelling, importation, distribution and storage of natural products in Canada. The GMP guidelines outline the requirement of specifications for the NHPs which should contain detailed information on purity, information and identity of each medicinal ingredient, quantity per dosage unit and its potency on the product label. The manufacturers of NHPs are required to maintain records of master production documents, list of all ingredients in each batch/lot of NHP, record of analytical testing and specifications of NHP at the site of manufacture. The manufacturer should also maintain the information and documents for every batch of NHP produced for sale along with the list of all other NHPs manufactured in that premises. Most importantly, the documents related to sanitation have to be maintained at the site [32].

The NHPs are classified as drugs according to the Food and Drugs Act. The act has two components, firstly the function of NHP and secondly the substance used as NHP. The gazette also provides regulations for clinical trials involving human subjects, which emphasizes the requirements for proper documents and protocols by means of good clinical practices which are conducted by qualified investigators [32].

The labeling and packaging requirements prescribed by the regulations ensure that information on the labels should assist the consumer to meet their needs and expectations and also the merits and limitations of the chosen products. The label should recommend the health claim with cautions, warnings and contraindications along with any known ADRs. The label should carry natural product number 'NPN' which is issued with the product license. In case of homeopathic medicines the prefix 'DIN-HM' should be mentioned on the label [32].

The Natural Health Products Directorate, in its guidance document of November 2003, requires applicants for a license to provide evidence to support the quality of finished natural health products. This document aims to critically assess the identity, purity, quantity, potency and tolerance limits of the NHPs [33].

1.5.1.5 European Union Regulations

Previously, regulatory standards in countries within member states of the European Union (EU) were varied and did not represent a homogenous group. Natural products, generally regarded as botanical or herbal medicinal products were categorized as either licensed medicinal products whose QSE is proven in accordance with Article 4.8a (ii) of Council directive 65/65/EEC, or products with simplified proof of efficacy based upon use of the specific herbal product in that country. The major difference between countries was observed in the classification of individual herbal medicines into one of the categories. Although the European Scientific Cooperative on Phytotherapy (ESCOP) founded in 1989 and WHO monographs are in use by member states, the Committee on Herbal Medicinal Products (HMPC) formed by the European Medicines Agency, Evaluation of Medicines for human use (EMEA) is considered as the preferable regulatory authority. The EU placed emphasis on scientific monographs for medicinal products through ESCOP, which published 50 monographs, which were submitted to the Working Group on Herbal Medicinal Products. The monographs were based on scientific data on individual medicinal plant, but needs assessment in the form of an expert report [34].

In 2004, the EU passed a new directive (2004/24/EU) regarding traditional herbal medicinal products to provide a high level of health protection and consumer access to medicines of their

choice, and also provide QSE of such products. In their efforts to ensure a single market, the EMEA introduced new legislation and procedures for traditional herbal medicines, which also encourages trade between member states. According to the directive, herbal preparations are expected to meet the quality requirements similar to medicines approved officially [35].

The legislation states that clinical trials are not required if sufficient knowledge exists on a particular product, thereby avoiding unnecessary testing. For other medicines, it recommends marketing authorization without proof of efficacy, if they are [35]:

a. Safe.
b. Have been used continuously as a traditional medicine for 30 years, of which, at least 15 years in EU, from the date of application.
c. The production standards comply with the pharmaceutical standards when compared to manufacture of herbal medicines.

The HMPC recently released a concept paper, Common Technical Document (CTD) for traditional herbal medicinal products. This document is a revision of pharmaceutical legislation for a simplified registration procedure for traditional herbal medicinal products and refers to the data requirements for such applications. In order to streamline the legislative provisions on the data requirements, it recommends the introduction of explanatory notes in the existing CTD format for submission of applications for registrations. The anticipated impact of this guideline is to improve the understanding on how applications for traditional herbal medicinal products are to be presented. This is expected to help reduce problems with assessment of applications, the resources needed and also facilitate the reduction of the inconsistencies in expectations of the designated authorities with respect to documentation [36].

1.5.1.6 United Kingdom Regulations

Herbal medicines in the UK are regulated by the Medicines and Health Products Regulatory Agency (MRHA), which is an executive agency of the Department of Health. Since the inception of the Traditional Herbal Medicinal Products Directive (THMPD) by the EU in March 2004, the framework to control herbal medicines in UK has benefited by the single piece legislation when compared to the Medicines Act 1968. The present regulations place more emphasis on the QSE of herbal products, firstly, through use of modern analytical methods which would allow quality assurance through reproducible fingerprinting of herbal products and secondly by collecting data from other well-established texts such as Ayurveda and Chinese medicine. The licensing requirements at present exempt the products already on sale. The new regulations require the

manufacturers to apply for a license for each product and to comply with official published standards [37].

The concerns and consequences of these regulations are expected to add extra financial burden on the manufacturer to obtain a license for sale and also demands the 30 year rule as per the EU directive. Interestingly, the US herbal products imported into UK/EU are subject to minimal quality control at present, but with new regulations, the products may have to be re-formulated to expected standards [38].

The THMPD Directive had to be implemented by the member states to establish a registration scheme by 30th October 2005 and the Article 2(2) of THMPD provides that where traditional herbal medicinal products were already on the market when THMPD was in effect, the member states were allowed a transitional period of up to seven years. However, the MHRA's current intention is to allow the transitional period to run until 2011 in the UK [39].

The MRHA, in its efforts to educate herbal product manufacturers, posted a comprehensive THMPD Registration Dossier Requirements in November 2004. The document defined a herbal product as "any medicinal product exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations" and herbal substances defined as "mainly whole, fragmented or cut, plants, parts of plants, algae, fungi, lichen in an unprocessed state, usually dried form but sometimes fresh". Certain exudates that have not been subjected to a specific treatment are also considered to be herbal drugs [39].

Furthermore, it describes a simplified registration procedure and guides the applicant on the labelling and stability studies for herbal products. The labelling requirements as per Article 16i, should provide a high degree of consumer protection, and facilitate the consumer to use the medicinal product correctly on the basis of full and comprehensible information [39].

Stability studies for herbal products are required to be conducted as per Directive 2003/83EEC. The directive indicates a comprehensive study programme based on validated analytical methods and appropriate fingerprinting of the herbal constituents in the product. In case of a constituent of known therapeutic activity, the variation should not exceed ±5% of the initial assay, unless justified, and also considers a case by case approach due to the complexity of the herbal products [39].

1.6 CONCLUSIONS

Undoubtedly, the popularity and commercialization of herbal products are going from strength to strength. In the present scenario with rapid globalization, herbal products are playing a vital part in the global economy. From a modest dietary supplement, herbal products are being transformed into various medicinal products due to inherent pharmacological effects or sometimes even due to unsubstantiated claims by dubious manufacturers. Herbal product adulteration, substitution of active principles, formulation as inappropriate dosage forms, manufactured under non-cGMP conditions (i.e. improper QC and quality assurance), are some of the major issues associated with herbal products. This has prompted implementation of revised regulations in an effort to ensure QSE of herbal products by many countries around the world. However, there exists a major gap in such regulations between developed and developing countries, where it is estimated 80% of the population use traditional medicine as a form of primary health care.

Countries such as South Africa and the UK are in the process of integrating CAMs into the health care system through credible implementation of knowledge generated on herbal medicines based on the traditional use of such botanicals. These efforts are commendable, but require sound financial support and extensive research. There are reports where integrated medicine is practiced by medical practitioners in the USA. Patients in many instances are reported to take prescription medicine along with OTC medicine and herbal medicine, leading to possible adverse reactions. The pharmacist is well-equipped to provide effective counselling in this area and well proposed regulations should be considered to enhance the consultations between the patients and health practitioners to ensure continued safety and awareness.

Regulations play an important role in ensuring quality and safety of herbal products. Herbal products have generally been considered safe and most of these products are available over the counter. Misconceptions of their inherent safety have resulted in serious, sometimes disastrous ramifications. Stringent criteria are being developed and implemented in an effort to improve the situation and also to harmonize the regulations between international regulatory bodies. The challenge for most regulatory bodies is to strike a balance between maintaining availability of herbal products for consumers at an affordable price and also ensuring best possible levels of QSE.

CHAPTER 2

SCELETIUM SPECIES: PLANT AND PRODUCTS

2.1 INTRODUCTION

The name *Sceletium* is derived from the Latin word *Sceletus* meaning skeleton. The derivation of the name is due to the prominent lignified leaf vein structure that is observed in dried leaves of this genus which give a skeletal appearance. The *Sceletium* plant (Figure 2.1a) can easily be identified by its persistent dry 'skeletonised' leaves. The dry leaves enclose the young leaves (Figure 2.1b) during the dry season, to protect them from adverse environmental conditions. The flowers (Figure 2.1c) in *Sceletium* range from white, pale to bright yellow and/or pale pink [40].

Figure 2.1a: Sceletium plant

2.1 b: Dried leaves enclosing young leaves

2.1 c: Sceletium flower

The genus *Sceletium* belongs to the family, Aizoaceae, which occurs in the Western, Eastern and Northern Cape province of South Africa, more prominently occurring in the Little, Great and Upper Karoo regions. It is also reported to be found in the Namaqualand Rocky Hills, Knersvlakte, Ceres Karoo and some moister parts of Western Cape [41].

There are about eight reported species of this genus [40] viz.:

1. *Sceletium crassicaule* (Haw.)L.Bolus
2. *Sceletium emarcidum* (Thunb.) L.Bolus ex H.J.Jaccobson
3. *Sceletium exalatum* Gerbaulet
4. *Sceletium expansum* (L.) L.Bolus [nt]
5. *Sceletium rigidum* L.Bolus
6. *Sceletium strictum* L.Bolus
7. *Sceletium tortuosum* (L.) N.E.Br. [nt]
8. *Sceletium varians* (Haw.) Gerbaulet

2.2 COLLECTION OF *SCELETIUM*

Since the distribution of this plant is mainly in North-Western Cape regions of southern Africa, initial efforts to source the plant from its natural habitat were unsuccessful. However it was observed that the plants were mostly grown in greenhouses in those regions. Hence, the availability of the plants depended on the cultivators, and most of the samples were gratefully supplied as gifts by the *Sceletium* cultivators in their effort to identify and ascertain the quality of the plant material that they had. The contributors to this collection of *Sceletium* plants have been gratefully acknowledged. Plants provided were not always flowering. The samples were potted and cultivated. Upon flowering, samples were pressed to provide herbarium specimens for future references as an achival record. The specimens were deposited at the Selmar Schonland Herbarium (GRA), Grahamstown, South Africa. Specimens have been submitted under the author's name and designated "SP" (the author's initials) along with a collection number.

2.3 IDENTIFICATION OF *SCELETIUM*

The specimens were studied and identified using the identification key of Gerbaulet [41]. Based on the identification key, the venation pattern which differs between species is one of the important taxonomic identification features. The main differences are found in the secondary veins that branch off from the middle vein towards the leaf margin. Based on the venation type, the species is mainly classified as either *emarcidum* or *tortuosum* types (Figure 2.2a). In the *emarcidum* type (Figure 2.2b) the leaf is more flat and the dried leaf venation pattern shows a central main vein with the curved secondary vein which branches off the main vein, reaching the leaf margins. The other species in this group are *S. exalatum and S. rigidum*.

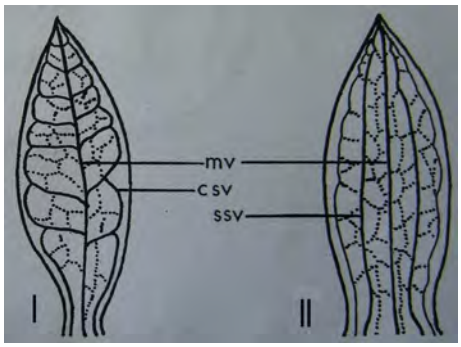


Figure 2.2a: Venation pattern of dry leaves in Sceletium. I- emarcidum type II- tortuosum type; csv=curved secondary vein, mv=middle vein, ssv=straight secondary vein [41]

2.2b: Skeletal leaf of S. emarcidum (SP01, GRA)

2.2c: Skeletal leaf of S. tortuosum (SP02, GRA)

In plants of the *tortuosum* type (Figure 2.2c), the dry leaves are more concave and usually show about three to five or sometimes up to seven major parallel veins. The secondary veins run straight up to the apex on both sides of the middle vein. The other species in this group are *S. crassicaule, S. expansum, S. strictum and S. varians.*

Of the six plant specimens which were received as gift samples, it was observed that three samples were of the *emarcidum* type and the other three were of the *tortuosum* type. These specimens are discussed in more detail below. The *emarcidum* type leaf was observed in the following samples:

1. *S. emarcidum* collected from a greenhouse, Hermanus, South Africa, (Specimen number SP01, GRA, Figure 2.3).
2. S. *rigidum*, sample received as a gift from Vanwyksdorp, South Africa, (Specimen number SP02, GRA, Figure 2.4).
3. *S. exalatum*, sample submitted for identification, from Somerset West, South Africa, (Specimen number SP03, GRA, Figure 2.5).

Figure 2.3: Specimen identified as S. emarcidum (SP01, GRA)

Figure 2.4: Specimen identified as S. rigidum (SP02, GRA)

Figure 2.5: Specimen identified as S. exalatum (SP03, GRA)

The *tortuosum* type of leaf was observed in the following samples.

1. *S. tortuosum*, which was collected from a greenhouse in Robertson, South Africa, (Specimen number SP04, GRA, Figure 2.6).
2. *S. expansum*, sample submitted for identification by cultivator, (Specimen number SP05, GRA, Figure 2.7).
3. *S. strictum*, sample submitted for identification by cultivator, (Specimen number SP06, GRA, Figure 2.8a, b).

During identification of SP06 (GRA), it was observed that there were no skeletonised leaves (Figure 2.8a) and it was suspected that this sample did not belong to the *Sceletium* species. It was about three months later when the skeletonised leaves were observed (Figure 2.8b). This observation was attributed to the fact that the initial submitted sample, grown in a greenhouse, could have had excessive quantities of water. After receipt of the sample in our laboratories, the sample was watered only once in a week. This demonstrates the difficulty of identifying specimens when growth conditions are different to natural wild conditions.

Figure 2.6: Specimen identified as S. tortuosum (SP04, GRA)

Figure 2.7: Specimen identified as S. expansum (SP05, GRA)

Figure 2.8a: Specimen identified as S. strictum (without dried leaves) (SP06, GRA)

Figure 2.8b: S. strictum (with dried leaves)

2.4 CHEMISTRY OF *SCELETIUM* ALKALOIDS

The preliminary studies on *Sceletium* were done by Meiring in 1896 suggesting the presence of alkaloids and this was confirmed by Zwicky in 1914. In a detailed study by Zwicky on about 40 species of the genus *Mesembryanthemum*, more than 50% of the plants tested positive for alkaloids. Due to this large number, the genus *Mesembryathemum* was abandoned and some of the species were reassigned to genus *Sceletium*; family Azioaceae. Further studies on *S. expansum* and *S. tortuosum* reported by Zwicky in 1914, yielded a non-crystalline alkaloid which was named mesembrin with the reported molecular formula, $C_{16}H_{19}NO_4$ [42].

Rimington and Roets [43] re-investigated this plant in 1937 and attempts to crystallize the alkaloid as a free base or an hydrochloride salt were unsuccessful. In their experiments, they managed to obtain a crystalline picrate and platinichloride from the methylated free base and the compound was deduced for its molecular formula based on combustion analysis. The molecular formula for mesembrin was reassigned as $C_{17}H_{23}NO_3$, which is presently know as mesembrine and it was suggested that the molecule belonged to the tropane ester alkaloid group.

Bodendorf and Krieger [44], in their work in 1957, re-visited the molecule and crystallized the mesembrine base to its hydrochloride salt, along with isolation of two more bases, namely mesembrinine, presently known as mesembrenone, which had two hydrogen atoms less and the structure was closely related to mesembrine. The other base was called Channine, which was described as a phenolic base and it was also reported that the three compounds were optically inactive.

Poplek and Lettenbauer in 1967 reported the levels of *Sceletium* alkaloids at 1% to 1.5%, which consisted of 0.7% mesembrine and 0.2% mesembrinine [45]. The structure of mesembrine, deduced from their study, was reported as *N*-methyl-3a-(3',4'-dimethoxyphenyl)-6-oxo-*cis*octahydroindole, which provided the foundation for continued studies on this group of alkaloids [42].

Jeffs *et al*, in 1974 [46] worked further on *S. namaquense* and *S. strictum* and reported five new alkaloids namely, *Sceletium* alkaloid A4, N-formyltortuosamine, 4'-O-demethylmesembrenone, Δ^7mesembrenone and sceletenone. It was also reported that in a concurrent study by Wiechers *et al* on *S. tortuosum*, another base, tortuosamine, was isolated and had a close structural relation to *Sceletium* alkaloid A4.

Arndt and Kruger in 1970 [47] reported three new alkaloids, joubertiamine, dihydrojoubertiamine and dehydrojoubertiamine from *S. joubertii*. The basic structure was reportedly close to mesembrane and not related to the mesembrine type of alkaloids. The above alkaloids were also isolated and reported in another *Sceletium* species, *S. subvelutinum*, by Herbert and Kattah 1990 [48], in their study of the biosynthesis of *Sceletium* alkaloids.

The alkaloids which have been isolated from *Sceletium* species are broadly classified into four structural classes. The major subgroup being the 3a-aryl-cis-octahydroindole skeleton which is referred to as the mesembrine group (Figure 2.9a) which includes Δ^4 series and Δ^7 series based on the double bond at position 4–5 (Figure 2.9b) and 7–7a (Figure 2.9c), respectively. *Sceletium* alkaloid A4 (Figure 2.9d) constitutes the lone member of the second subgroup. The third subgroup is closely related to the second, which is the alkaloid, tortuosamine type (Figure 2.9e) and the fourth group is the joubertiamine type (Figure 2.9f) which is closely related to the mesembrine series [49].

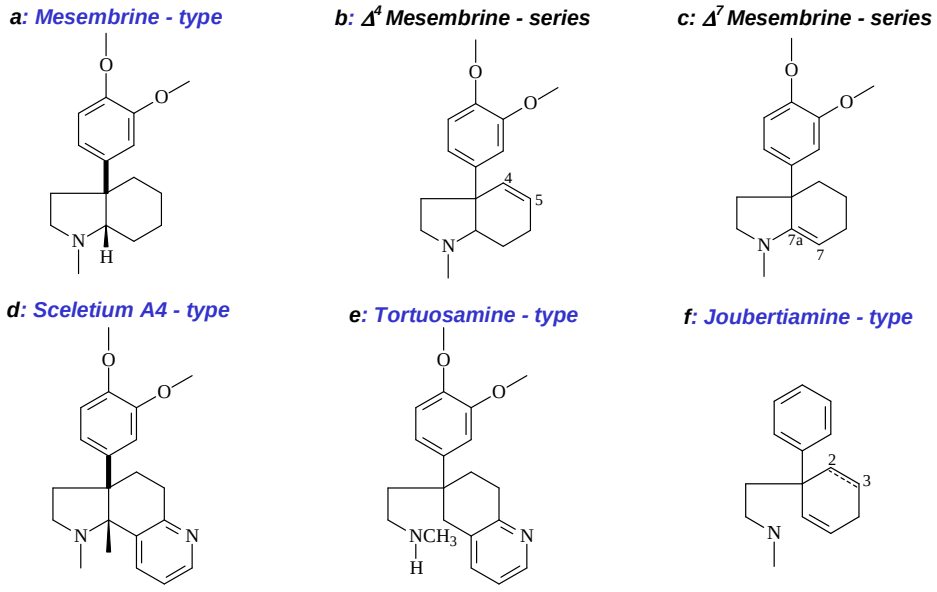
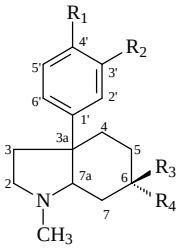


Figure 2.9 a-f: Structural classes of Sceletium alkaloids

Of the above subgroups, the mesembrine type is the largest, consisting of about 15 alkaloids. The class derives its name from mesembrine, which was the first structurally characterized molecule [42].

2.4.1 Mesembrine type

The major alkaloid in mesembrine type is (–)-mesembrine, reported to be present in up to 1% in *S. namaquence* and occurs as a partial racemate in *S. strictum* and *S. tortuosum* in smaller amounts [42]. The reported alkaloids in this sub-group are listed in Table 2.1, 2.2 and 2.3 [49].

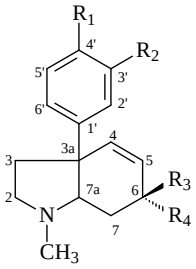


(I)

Table 2.1 Mesembrine type (I) *Sceletium* alkaloids

No.	R1	R2	R3, R4	R3	R4	Compound
1	OMe	OMe	O	-	-	mesembrine
2	OMe	OMe	-	OH	H	mesembranol
3	OMe	OMe	-	H	OH	epimesembranol
4	OMe	OMe	-	OAc	H	mesembranol acetate
5	OH	OMe	-	OH	H	4'demethyl mesembranol
6	OMe	OMe	-	OMe	H	mesembranol methlyether
7	OMe	OMe	H	-	-	mesembrane

2.4.2 Δ^4Mesembrine type

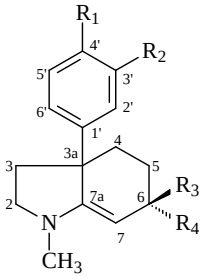


(II)

Table 2.2 Δ^4Mesembrine type (II)*Sceletium* alkaloids

No.	R1	R2	R3,R4	R3	R4	Compound
8	OMe	OMe	O	-	-	mesembrenone
9	OMe	OMe	-	OH	H	mesembrenol
10	OMe	OMe	-	H	OH	6-epimesembrenol
11	OMe	OMe	-	OAc	H	mesembrenol acetate
12	OH	OMe	-	OMe	H	-
13	OMe	H	O	-	-	4'-O-methyl sceletenone
14	OH	H	O	-	-	sceletenone

2.4.3 Δ^7Mesembrine type



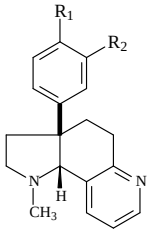
(III)

Table 2.3 Δ^7Mesembrine type (III) *Sceletium* alkaloid

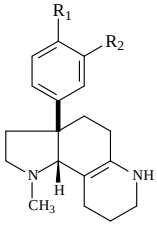
No.	R1	R2	R3,R4	R3	R4	Compound
15	OMe	OMe	O	-	-	Δ^7mesembrenone

2.4.4 *Sceletium* A4 type

Sceletium A4 alkaloid (16) is reported to occur in S. *namaquense* as an optically active crystalline base. The other reported alkaloid which is closely related to this structure is a non-crystalline optically active compound mentioned as dihydropyridone base (17) [42].



(16)



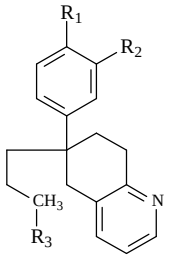
(17)

Table 2.4 ***Sceletium*** **A4 type alkaloids**

No.	R1	R2	Compound
16	OMe	OMe	*Sceletium* A4
17	OMe	OMe	dihydropyridone base

2.4.5 Tortuosamine type

The reported alkaloids in this subclass are tortuosamine (18), N-formyltortuosamine (19) and N-acetyltortuosamine (20). Tortuosamine, a non-crystalline optically active base was isolated from *S. tortuosum* [42].



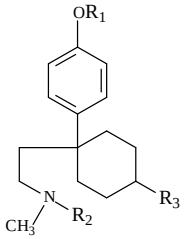
(IV)

Table 2.5 Tortuosamine type (IV) ***Sceletium*** **alkaloids**

No.	R1	R2	R3	Compound
18	OMe	OMe	H	tortuosamine
19	OMe	OMe	CHO	N-formyltortuosamine
20	OMe	OMe	COMe	N-acetyltortuosamine

2.4.6 Joubertiamine type

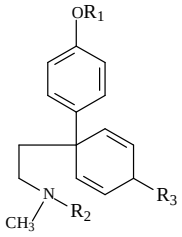
These alkaloids are reported to occur principally in *S. joubertii* and have also been reported to occur in *S. subvelutinum*. These alkaloids are further classified as dihydrojoubertiamine (V) (Table 2.6), dehydrojoubertiamine (VI) (Table 2.7) and joubertiamine (VII) (Table 2.9) [42].



(V)

Table 2.6 Dihydrojoubertiamine type (V) *Sceletium* alkaloids

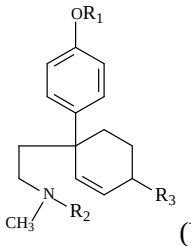
No.	R1	R2	R3	Compound
21	H	Me	O	dihydrojoubertiamine
22	H	Me	Me	O-methyldihydrojoubertiamine



(VI)

Table 2.7 Dehydrojoubertiamine type (VI) *Sceletium* alkaloid

No.	R1	R2	R3	Compound
23	H	Me	O	dehydrojoubertriamine



(VII)

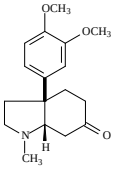
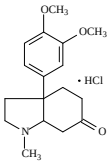
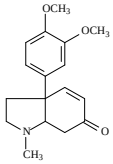
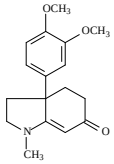
Table 2.8 Joubertiamine type (VII) *Sceletium* alkaloids

No.	R1	R2	R3	Compound
24	H	Me	O	joubertiamine
25	Me	Me	O	O-methyljoubertiamine

2.4.7 Physico-Chemical Characteristics of some *Sceletium* Alkaloid Subclasses

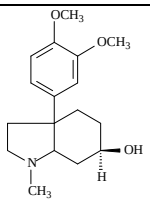
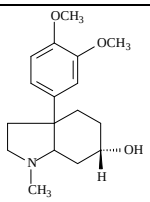
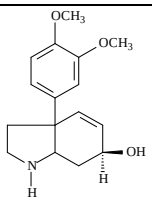
2.4.7.1 Mesembrine type (Table 2.9 and 2.10)

Table 2.9 Physico-chemical characteristics of Mesembrine type *Sceletium* alkaloids (1)

Structure				
Alkaloid	(–)-Mesembrine	(–)-Mesembrine HCl	Mesembrenone	Δ^7 Mesembrenone
MW	289.36	325.80	287.36	287.36
MF	$C_{17}H_{23}NO_3$	$C_{17}H_{23}NO_3$.HCl	$C_{17}H_{21}NO_3$	$C_{17}H_{21}NO_3$
Description	Pale yellow oil	Needle shaped crystals	Low melting solid	-
OR $[\alpha]_D^{20}$	–55.4° (MeOH)	–8.4° (MeOH)	raceimic	-
BP	*186-190°C	-		-
MP		205-206°C	†88-89°C	-
Reference	[42], *[50]	[42]	[42], †[45]	[42]

MW=Molecular weight, MF=Molecular formula, OR=Optical rotation, BP=Boiling point, MP=Melting point, MeOH=Methanol

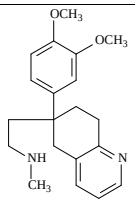
Table 2.10 Physico-chemical characteristics of Mesembrine type *Sceletium* alkaloids (2)

Structure			
Alkaloid	(–)-Mesembranol	Epimesembranol	(–)-N-Demethylmesembranol
MW	291.39	291.39	275.15
MF	$C_{17}H_{25}NO_3$	$C_{17}H_{25}NO_3$	$C_{16}H_{23}NO_3$
Description	Cubic crystals	Brown colored oil	
OR $[\alpha]_D^{20}$	–32°($CHCl_3$), –30° (C_2H_5OH)†	–3.2° (C_2H_5OH)†	–13°
BP	-	-	-
MP	144-145°C		178-185°C
Reference	[42], †[45]	[42], †[45]	[42]

MW=Molecular weight, MF=Molecular formula, OR=Optical rotation, BP=Boiling point, MP=Melting point, $CHCl_3$=Chloroform, C_2H_5OH= Ethanol

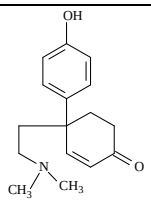
2.4.7.2 Tortuosamine type

Table 2.11 Physico-chemical characteristics of Tortuosamine

Structure	Alkaloid	MW	MF	OR	BP	MP	Reference
	Tortuosamine	326	$C_{20}H_{26}N_2O_2$	-	-	-	[42]

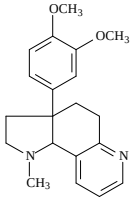
2.4.7.3 Joubertiamine type

Table 2.12 Physico-chemical characteristics of Joubertiamine

Structure	Alkaloid	MW	MF	OR $[\alpha]_D^{20}$	BP	MP	Reference
	Joubertiamine	325.80	$C_{17}H_{25}NO_3$	–32°($CHCl_3$)*, –30° (C_2H_5OH) †	-	-	*[42], †[45]

2.4.7.4 *Sceletium* A4

Table 2.13 Physico-chemical characteristics of *Sceletium* A4

Structure	Alkaloid	MW	MF	OR $[\alpha]_D^{20}$	BP	MP	Reference
	*Sceletium* A4	324.18	$C_{20}H_{24}N_2O_2$	*+131°	-	†153-154°C	*[42], †[46]

MW=Molecular weight, MF=Molecular formula, OR=Optical rotation, BP=Boiling point, MP=Melting point

2.5 USES OF *SCELETIUM*

The historical and traditional preparation of this plant has been reported as *Kougoed* or *Channa*, which refers to a preparation method used by the native Bushmen of Namaqualand. The traditional preparation is made by a fermentation process, which is purported to enhance the stimulant effect of the plant [51].

Smith *et al* 1996 [51], in their review on *Sceletium*, described the techniques of preparation and use of *Kougoed* and reported on subjective experiences of users. It is noted that the traditional preparation by the Bushmen of Namaqualand involved a fermentation process**,** in which whole plant material *kougoed* is bruised and crushed between two stones. Then the mass is preserved to ferment in sheep skin bags and chewed or smoked to produce a state of intoxication. It is also chewed frequently especially when thirsty, indicating that it could be a huger and thirst suppressant. This observation led to commercial interest to cultivate this plant. Those authors [51]concluded that the fermentation process was an effective method to remove the oxalates in the plant and retain the alkaloids which are narcotic-anxiolytic in nature and not hallucinogenic. In addition to its traditional use, it is mentioned that it is also used as a tea and snuff. The use as snuff, described to me by one of the *Sceletium* cultivators, was considered to be an effective method to assess the potency of the processed *Sceletium* powder.

At present, this plant is under patent (US patent 6,288,104; September 11, 2001, by Gericke *et al.* [52]). The patent includes the use of mesembrine and related compounds as serotonin-uptake inhibitors, pharmaceutical compositions comprising such compounds or dry material or an extract of plants from the *Mesembryanthemaceae* family including *S. tortuosum (*Aizoaceae*)*. According to the invention, dosage forms consisting of plant material or an extract containing 20 μg to 2 mg, or between 50-500 μg, or between 100-300 μg of active *Sceletium* alkaloid comprising of mesembrine, mesembranol and mesembrenone, or a mixture of any two components are described. The alkaloid (–)-mesembrine is the preferred active compound. The dose is administered once a day as aqueous ethanolic tinctures, tablet, capsules, nasal sprays and skin patches. The patent also suggests that the formulations may be developed to administer as a sublingual, transdermal or an intra-nasal delivery system. The dosage forms described by the invention is purported to be useful in the treatment of mild to moderate depression, psychological and psychiatric disorders, anxiety, alcohol and drug dependence, bulimia nervosa and obsessive-compulsive disorders [52].

2.6 SOURCES OF *SCELETIUM*

As discussed in Section 2.5, *Sceletium* is used for its psychoactive preparations and historically by the Khoisan tribe of southern Africa. Reports of trade and commercial interests of this plant, date back as early as 17th century [51].

Based on historical records of this plant mentioned by Smith *et al.*, it is understood that this plant is highly revered and held in great esteem by the tribes who collected and hawked it frequently for long distances, which was exchanged for cattle and other commodities [51]. Subsequently, the Dutch colonists further showed commercial interest in this plant and many plants of this family were also introduced to European cultivation [51]. Since then, not much has changed as most of the plants and their products have remained in commercial interests with claims of some pharmacological activity to the extent that the intellectual property rights have been issued for the use of *Sceletium*.

From my experiences with the procurement and collection of these plants, the wild grown plants are almost extinct due to over-harvesting and in some cases it has been mentioned that there are instances where the roots are roasted over fire and consumed. Also, Smith *et al* [51] described a rapid *Kougoed* preparation, where a small fire is made and doused, the ashes are placed in a hollow made in sand and a freshly picked whole plant is placed within and covered with hot sand. The effects from this preparation have been reported to be same as the conventional preparations. These activities whereby the roots are removed indicate that the further propagation of this plant is in danger.

The marketed *Sceletium* is mostly organically grown in greenhouses and cultivated on farms. The samples for this project were mainly obtained from farmers cultivating for commercial production of *Sceletium* products and greenhouses propagating the plants for sale.

Figure 2.10a: Sceletium farm: Northern Cape
Photo courtesy: Mr. L. Rabbets

Figure 2.10b: Sceletium farm: Lushof
Photo courtesy: Mr. F van der Walt

Sceletium farming is presently being exploited on a large scale (Figure 2.10a & b). The major issue with such commercial cultivation is the difficulty in the selection of the proper species which contain the reported alkaloids. During this study, some of the plant specimens collected and supplied from different sources were found to be of incorrectly named species and in most cases the cultivators were not knowledgeable of the plant species they were growing. It was assumed that if the plant has 'skeletonised' leaves, it was considered as *S. tortuosum*. A few cultivators who were interested in finding the right species for cultivation, referred to the only know literature on the identification key to the species by Maike Gerbaulet [41]. It is also important to note that this identification key is applicable to *Sceletium* which grows in the wild under natural conditions. However, with the plants growing in greenhouse controlled conditions, the morphology of leaves and pattern change with growth. It was observed with one such specimen (SP06) that it was difficult to identify as the leaves of this specimen were different from other *Sceletium* plants.

2.7 *SCELETIUM* PRODUCTS

Sceletium is marketed as plant powders, tablets, capsules and sometimes as extracts. One *Sceletium* product is marketed as an alcoholic mother tincture. Most of these products are recommended for chewing, smoking or for use as snuff. The marketing of these products is through the internet and some health shops, but the majority is through the net. Advertisements of the products make extraordinary claims relating to product excellence and the power it has to intoxicate and other purported effects of this plant. The label claims of *Sceletium* products vary in terms of the description of its contents whereas information regarding the alkaloid content is generally not mentioned.

2.7.1 *Sceletium* Plant Products

Sceletium powder samples were obtained mostly from *Sceletium* plant cultivators (Figures 2.11, 2.12, 2.13, 2.15, 2.16, and 2.17), who processed the plant for sale as powder and for use in the manufacture of its products. According to the cultivators who provided these products, the *Sceletium* plants were prepared by fermentation process. One sample of *S. emarcidum* powder (Figure 2.14) was purchased through the internet from a company based in Australia (www.herbalistics.com.au). A list of products is shown in Table 2.14.

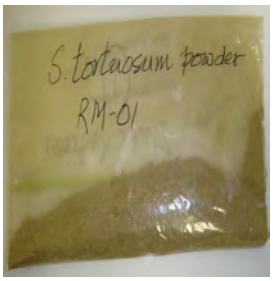


Figure 2.11: SRM01

Figure 2.12: SRM02

Figure 2.13: SRM03

Figure 2.14: SRM04

Figure 2.15: SRM05

Figure 2.16: SRM06

Figure 2.17: SRM07

Table 2.14 List of *Sceletium* plant powder samples

Product code	***Name***	***Source***
SRM01	*S. tortuosum* powder	Mr. R Grobellar
SRM02	*S. tortuosum* powder	Mr. R Grobellar
SRM03	*S. tortuosum* powder	Cederberg, Western Cape
SRM04	*S. emarcidum* powder	*www.herbalistics.com.au* (Australia)
SRM05	*S. tortuosum* powder	Ekogia Foundation, Cederberg, Western Cape
SRM06	*S. emarcidum* powder	Hermanus, Western Cape
SRM07	*S. tortuosum* powder	Lushof, Western Cape

2.7.2 *Sceletium* Formulations

Sceletium tablets manufactured by Big Tree® (Figure 2.18) was the only formulation available in the Eastern Cape province of South Africa. As per the label claim, each tablet contains 50 mg of *Sceletium tortuosum* (probably powdered plant material). The website (Figure 2.19) gives an introduction to *Sceletium tortuosum*, its preparation and the way it can be used. The alkaloids mesembrine, mesembrenone, mesembranol and tortuosamine are stated to be in the concentrations of 0.05% to 2.3%, of which the major alkaloid mesembrine is claimed to be a very potent serotonin re-uptake inhibitor. The claims of *Sceletium* products include their successful use in clinical practice with "excellent results" for treatment of stress and tension.

Figure 2.18: Sceletium tortuosum tablets, Product A

Figure 2.19: Webpage of Big Tree Health Products www.bigtreehealth.com/products/Sceletium-tortuosum.asp, date accessed 20-06-2006

Further, there are also claims that the plant has been organically grown as the plant in wild is almost non-existent. Interestingly, the manufacturer claims that the label complies with the "International labeling requirements as per guidelines of US-FDA" (Figure 2.20).

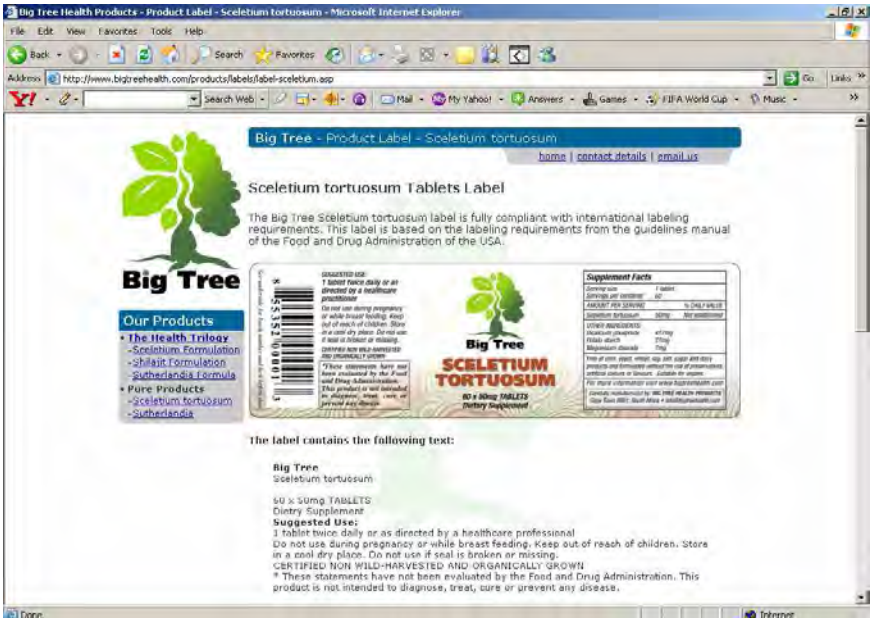


Figure 2.20: Sceletium tortuosum tablet label, claiming to comply with USFDA regulations for labeling www.bigtreehealth.com/products/labels/label-Sceletium.asp, date accessed 20-06-2006

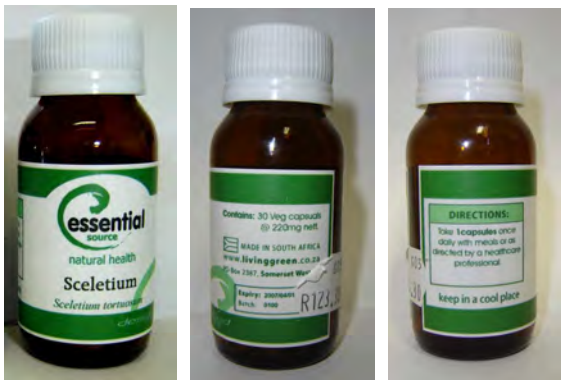
The first product (Product A, Batch no. 7161) was purchased in September 2004 costing ZAR 127.00 at a local health shop in Grahamstown and the second product was purchased (Product B, Batch no. 9332) in September 2005 at a price of ZAR 202.00. Meanwhile, on a visit to another health shop in a nearby city, Port Elizabeth, the price of the same product with identical batch number was purchased at ZAR 98.00. A further product (Product C, Batch no. 9961) was purchased at ZAR 160.00. The direction for use recommends "1 Tablet twice daily or as directed by a health practitioner".

The capsule formulations were purchased from health shops in Cape Town. The capsules were manufactured by Herbal Care produced for Simply Natural (Canal Walk, Cape Town) and was purchased at ZAR 53.00 (Product D, Figure 2.21). It has a label claim of "Sceletium Formulation 30 x 250 mg capsules containing Sceletium 25 mg and *Buchu* 5 mg per capsule". *Buchu* is the common name in South Africa for the plant, *Agathsoma betulina* (Rutaceae), used as an herbal medicine for its essential oils which are reported to act as an antiseptic and urinary tract disinfectant [53]. The label has a caution "Do not use with other psychiatric medication" and "Do not use with alcohol". The recommended dose of this product is 1-3 capsules per day.

Figure 2.21: Sceletium capsules. Manufactured by: Herbal Care. Product B

The *Sceletium* capsules manufactured by Essential Source (Somerset West, South Africa) were purchased on two occasions. One product was purchased in December 2005, costing ZAR 159.00 (Product C, Figure 2.22) and the second product was purchased in September 2006 costing ZAR 123.00 (Product D, Figure 2.23a). The labels of these two products were found to be different. The first product had a label claim of "*Sceletium tortuosum* 30 veg caps@200 mg", without Directions of use. The second product label claimed **"***Sceletium tortuosum* 30 veg capsuals@220 mg nett"[e] (Figure 2.23b) with the following directions: "Take 1 Capsules once daily with meals or as directed by health care professional"[f] (Figure 2.23c)

Figure 2.22: Sceletium tortuosum capsules, Product C



Figures 2.23a 2.23b 2.23c Sceletium tortuosum capsules, Product D

[e] As mentioned on the product label
[f] As mentioned on the product label

The other product purchased was a "*Sceletium tortuosum* Mother Tincture" manufactured by: Essential Source (Product E, Figure: 2.24) with a label claim of "Min 64% EtOH Extract"[g]. Direction of use mentioned "Take 4-8 drops in distilled water 3x daily when necessary" [h]. The "3x daily" probably means "three times daily". The label also suggests that the product is to be used in consultation with a licensed health care professional before using any herbal/health supplement.

Figure 2.24: Sceletium tortuosum Mother Tincture, Product E

[g] As mentioned on the product label
[h] As mentioned on the product label

2.7.3 *Sceletium* Products Marketed on the Internet

In addition to the products mentioned above, there are many other products of *Sceletium* being advertised, sold over the counter and especially on the internet. This makes the regulation of these products extremely difficult. Some of the webpage screens are given below so as to highlight the marketing and claims of these products.

African Drugs.com website (Figure 2.25) markets *Sceletium* products and one of their formulations advertised on the website has been used in this study. The website informs its American and European customers regarding the exchange rates of US dollar and the Euro and the pricing difficulties, which have forced them to indicate their prices in Euros.

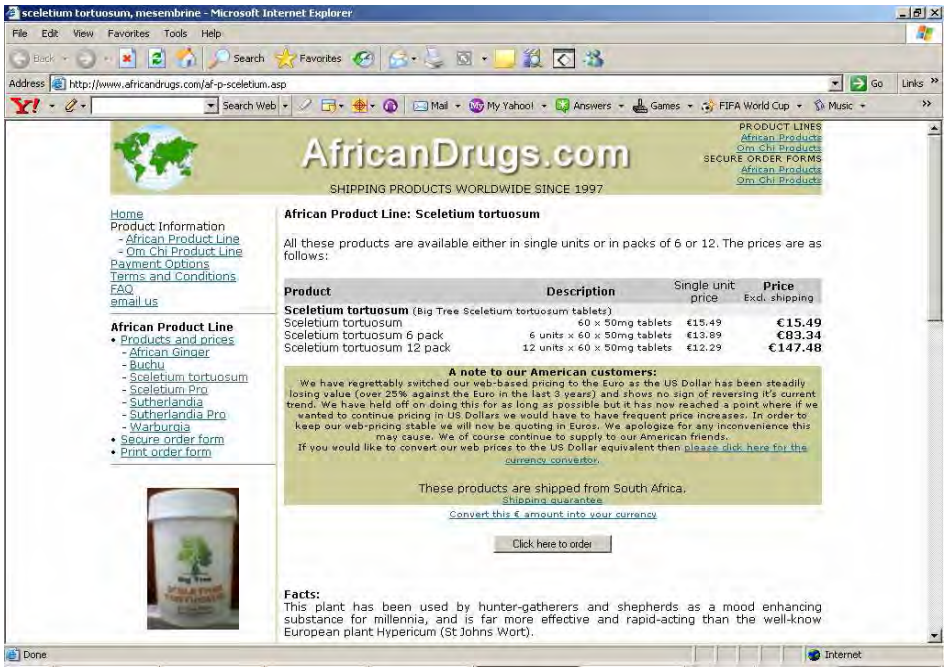


Figure 2.25: African Drugs.com webpage1 www.africadrugs.com/af-p-Sceletium.asp, date accessed 20-06-06

There is another product from this website, advertised as Sceletium Pro (Figure 2.26), which claims to be an improvement on the regular product. The formulation claims to contain another herbal component *Baccopa monneri* (purported to enhance memory), amino acid L-tyrosine, trimethyl glycine, folic acid and vitamin B12. Interestingly, a formula on the same lines for oral supplement containing *Kava-Kava*, *Sceletium*, adenosyl methionine and hydroxytryptophan has been patented by Holford and Associates Limited, UK in 2002 [54].

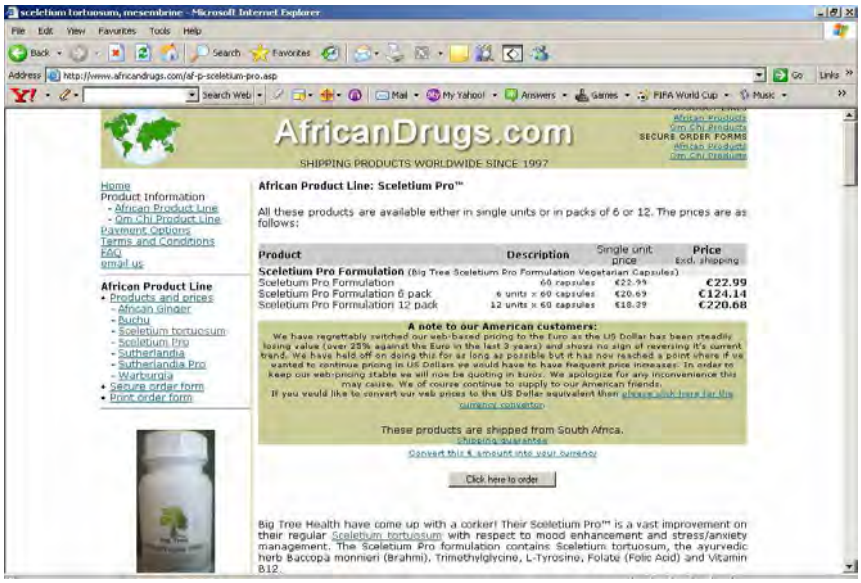


Figure2.26: African Drugs.com webpage2 www.africadrugs.com/af-p-*Sceletium*-pro.asp, *date accessed 20-06-06*

The website marijuanaalternatives.com (Figure 2.27) mentions some of the side effects and drug interactions related to *Sceletium*. It mentions that "very few people experience side effects" which include symptoms like slight nausea, anxiety and irritability after "initiating treatment" and can be corrected by administering a 50 mg dose at night, which reportedly has "no side effects". The website also claims that there are no confirmed drug interactions, but cautions the user that it may cause interactions with other psychiatric drugs, cardiac medicines and its safety in pregnancy has not been established. Interestingly, it mentions that *Sceletium* is related to the herbal medicine St. John's wort, which is sold as incense.

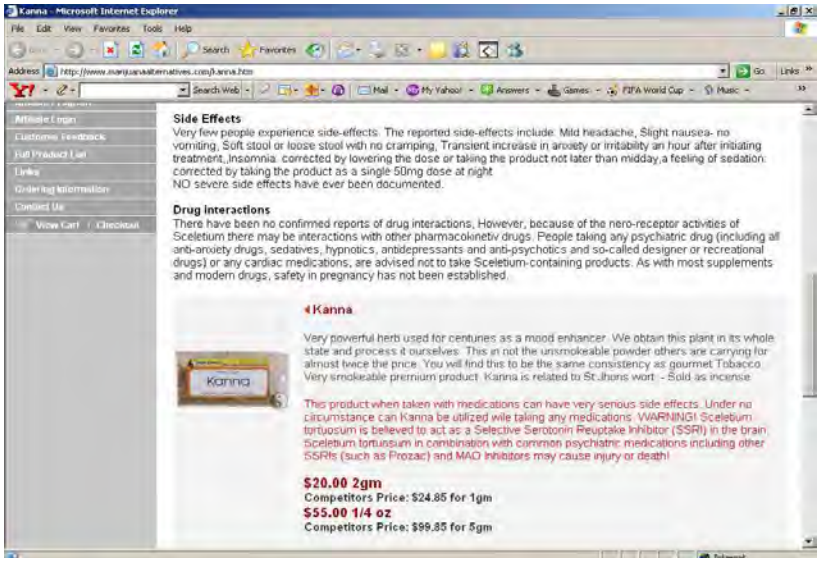


Figure 2.27: Marijuanaalternatives webpage www.marijuanaalternatives.com/kanna.htm, date accessed 20-06-06

The website, ethnoafrica.com (Figure 2.28), advertises to its potential buyers about inferior *Sceletium* products being marketed which are ineffective due to the fact that the mesembrine content is "far below standard". A chart is displayed giving the various percentage content of mesembrine from the *Sceletium* products available and with claims that the material that they market is the most superior as it has "1.71%". The site also claims that their tablets and capsules contain "PURE Sceletium 55 mg per tablet/capsule"

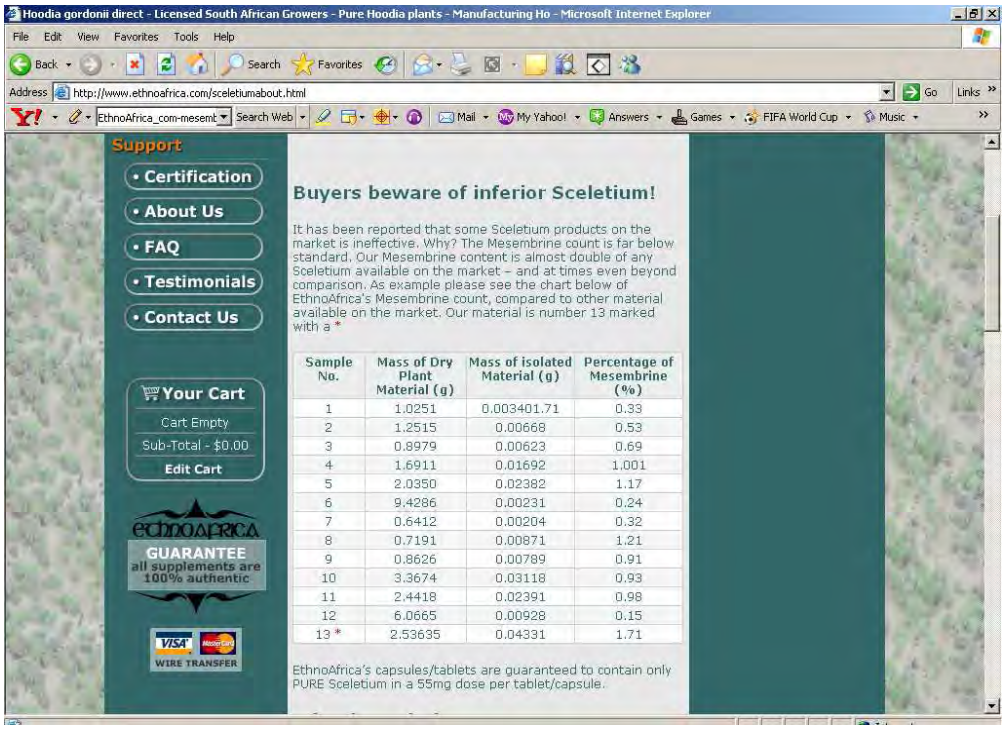


Figure 2.28: Ethnoafrica webpage www.ethnoafrica.com/*Sceletium*about.html , *date accessed 20-06-06*

This interesting website, maya-etnanobotanicals.com (Figure 2.29a), markets *Sceletium* lollipops at USD 4.6 along with other variations of *Sceletium* products which are claimed to be fermented. Information regarding the content and the nature of *Sceletium* used in manufacturing this lollipop product is not provided. The website also markets shredded leaves (Figure 2.29b) and shredded roots of *Sceletium tortuosum*, which have also been fermented.

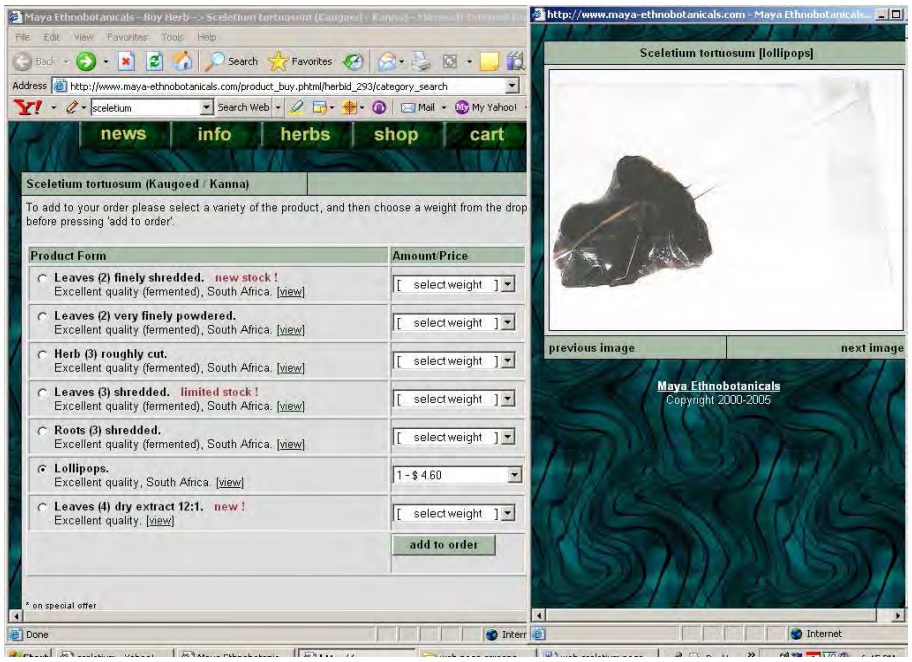


Figure 2.29a: Maya-etnanobotanicals webpage http://www.maya-ethnobotanicals.com/product_buy.phtml/herbid_293/category_search, date accessed 20-06-06

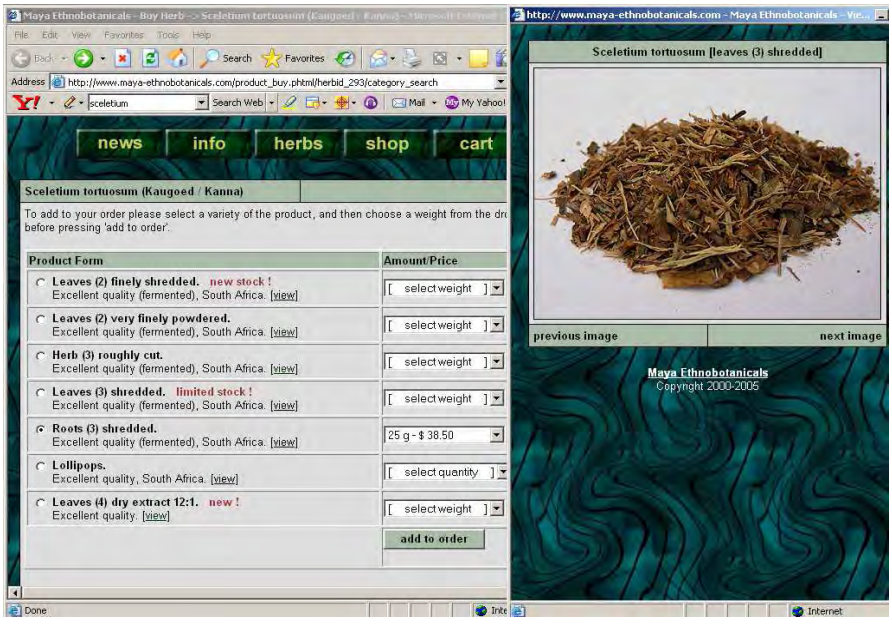


Figure 2.29b: Maya-etnanobotanicals webpage http://www.maya-ethanobotanicals.com/product_buy.phtml/herbid_293category_search, date accessed 20-06-06

The website, www.remedyfind.com (Figure 2.30), describes *Sceletium* as a South African plant and explains its history. The effects are explained and ratings provided for the effects of administration of *Sceletium* and its alkaloids. A "visitor" to this website has suggested that it has to be prescribed by doctors and has rated it as 8.5 on the scale of 10.

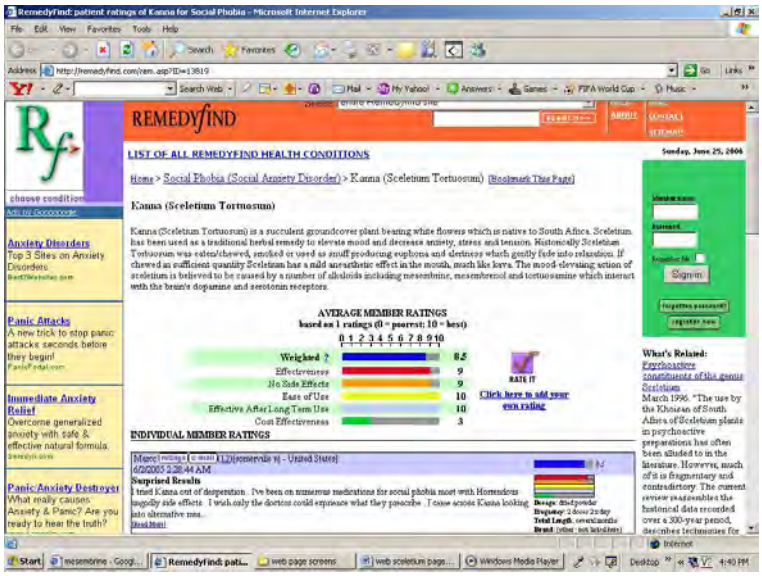


Figure 2.30: Remedyfind webpage www.remedyfind.com/rem?ID=13819 , date accessed 25-06-06

This website of Entheogen (Figure 2.31) notes a communication regarding extraction of *Sceletium* alkaloids using the alcoholic beverage Vodka. The extract provided no purported effects which prompted the user to try an alkali extraction. Another visitor to this website suggests using acetone for extraction.

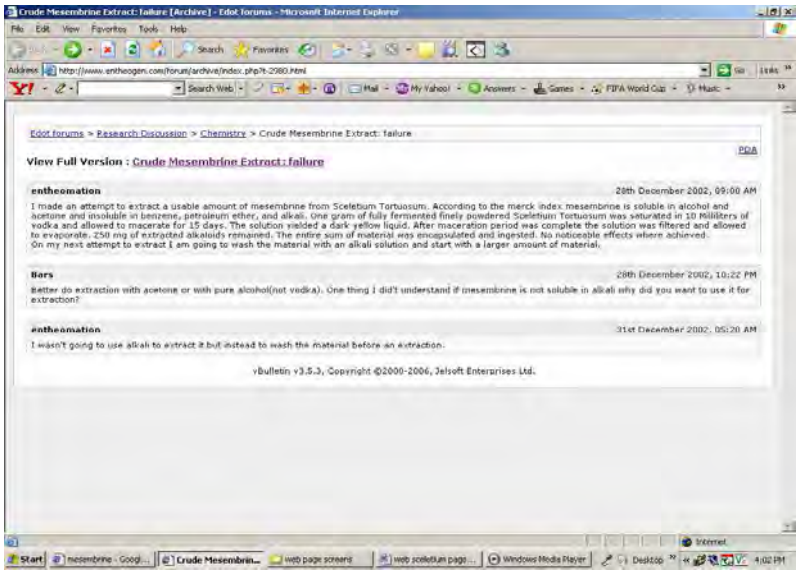


Figure 2.31: Entheogen webpage www.entheogen.com/forum/archive/index.php?t-2980.html , date accessed 20-06-06

Attempts to source products of *Sceletium emarcidum* were not successful in the health shops which sold the *Sceletium tortuosum* products. The availability of this product on the internet prompted me to place an order for 25g sample from Herbalistics, Australia, which was priced at $50 (Australian). The website (Figure 2.32) describes the plant and its cultivation, mentioning it as one of the *Sceletium* species prepared by a fermentation process as done by the Khoisan tribesmen.

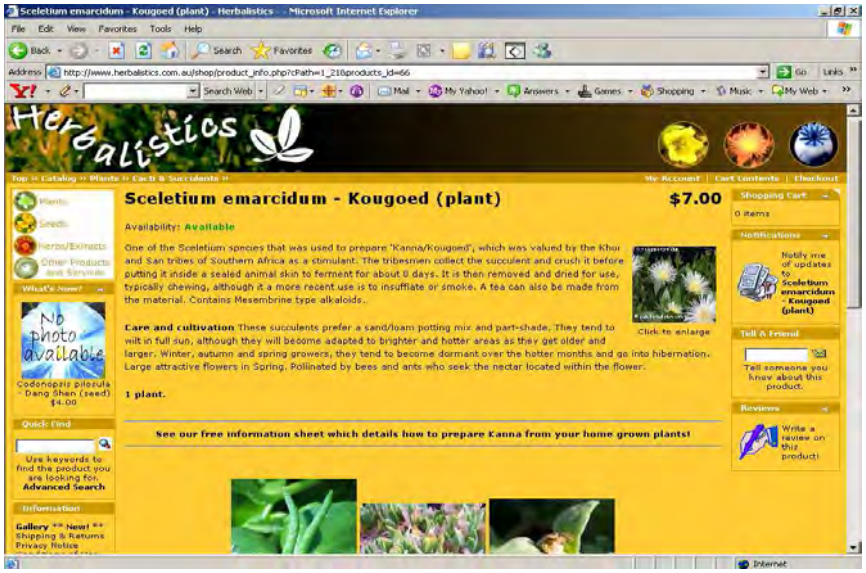


Figure 2.32: Herbalistics webpage 1 www.herbalistics.com.au/shop/product_info.php?cPath=1_21&products_id=66 , *date accessed 20-06-06*

The webpage for *Sceletium emarcidum* extract below (Figure 2.33) claims that the product is five times concentrated for an effective mood elevating experience.

Figure 2.33: Herbalistics webpage 2 *www.herbalistics.com.au/shop/product_info.php?cPath=2_18&products_id=238* , *date accessed 20-06-06*

Even though most of the *Sceletium* species are cultivated in South Africa, for unknown reasons, *S. emarcidum* products are not available here. However, these products are mostly advertised by companies based in Australia. Some of the companies have advertised the availability of *S. emarcidum* plants and extracts for sale (Figure 2.34).

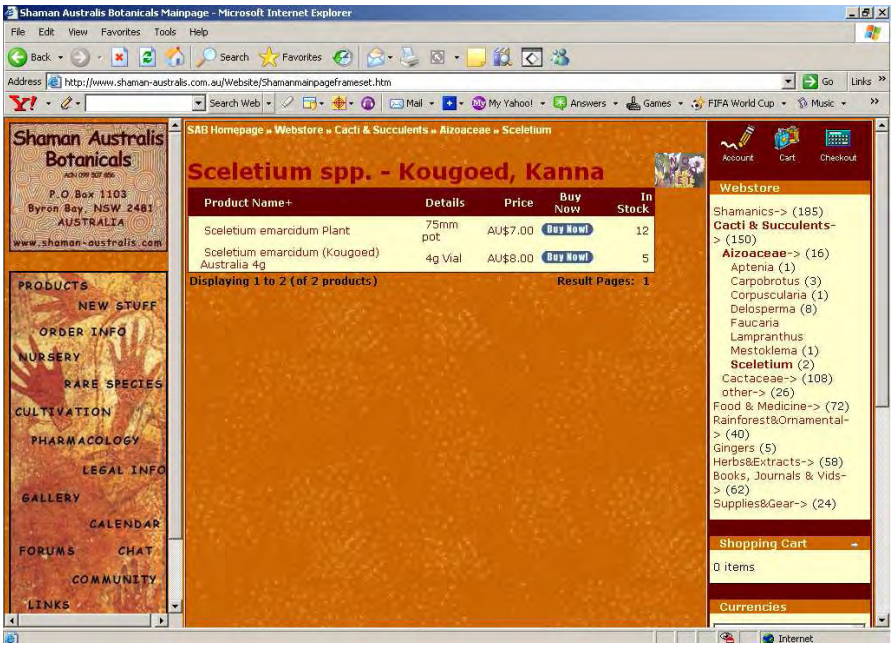


Figure 2.34: Shaman-australis webpage http://www.shaman-australis.com.au/Website/shamanmainpageframeset.htm , date accessed 20-06-06

During the process of sourcing *Sceletium* plants for this project, some of the South African cultivators who provided the *Sceletium* samples were not aware that the species they were cultivating is the *S. emarcidum* type.

Apart from *S. expansum, S. strictum, S. tortuosum, S. varians, S. joubertii and S. subvelutinum* which have been reported to contain alkaloids, the presence of alkaloids in other *Sceletium* species, such as *S. crassicaule, S. emarcidum, S. exalatum, S. rigidum* and *S. varians* are largely unknown.

This situation leads to a major issue associated generally with herbal products, that there could be products containing species which either are devoid of alkaloids or whose alkaloidal content is low. *S. emarcidum,* for example is currently being sold as *S. tortuosum* in South Africa and also on the international herbal market. The present study set in the above background aims to develop systematic and scientific methods to assure quality products of *Sceletium*. This is an important step to assist the cultivators in identifying and cultivating *Sceletium* species containing the relevant alkaloids which improves the quality of the final product(s).

CHAPTER 3

EXTRACTION, ISOLATION, SYNTHESIS AND CHARACTERIZATION OF *SCELETIUM* ALKALOIDS

3.1 INTRODUCTION

Natural products are known to contain complex chemical components. Hence, it is essential that active components in such products are identified and analyzed by validated methods to ensure product quality. The development and validation of the requisite analytical method and procedures for QC can only be achieved by testing the product using qualified reference substances. Although phytochemical components of *Sceletium* alkaloids have previously been isolated, synthesized and their chemical properties studied, only a few methods have been published for use in qualitative analysis [55-57]. The literature is conspicuously absent of analytical methods which can be applied for the quantitative determination of *Sceletium* components and QC of *Sceletium* products.

During the course of this research, efforts to identify a commercial source for these alkaloids, and more specifically the purported major alkaloidal component in *Sceletium* species i.e. mesembrine, were unsuccessful. Thus it was necessary to isolate and characterize relevant alkaloidal components from plant material and qualify them for use as reliable reference substances.

Several methods have been reported for the extraction and isolation of these alkaloids from *Sceletium* species. In 1937, Rimington and Roets [43] extracted *Sceletium* alkaloids by treating the plant material with boiling ether and further continuous extraction with boiling chloroform ($CHCl_3$). Charcoal was used to decolorize the solution and the solution was evaporated to dryness. The residue was stirred with dilute hydrochloric acid (HCl), some of the tarry material discarded and $CHCl_3$ added to the dry residue and subsequently filtered. The hydrochloride solution of the base was washed with petroleum ether which precipitated the crude alkaloidal salt that was found to be a slightly hygroscopic, pale buff colored powder. Attempts to crystallize the alkaloid as a salt or the free base were not successful. Salts of picrate and platinichloride were easily crystallized and the methylated free base was also reported to have been easily crystallized. From these crystalline compounds, the composition of the free base was deduced. This work gave mesembrine the reassigned molecular formula of $C_{17}H_{23}NO_3$ based on combustion analysis.

In 1957, Bodendorf and Krieger [44] extracted *Sceletium* alkaloids from 3 kg of coarsely powdered material with 9 liters of 96% alcohol for 14 days. The alcoholic extract was concentrated *in vacuo* and the residue was treated by stirring with dilute sulphuric acid (H_2SO_4), which precipitated the chlorophyll and other insoluble material. The sulphuric acid solution was alkalinized with caustic soda and further washed with ether, which yielded 33 g of a brown honey-like residue when evaporated. The caustic soda solution was acidified with H_2SO_4 and adjusted to pH 8 with ammonium hydroxide. The basic solution was extracted with ethyl acetate and the extract concentrated and washed with ether to remove the ether soluble components. Crystallization of the bases which were soluble in ether was not successful. Crystals of mesembrine hydrochloride were obtained by dissolving the base in isopropanol (IPA) using etherized hydrochloric acid. The hydrochloride crystallized as small needles, which had a melting point of 199-201°C and a yield of about 0.7%. The remaining alkaline solution was further evaporated to obtain amorphous products which were then suspended in water and re-precipitated with caustic soda solution. The basic components that precipitated were extracted several times with ether and the combined extracts were evaporated and purified over an aluminum oxide column. The eluted components were dissolved in IPA and crystallized by treating with etherized HCl to yield mesembrenone previously known as mesembrinine, which had a melting point of 179-181°C. The ether insoluble fraction was washed with ethyl acetate to obtain another alkaloid by re-suspending the base and crystallizing it from acetic ester. This alkaloid had a melting point of 179-180°C, and was called Channine.

Popelak and Lettenbauer, in 1967 [45] reported the isolation of some alkaloid bases. Mesembrine and mesembrinine hydrochlorides were isolated with yields of 0.7% and 0.2% from 'Channa'. They also noted that other alkaloids were present only in very small quantities. Channa was extracted by treatment with alcohol which was then distilled off and the residue was then treated with dilute H_2SO_4 and the insoluble materials were removed by filtration. The aqueous layer was alkalinized and first extracted with ether followed by further extraction with ethyl acetate which yielded an oily residue and a solid, respectively. The ether-soluble oily fraction was dissolved in IPA, which yielded mesembrine hydrochloride when treated with ethereal hydrochloride. The solid residue from ethyl acetate was crystallized with a mixture of ethyl acetate and IPA, which yielded the alkaloid Channine.

Arndt and Kruger [47] reported an extraction procedure of the aerial parts of *Sceletium joubertii* (3.30 kg) using 2% ethanolic tartaric acid, which yielded a crude alkaloid mixture of about 1.10 g. The crude mixture was repeatedly chromatographed on alumina, and silica gel impregnated with sodium carbonate to obtain the alkaloids.

Herbert and Kattah [48] in their biosynthesis study of alkaloids in *Sceletium subvelutinum* reported the isolation and purification of joubertiamine and related alkaloids. The extraction was carried out on whole plant material macerated with ethanol (500 ml). The macerate was filtered and the residue was extracted further by soxhlet extraction with ethanol. The combined extracts were filtered, evaporated and the residue was taken up into 300 ml $CHCl_3$, washed with a saturated aqueous sodium carbonate solution (3 x 100 ml) and extracted with 1M HCl (3 x 150 ml). The volume of the combined acid extracts were reduced, filtered and the filtrate was basified with sodium carbonate and extracted with $CHCl_3$ (5 x 100 ml) and the combined extracts were dried. Crude alkaloid was obtained when the solvent was evaporated, which was 0.047% of the weight of wet plant material. The alkaloid mixture was further purified by chromatography on a silica gel column, eluted with chloroform: methanol: conc. ammonia (80 ml: 20 ml: 20 drops) and then further by either normal phase HPLC with a silica column eluted with the same mobile phase or alternatively with a reverse phase (PLRP-S 100A 10 micron 300 x 7.5 mm) column eluted with acetonitrile: water and conc. ammonia to separate the following alkaloids: O-methyldehydrojoubertiamine, O-methyljoubertiamine, O-methyldihydrojoubertiamine, dehydrojoubertiamine, joubertiamine, N,N-dimethyltyramine and dihydrojoubertiamine.

Jeffs *et al.* [58] reported the extraction of alkaloids from *Sceletium namaquense* where 3.5 kg dried plant material was subjected to 17 hour soxhlet extraction with ethanol (95%) (15 L) and the extracted material was further macerated with ethanol (10 L) in portions using a blender. The combined ethanol extracts were acidified with 5% tartaric acid after concentrating to 2.5 L. The acidic solution was extracted with ether (5 x 500 ml) and the ether extract was discarded. The aqueous layer was basified with sodium carbonate and further extracted successively first with chloroform (10 x 500 ml) and then with chloroform:methanol (4:1 mixture) (1 x 300 ml). The organic extracts were combined and concentrated to 1.5 L and filtered to remove small quantities of insoluble material. The chloroform layer was first extracted with 1N NaOH (3 x 200 ml) and then washed thoroughly with water. The chloroform layer was evaporated to obtain 120 g of non-phenolic alkaloids. The sodium hydroxide layer was adjusted to pH 9 with carbon dioxide (CO_2) and extracted with $CHCl_3$ (5 x 100 ml), which yielded 20 g of phenolic alkaloids. The non-phenolic alkaloid fraction (120 g) was refluxed with ether (3 x 500 ml), which extracted the ether-soluble alkaloids (60 g), mesembrine and mesembrenone. Further, the ether–insoluble residue (60 g) was dissolved in 1200 ml of $CHCl_3$:MeOH (3:1 mixture) which was passed through a 1400 g silica gel (170-200 mesh) column. The evaporated eluate yielded 50 g of alkaloidal material which was dissolved in chloroform and chromatographed over neutral alumina (activity IV) using a linear solvent gradient of $CHCl_3$-$CHCl_3$:MeOH (4:1) and 15 ml fractions were collected. The fractions collected at different intervals yielded mesembrine, mesembrenone, *Sceletium* A4, N-formyltortuosamine, Δ^7mesembrenone, tortuosamine and unidentified alkaloids.

Smith *et al.* [55] extracted the *Sceletium* alkaloids from 25 g plant material with 95% ethanol (200 ml) for 12 hours in a soxhlet apparatus. The ethanol was evaporated from the extract using a rotary evaporator. The residue was re-dissolved in 2N HCl (20 ml), which was partitioned against three washes of diethyl ether (150 ml) to remove fatty material and pigments. The aqueous solution was applied to columns packed with 60 ml Extrelut® N20 pre-packed columns and left for 20 minutes. The column was eluted with 40 ml of dichloromethane:isopropanol (85:15) and the eluate was discarded. The column was basified using ammonia gas and the alkaloids were eluted with a further 40 ml of dichloromethane:isopropanol (85:15). In view of the very low concentrations of alkaloids in the plant material, the extracts of three lots of 25 g plant material were pooled and the solvent volume was reduced to 2 ml using a rotary evaporator. This 2 ml aliquot was further subjected to column chromatography on silica gel slurried in dichloromethane. Successive elutions were carried using 35 ml each of dichloromethane, ethyl acetate, acetone, acetonitrile, methanol and acetic acid. Each of these eluates were concentrated to 2 ml and analyzed by gas chromatograph fitted with a Nitrogen-Phosphorous specific detector (NPD). The acetone fraction yielded 3 mesembrine alkaloids, a small amount of mesembrine alkaloid was found in the acetonitrile fraction whereas no alkaloids eluted in any of the methanol, acetic acid, dichloromethane or ethyl acetate fractions. All the alkaloids eluted with acetone when the elution volume was changed from 35 to 70 ml.

Gericke *et al.* [52] in their US patent application described the extraction of alkaloids from dried plant material or alcoholic extracts which were extracted by mixing with 15 ml 0.05M sulphuric acid at room temperature for 20 minutes and filtered. The retained solids were re-extracted with 5 ml 0.05M H_2SO_4. The combined aqueous phases were applied to glass columns with a coarse grade Celite® which was alkalinized with ammonia and extracted with 100 ml of dichloromethane (DCM). The DCM extracts were passed over anhydrous sodium sulfate to dry and the solvent evaporated, which yielded a pale brown oil containing alkaloids. The procedure mentions that the alkaloids can also be extracted with hot or cold water instead of H_2SO_4 or with methanol, ethanol, acetonitrile, chloroform or with dichloromethane. Yield figures for mesembrine are reported to be variable but were observed to be between 15 and 35 mg per gram of dry leaves.

3.2 EXTRACTION OF *SCELETIUM* ALKALOIDS

3.2.1 Reagents and Materials

HPLC grade methanol (215) and acetonitrile (200) were obtained from Romil Ltd. (Cambridge, Great Britain). Platinum oxide (IV) was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Silica gel 60 chromatography grade (70-230 mesh ASTM), chloroform-D1 (99.8%), TLC aluminium sheets 20 x 20 cm, Silica gel 60 F_{254}, acetic acid and charcoal were purchased from Merck KGaA (Darmstadt, Germany). Ethyl acetate was purchased from Burdick and Jackson (Muskegon, Michigan, USA). Diethylether, isopropyl alcohol, acetone, dichloromethane, and ammonium hydroxide 25% solution were acquired from Associated Chemical Enterprises (Pty) Ltd. (Southdale, South Africa). Orthophosphoric acid was obtained from Merck Chemicals (Pty) Ltd. (Wadeville, South Africa). Water was purified in a Milli-Q® system, Millipore (Bedford, USA) and Millex HV® hydrophilic PVDF (0.45 µm) membrane filters were purchased from the same source. Bismuth subnitrate was obtained from Hopkins and Williams (Essex, England). Anhydrous sodium sulphate, sodium nitrite and potassium iodide were purchased from BDH Chemicals Ltd. (Poole, England). Diaion® HP-20 was obtained from Supleco (Bellefonte, PA, USA) and pH indicator paper was purchased from Test Papers Ltd. (Herts, England).

3.2.2 Instrumentation

Semi-preparative HPLC was constructed by assembling a SpectraSYSTEM P2000 pump connected to an AS 1000 auto sampler and data integrator SP4290 (Thermo Separation Products, Riviera Beach, FL, USA). The detection was carried out on a Linear 206 PHD detector (Linear instruments Corp. Nevada, USA). The separation of alkaloids was achieved on a Luna® C_{18} (2) column (5 µm, 250 mm x 10 mm i.d.) (Phenomenex®, Torrence, CA, USA). The isolation process was monitored using an Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA). The LCMS analyses were carried out using a Finnigan MAT LCQ ion trap mass spectrometer (Finnigan, San Jose, CA, USA) coupled to a SpectraSYSTEM P2000 pump connected to an AS1000 auto sampler and UV1000 variable-wavelength UV detector (Thermo Separation Products, Riviera Beach, FL, USA).

3.3 EXPERIMENTAL

3.3.1 Raw Material Collection

The preliminary work was done on *S. tortuosum* plant powder, obtained as a gift sample (Figure 3.1) from Bioharmony, Wynberg, South Africa and used for extraction, which was initially carried out according to the method described by Smith *et al.* [55] in order to identify any alkaloid(s) present in the sample.

The scale-up of the developed extraction procedure to isolate alkaloids in larger quantities was carried out on a dried plant sample gratefully supplied by Mr. R. Grobellaar [59].

Figure 3.1: Sceletium tortuosum- dried plant powder supplied by Bioharmony

3.3.2 Extraction

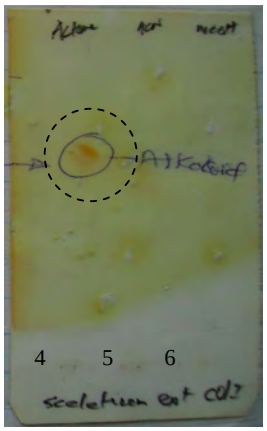
3.3.2.1 Preliminary Extraction work

The extraction procedure was based on a general method, where the plant material was extracted with solvent, which was evaporated and treated with an aqueous acid. The aqueous acid was basified with an alkali and the alkaloid bases extracted with a suitable water immiscible non-polar solvent. Various isolation processes have been reported for the relevant alkaloids, where different techniques and procedures have been used. The preliminary extraction work was carried out according to the procedure described in the patent literature [52] and reported by Smith *et al.* [55].

The reported procedures were carried out and the presence of alkaloids was confirmed in the pre-column extracts, which were tested by the TLC method described by Smith *et al.* [55]. The fractions collected from column chromatography were tested by TLC for the presence of alkaloids by viewing under UV_{254}. The acetone fraction showed one primary spot at retention factor (Rf) of 0.67 and two faint secondary spots below the primary spot, of which one spot was more intense under UV_{366}. The acetonitrile fraction showed a very faint spot when observed under UV_{366} but no spots under UV_{254}. The fractions were further chromatographed by TLC and sprayed with Dragendorff's reagent [60] for visual identification of alkaloids. The acetone fraction showed an orange spot indicating the presence of alkaloids, whereas other fractions tested negative (Figures 3.2a and 3.2b). Attempts to isolate pure mesembrenone (mesembrinine) by solvent extraction were not successful.

Although, these procedures yielded alkaloids, it was found to be cumbersome and expensive due to the use of materials such as Celite® and Extrelut® N20 pre-packed columns as well as the use of ammonia gas to basify the column. Acetic acid elution is not recommended due to its pungent nature.

Figure 3.2a: Column fractions analyzed as per Smith et al. [55] TLC method. Visualized: UV_{254}



1. *DCM fraction*
2. *Ethyl acetate fraction*
3. *Acetone-1 fraction*
4. *Acetone-2 fraction*
5. *Acetonitrile fraction*
6. *Methanol fraction*

Figure 3.2b: Alkaloid identification of acetone, ACN and MeOH fraction. Visualized: Dragendorff reagent

The developed extraction procedure for alkaloids discussed in this chapter is relatively simple and inexpensive. The preliminary extraction of alkaloids was carried out by simple solvent extraction and the isolation procedures involved chromatographic techniques.

3.3.2.2 Soxhlet Extraction

Figure 3.3: Soxhlet extraction of Sceletium plant material

A further *Sceletium* plant sample provided as a gift from Mr. R. Grobellar was tested and confirmed for alkaloids by a TLC method which is described in Section 3.4. The dried plant material (25 g) was extracted with absolute alcohol (250 ml) by refluxing for 8 hours in a soxhlet extractor (Figure 3.3) connected to a water condenser. The alcohol extract was evaporated under reduced pressure using a rotary evaporator. The resinous brown mass was suspended in dilute HCl (50 ml) and shaken to disperse. The aqueous acid extract was transferred to a separating flask and washed with diethyl ether (3 x 50 ml) to remove the fatty pigments. The aqueous acidic layer was further treated with 25% ammonium hydroxide solution (10 ml) to render the pH of the solution basic and tested by color change of red litmus paper to blue. The basic layer was further extracted with DCM (4 x 20 ml) and the DCM layer collected through anhydrous sodium sulfate into a round bottom flask. The solvent was

evaporated under vacuum to obtain an amber colored resinous mass. The presence of alkaloids in the resinous residue was confirmed by TLC (*vide infra*) (Figure 3.4) and column chromatography was subsequently used to isolate the relevant alkaloids.

3.3.2.3 Column Chromatography

Column chromatography was carried out on a glass column (17 cm x 2 cm i.d.) packed by suspending silica gel (25 g) in 100 ml of DCM and stirred to form a slurry. The gel was carefully transferred into the column to ensure uniformity and absence of air traps as well as column cracks. The column was washed with DCM (20 ml) with care being taken to retain the solvent level above the silica packing. The alkaloid residue was dissolved in 5 ml of DCM and carefully transferred onto the column to obtain a uniform band above the silica gel packing. The column was eluted with solvents in the following order: DCM (40 ml), acetone (2 x 40 ml), methanol (MeOH) (40 ml) and acetonitrile (ACN) (40 ml). The collected eluents were spotted on a TLC plate to identify the alkaloids. It was noted that the ACN fraction eluted a very dark and distinct band when compared to the other eluents, acetone and MeOH, which were pale yellow in colour.

The collected eluents were tested by the developed TLC method to identify the presence of alkaloids. The TLC plate was first observed under UV_{254} which showed extensive related substances (*acetone-Track 3 and acetonitrile-Track 4*) and further sprayed with Dragendorff's reagent (Figure 3.4). The acetone fraction and the acetonitrile fractions were found to contain alkaloids. Interestingly, the spot observed from the ACN fraction (*Track 4*) was observed to have different Rf value from that of acetone fraction, which indicated that this alkaloid could be structurally different.

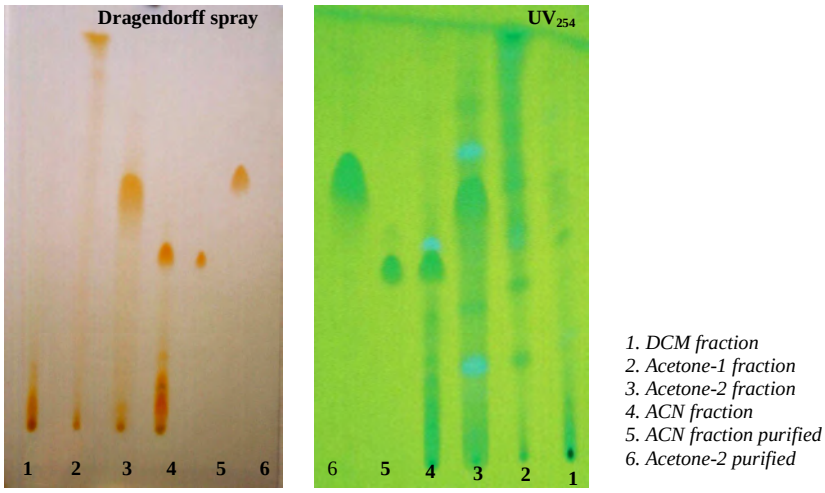


Figure 3.4: TLC plate of the column fractions by developed TLC method observed under UV_{254} and subsequently sprayed with Dragendorff's reagent for positive identification of alkaloids

The acetone and acetonitrile fractions were exposed to a stream of nitrogen at 50°C to evaporate off solvent and the resulting residues were found to be dark in color. These were then dissolved in 10 ml of acetone and treated with charcoal (100 mg) at 40°C and shaken to disperse the contents. The acetone residue after charcoal treatment was pale yellow in colour. The acetonitrile residue which was dark brown in colour (*Track 4*) was suspended in acetone and shaken, which yielded insoluble material which was removed by filtering and the filtrate was evaporated and treated with charcoal which was shaken at 40°C. The charcoal was filtered off and the clear filtrate was collected and evaporated. These fractions were further purified by preparative TLC by applying 2-3 ml quantities on a 20 x 20 cm TLC plates. The plates were observed under UV_{254} and the bands were marked. The marked band of silica gel was scrapped off the TLC plate. The collected silica gel was suspended in 20 ml acetone, sonicated for 5 minutes and filtered through a 0.45 µm PVDF membrane filter and the clear filtrate was evaporated to obtain a clear pale yellow oily residue (*Track 5*). The acetonitrile residue was also subjected to the same process to obtain a pale brown residue (*Track 6*).

The acetone fraction was tested on an HPLC-PDA system (Figure 3.5) for its chromatographic purity that was about 85% of principal peak at RT 7.19 minutes and 15% secondary peak at RT 6.54 minutes. The compounds showed peak maxima at 225 and 280.5 nm and 229.7 and 279.3 nm for secondary and primary peaks, respectively. The NMR analysis of this fraction also concluded that the isolated compound was not pure and contained another compound that indicated a structure which could probably be mesembrenone.

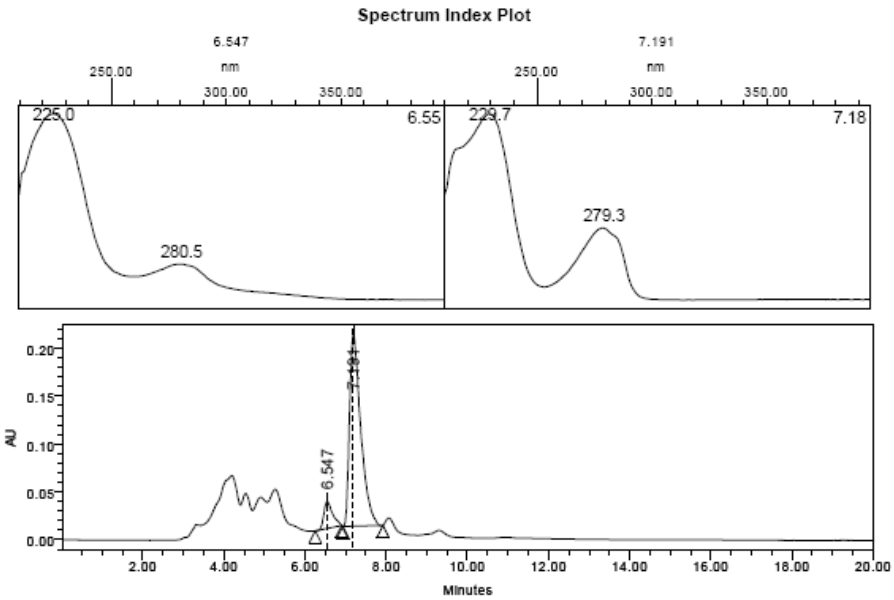
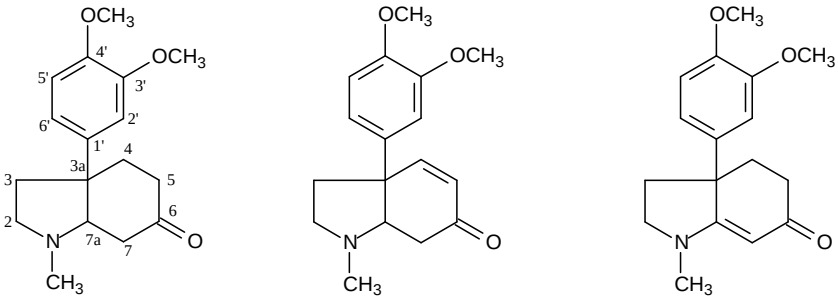


Figure 3.5: HPLC-PDA of acetone fraction- spectrum index plot (top) and chromatogram (bottom)

The acetone residues were chromatographed on TLC plates. Upon close observation under UV light, it was seen that a very slightly distinct band eluted below and very close to the principal band. Even though the bands were not well separated, only top portions of the bands were marked and scrapped off, dissolved and extracted with acetone. The acetone was evaporated under a stream of nitrogen at 50°C and the residue was subjected to NMR in order to determine its structure. The NMR data confirmed the structures of the isolated compounds as mesembrine (Figure 3.6a), which was the major alkaloid and mesembrenone (Figure 3.6b). In this process, the yields of the isolated compounds were very low as a result of loss due to selection and marking of bands on the TLC plate.

Mesembrine **Mesembrenone** **Δ^7 Mesembrenone**



a *b* *c*

Figure 3.6 a-c: Structure of the isolated compounds- mesembrine (a), mesembrenone (b) and Δ^7 mesembrenone (c)

The ACN fraction was also tested for its characteristics by HPLC-PDA system. The principal peak (RT 5.00 minutes) showed a UV maximum at 298.2 nm (Figure 3.7) and this fraction was further purified by preparative TLC. The structure of the isolated compound was confirmed by NMR analysis, and was found to be Δ^7mesembrenone (Figure 3.6c). The NMR data of the isolated compounds are discussed in the spectral data section (*vide infra).*

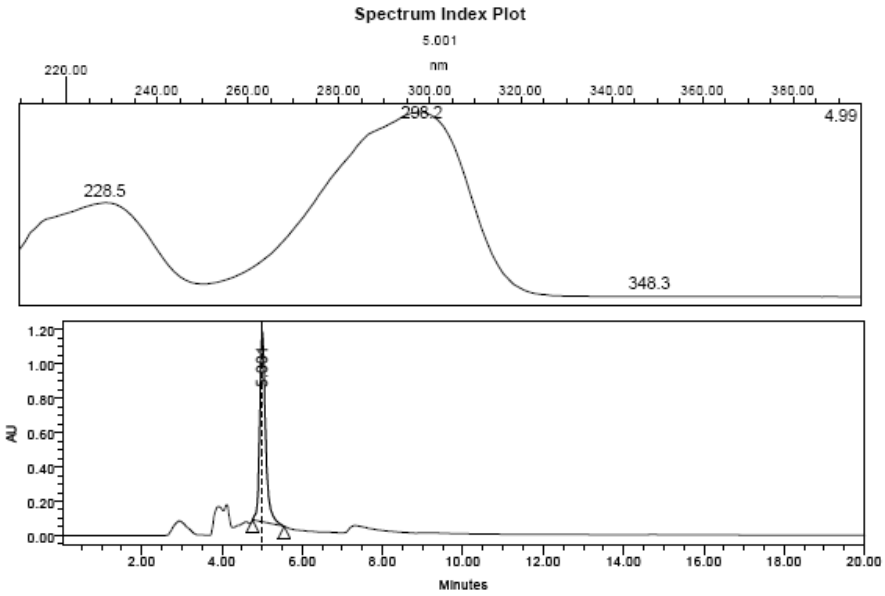


Figure 3.7: HPLC-PDA of ACN fraction-spectrum index plot (top) and chromatogram (bottom)

The isolated alkaloids provided the important analytical markers for further extraction work and all subsequent extractions were scaled-up and the process modified to isolate and purify larger quantities of the relevant alkaloids for use as analytical markers. The scale-up extraction process was performed on 800 g of the previously obtained *Sceletium* powder. The batch size was increased to 50 g (14 batches) and quantities of all the solvents and chemicals were increased proportionately. The preparative TLC for isolating mesembrine and mesembrenone was found to be tedious and time consuming. Hence, the TLC process was replaced by semi-preparative HPLC using a 250 mm x 10 mm C_{18} semi-preparative column connected to a HPLC pump and UV detector set at 280 nm. An isocratic mobile phase consisting of a mixture of water: acetonitrile: ammonium hydroxide solution (25%), (50:50:0.05, v:v:v), was used at a flow rate of 1 ml/min. The sample, obtained from the acetone fraction from the previously described column chromatographic procedure, was dissolved in methanol (25 mg/ml) and injected using an auto-sampler with a 100 µl loop size. The sample was monitored for the peaks corresponding to mesembrenone and mesembrine. The fractions were collected at the RTs observed for these peaks. The collected fractions were accumulated in a volume of about 250 ml and the solvent was evaporated under vacuum using a rotary evaporator. The

residue was dissolved in acetone and transferred into an appropriate glass container and evaporated under a stream of nitrogen at 50°C in a water bath. The collected fraction-1 yielded a pale yellow oily compound, mesembrenone (105 mg) (Figure 3.8a), whereas fraction-2 yielded a pale yellow viscous oily compound, mesembrine (3 g). The collected residues from the two fractions were subjected to NMR analysis for structural confirmation.

The ACN fractions from the column chromatography were collected and treated by the same process used for the preliminary extraction and isolation process. This was possible since the ACN fraction exclusively yielded a single alkaloid, Δ^7mesembrenone. The purified sample of Δ^7mesembrenone (120 mg) (Figure 3.8b) was subjected to NMR analysis to confirm its structure.

Figure 3.8a: Mesembrenone

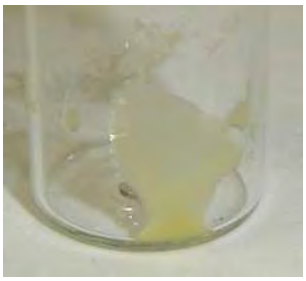


Figure 3.8b: Δ^7Mesembrenone

During the column chromatography procedures used to extract the relevant alkaloids, samples were collected for subsequent analysis by liquid chromatography-mass spectrometry (LCMS) to characterize and confirm the nature of compounds present in the various fractions. An LCMS method was developed (*vide infra)* (Chapter 5) and used for this analysis. The compounds resulting from the soxhlet extraction, prior to being subjected to the column chromatographic purification processes, were found to contain Δ^7mesembrenone (RT 7.05 minutes, *m/z* 288 $[M+H]^+$), mesembrenone (RT 10.34 minutes, *m/z* 288 $[M+H]^+$), with the major alkaloid being mesembrine (RT 15.03 minutes, *m/z* 290 $[M+H]^+$) (Figure 3.9)

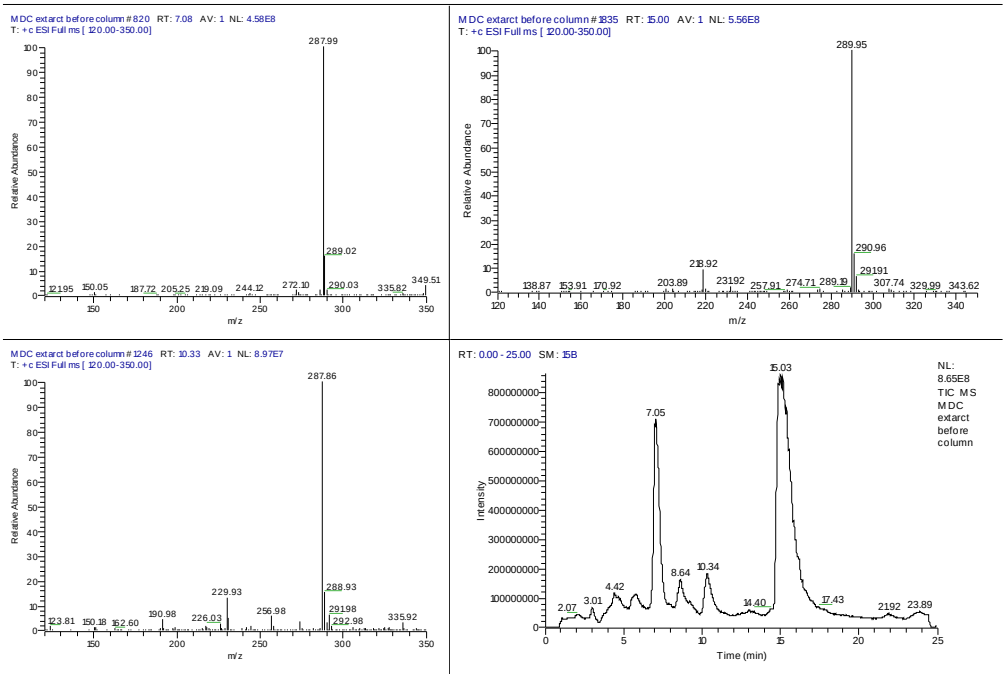


Figure 3.9: Total ion current (TIC) chromatogram of pre-column Sceletium extract (bottom right), ion spectrum of Δ^7mesembrenone (top left), mesembrenone (bottom left) and mesembrine (top left)

Confirmation of the presence of the alkaloids, mesembrenone (RT 9.42 minutes, *m/z* 288 $[M+H]^+$) and mesembrine (RT 13.7 minutes, *m/z* 290 $[M+H]^+$) in the acetone fraction was established by LCMS. (Figure 3.10)

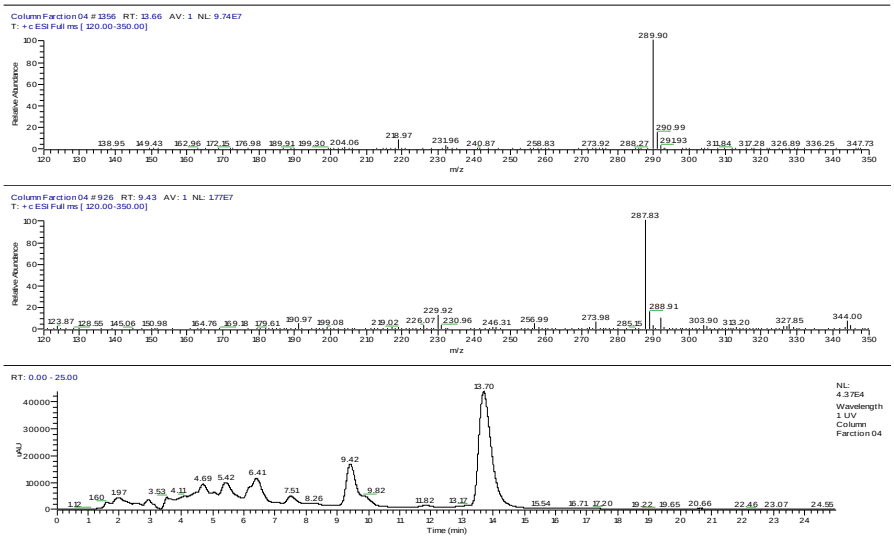


Figure 3.10: HPLC-UV chromatogram of acetone fraction (bottom), ion spectrum of mesembrenone (middle) and mesembrine (top)

Similarly, LCMS confirmed that the ACN fraction contained Δ^7mesembrenone (RT 6.94 minutes, *m/z* 288 $[M+H]^+$) (Figure 3.11).

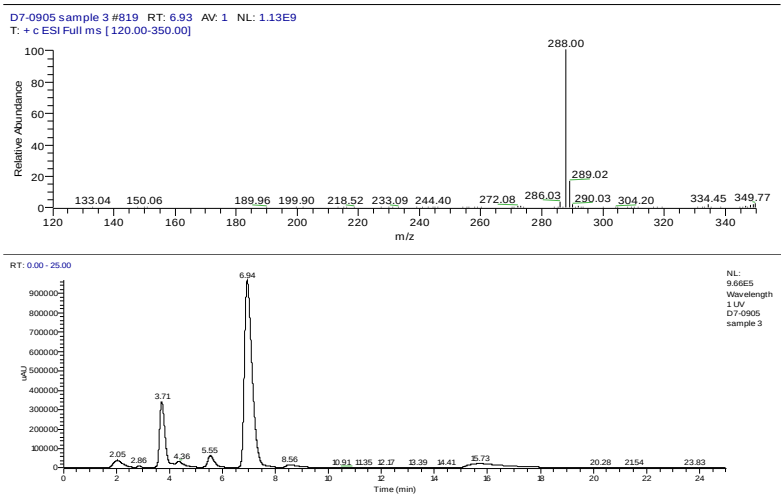


Figure 3.11: HPLC-UV chromatogram of ACN fraction (bottom), ion spectrum of Δ^7mesembrenone (top)

When the silica gel column was further eluted with additional acetonitrile (50 ml) the collected fraction upon analysis was found to contain a compound with a mass of *m/z* 292 $[M+H]^+$ at RT 7.04 minutes (Figure 3.12), which corresponds to another alkaloid, mesembranol (MW=291). However, the yield from this fraction after purification was very low, making isolation of this compound impractical. Hence, catalytic reduction of mesembrine to mesembranol was considered to be a better option and is discussed later in this chapter.

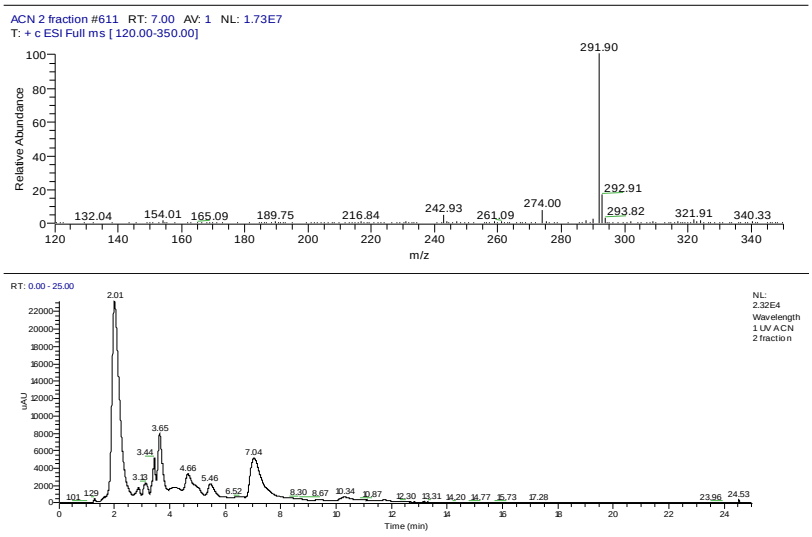


Figure 3.12: HPLC-UV chromatogram of ACN-2 fraction (bottom), ion spectrum of mesembranol (top)

3.3.2.4 Conversion of Mesembrine to Mesembrine Hydrochloride

The process was carried out firstly by production of HCl gas for the preparation of acidic ether. HCl gas was produced separately in a three necked round bottom flask containing NaCl (10 g) which was reacted with H_2SO_4 (15 ml) by careful addition through a separating funnel. The liberated HCl gas was collected into diethyl ether through a glass tube. Separately, mesembrine base (360 mg) (Figure 3.13a) was dissolved in IPA (3 ml) and acidic ether was slowly added to the IPA solution, which precipitated the hydrochloride salt as an amorphous pale white powder. The IPA was removed by filtration and the salt was dissolved in methanol (3 ml) and evaporated under a stream of nitrogen at 50°C. This process produced a crystalline-glassy material. The salt was redissolved in IPA (5 ml), allowed to remain overnight, and needle shaped crystals (Figure 3.13b) were formed. The solvent was carefully removed and the crystals of mesembrine hydrochloride (220 mg) were air dried. Using a Metler® Melting point apparatus the melting point of these crystals was found to be 208.2°C.

Figure 3.13a: Mesembrine base

Figure 3.13b: Needle shaped crystals of mesembrine hydrochloride

The compound was subjected to LCMS-Electro spray ionization (ESI) positive mode (+) and the ion was found to be *m/z* 290 $[M+H]^+$ corresponding to mesembrine whose mass is 289 (Figure 3.14).

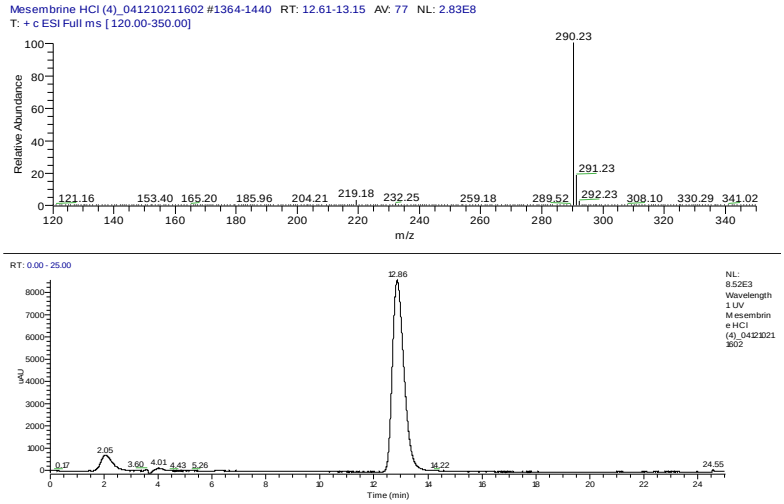


Figure 3.14: HPLC-UV chromatogram (bottom) and ion spectrum (top) of mesembrine hydrochloride

3.3.3 Synthesis of Mesembranol and Epimesembranol

3.3.3.1 Catalytic Hydrogenation

Catalytic hydrogenation is a process that involves reduction of functional groups with hydrogen (H_2) gas. The method involves the addition of hydrogen gas in the presence of a catalytic transition metal for the reaction to occur, and the reaction proceeds by adsorption of hydrogen and the substrate onto the metal. The extent of reducing a functional group depends on the nature and amount of catalyst and the procedure of hydrogenation. The most commonly used catalysts are platinum, palladium, nickel rhodium, iridium and ruthenium.

Bodendorf and Krieger [44], produced mesembranol from mesembrine dissolved in IPA, which was mixed with 50 mg of pre-reduced platinum oxide and hydrogenated by passing H_2 gas through the mixture for about 24 hours. Following removal of the catalyst, the solvent was evaporated, resulting in a residue of greasy material from which mesembranol was crystallized from ethyl acetate. The melting point of this product was found to be 142°C.

Jeffs *et al.* [61] reported the catalytic hydrogenation of 235 mg of mesembrine in 10 ml of IPA which was rapidly mixed with a solution of 200 mg of pre-reduced platinum catalyst in 10 ml of IPA. The mixture was stirred under 1 atm of H_2 and the progress of the reaction monitored by periodic analysis of the mixture. The reaction was found to be complete in 120 minutes which gave mesembranol as the exclusive product. Crystallization of the hydrogenation product from acetone gave 173 mg of pure (–)-mesembranol, mp 145-146°C, which was identical in every respect to an authentic sample.

In another procedure, the reduction of mesembrine with sodium borohydride ($NaBH_4$) was carried out with (–)-mesembrine (200 mg) in methanol (10 ml), which was added to a solution containing NaBH4 (200 mg) in methanol (2 ml). The mixture was refluxed for 30 min, cooled and 5 ml of 10% sodium hydroxide solution was added. The resulting solution was concentrated to 10 ml and the aqueous concentrate was extracted with ether, to yield an oily residue (194 mg), which contained mesembranol (40%) and 6-epimesembranol (60%).

The (–)-mesembranol was crystallized from the mixture using ethyl acetate and re-crystallized to obtain pure (–)-mesembranol, mp 143-145°C which was analyzed by mass and infrared spectroscopy to confirm its structure.

3.3.3.2 Synthesis of Mesembranol

Mesembranol was synthesized by catalytic hydrogenation of mesembrine using the procedure of Bodendorf and Krieger [44]. Mesembrine (185 mg) was dissolved in 4 ml of IPA, transferred to a reaction vessel containing platinum (IV) oxide (20 mg) equipped with air-tight neck and Neoprene® bung. Vacuum was applied to the flask prior to introduction of H_2 gas and the reaction was monitored by TLC, which continued for about 12 hours to effect complete conversion of mesembrine to mesembranol (Figure 3.15).

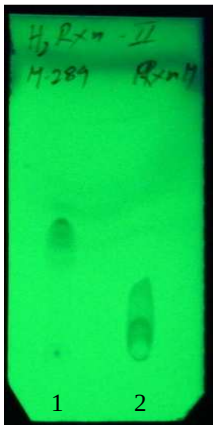


Figure 3.15: TLC of reaction mass after 12 hours of H_2 gas reduction

1. Mesembrine
2. Reaction mass
Visualization=UV_{254}

The reaction mass was subjected to LCMS analysis which provided the purity of the formed mesembranol (84.5%) and epimesembranol (15.5%) (Figure 3.16a and 3.16b). The reaction mass was applied to a short glass column filled with 1 ml of Celite® to remove the catalyst. The collected column eluate was evaporated on a water bath at 50°C under a stream of nitrogen. The residue was dissolved in ethyl acetate (3 ml) and allowed to stand for 12 hours, after which cubic crystals were observed. The excess ethyl acetate was removed and the crystals were washed twice with methanol: ethyl acetate (1:1) mixture (2 x 2 ml). The crystalline mesembranol (156 mg) (Figure 3.17) was further analyzed to confirm the structure. The melting point of this product was found to be 146-148°C and HPLC analysis indicated the purity to be about 99.9%.

The structure of the crystalline compound was subjected to 2D-NMR and X-ray crystallography which is discussed later in this chapter.

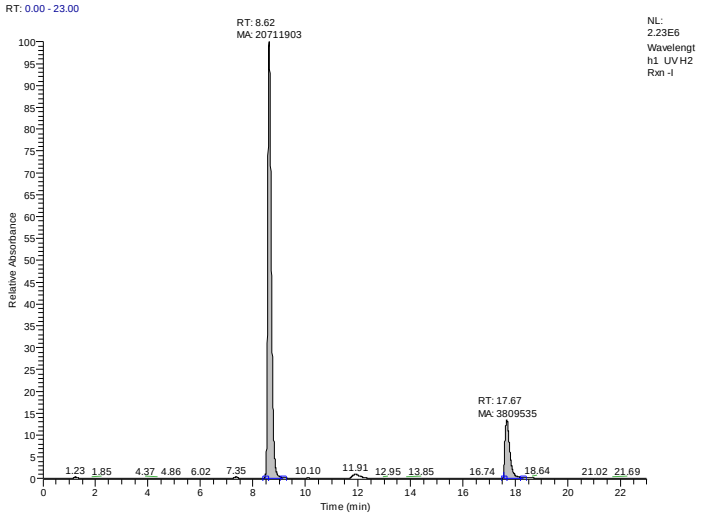


Figure 3.16a: HPLC-UV chromatogram of H_2 reaction mass RT 8.62= mesembranol and RT 17.67= epimesembranol

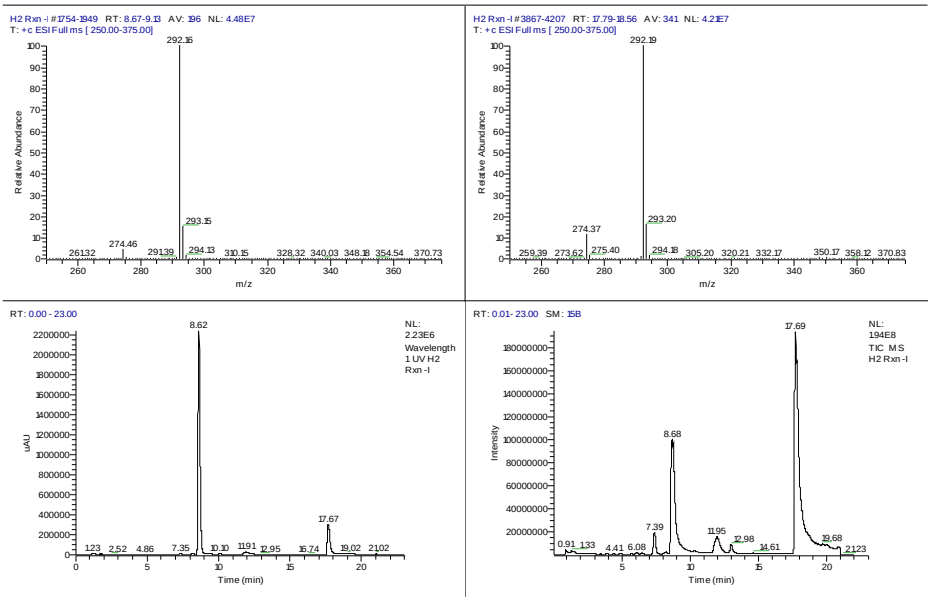


Figure 3.16b: LCMS of H_2 reaction mass. TIC chromatogram of (bottom right), HPLC-UV chromatogram (bottom left), ion spectra, m/z 292.16= mesembranol (top left) and m/z 292.19= epimesembranol (top right)

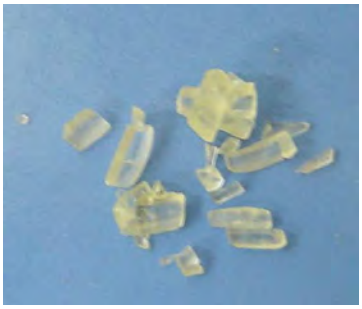


Figure 3.17: Mesembranol crystals

3.3.3.3 Synthesis of Epimesembranol

The reduction procedure of Jeffs *et al.* [61] was followed using $NaBH_4$ and the purification procedure was done in-house using a Diaion ® HP-20 column.

Mesembrine (48 mg) was dissolved in methanol (2 ml) and $NaBH_4$ (15 mg) powder was added to the solution under stirring. The reduction process was monitored by TLC for 1 hour and 20 minutes and the reaction was stopped when the mesembrine spot was no longer observed by testing the reaction mass on TLC (Figure 3.18).

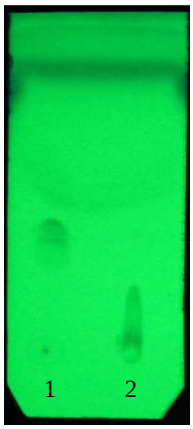


Figure 3.18: TLC of reaction mass after 80 minutes of $NaBH_4$ reduction

1. *Mesembrine*
2. *Reaction mass*

Visualization: UV_{254}

The product was purified by passing the reaction mass through a Diaion® HP-20 column (2 ml) that had been pre-equilibrated with acetone (10 ml) and then methanol (10 ml). The column eluent was collected and diluted with water (2 ml) and then recycled on the same HP-20 column. The diluted eluent was then collected and diluted further with water (4 ml) to achieve a final concentration of 25% methanol and this diluted eluent was again passed through the HP-20 column one more time. The column was then washed with water (10 ml) and eluted with methanol (10 ml) and then acetone (10 ml). The methanol fraction was evaporated under reduced pressure to afford mesembranol epimers (41.7 mg). The epimer residue was tested for purity by LCMS method and the purity of mesembranol and its epimer was found to be 27% and 73% respectively (Figure 3.19a and 3.19b).

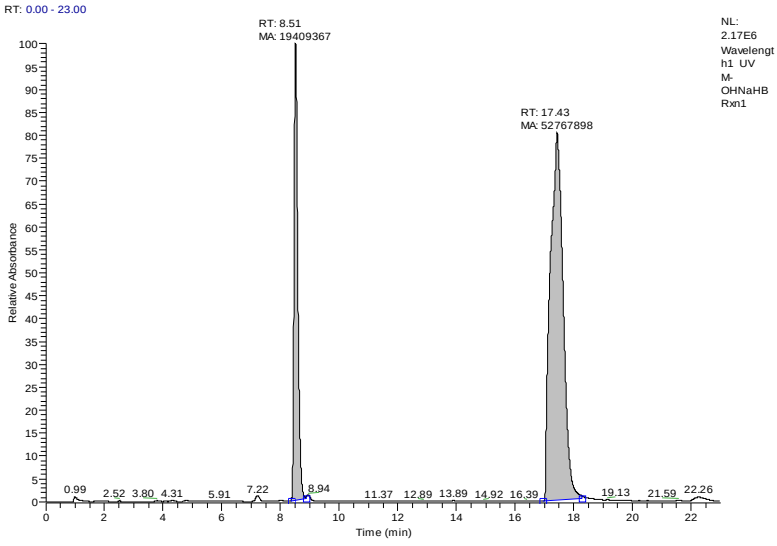


Figure 3.19a: HPLC chromatogram of $NaBH_4$ reaction mixture RT 8.51=mesembranol (27%), RT 17.43= epimesembranol (71%)

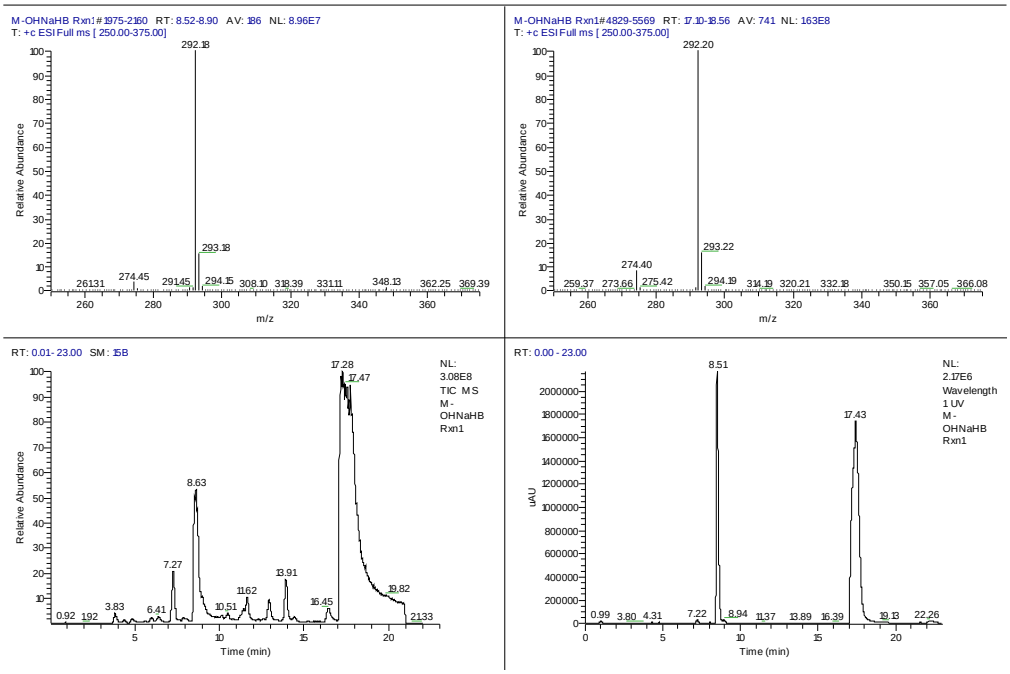


Figure 3.19b: LCMS of $NaBH_4$ reaction mass. TIC chromatogram of (bottom right), HPLC-UV chromatogram (bottom left), ion spectra of mesembranol m/z 292.17 (top left) and epimesembranol m/z 292.2(top right)

The epimers were separated by semi-preparative HPLC using a similar procedure that was previously described for the separation of mesembrenone and mesembrine. The fractionation process yielded a greasy brown compound.

The compound was purified by dissolving in methanol and loaded onto a column containing silica gel (1 g). The column was washed with DCM (5 ml) and the eluate was discarded. The column was eluted with methanol (2 x 5 ml), which was collected and evaporated under a stream of nitrogen at 50°C in a water bath to obtain a pale yellow oily compound epimesembranol (22 mg) (Figure 3.20). The process was repeated with 100 mg mesembrine to obtain a second batch of epimesembranol, whose structure and purity were confirmed by NMR and LCMS analyses, respectively to obtain a combined mass of epimesembranol (88 mg).

Figure 3.20: Epimesembranol

3.4 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a well established separation technique applied for qualitative and quantitative determination of the substances separated on a thin layer of silica gel and visualized under UV light or by spraying with a specific reagent for certain classes of chemical compounds. This technique is fast and efficient for finger-printing which allows quick identification and also provides a qualitative estimate of the content by comparison with a known concentration of standard when analyzed under the same conditions. Accurate quantification, however, can be accomplished when this technique is used as high performance thin layer chromatography (HPTLC).

Popelak and Lettenbauer in 1967 [45] reported the separation of mesembrine alkaloids using ascending formamide paper chromatography. The paper was further eluted with solvent A (methyl ethyl ketone: heptane; 2:3) and solvent B (methyl ethyl ketone: xylene) in a chamber saturated with ammonia. The visualization was carried out by spraying the paper with Dragendorff's reagent, which showed about nine alkaloids.

Smith *et al.* in 1998 [55] reported a TLC method for the identification of alkaloids wherein the crude extract was streaked onto silica gel plates and run twice in dichloromethane:methanol (3:1) and sprayed with Dragendorff's spray reagent.

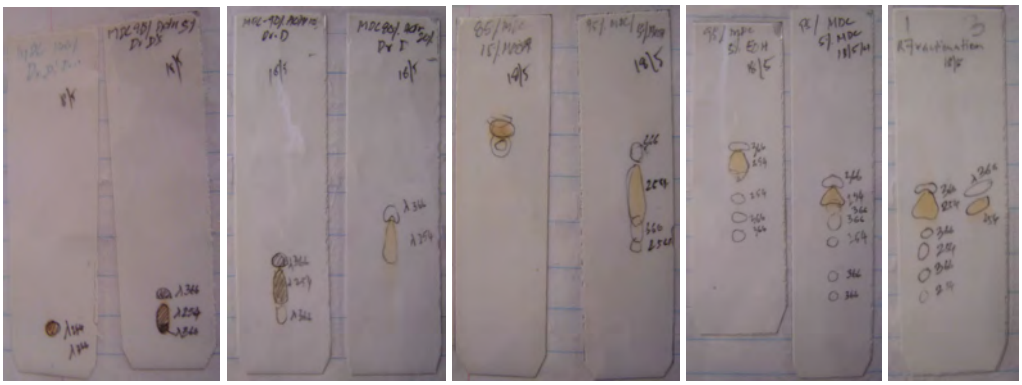
The method described in the US patent by Gericke *et al.* [52] has been suggested to be used only for rough screening purposes due to poor separation between mesembrine and mesembrenone alkaloids. However the TLC system suggested for routine screening was using Merck 60 F_{254} silica gel plates (the plates are dried at 100°C for 3 minutes) developed in $CHCl_3$:cyclohexane:diethyl amine (4:5:1) and studied under UV_{254} and UV_{365}. The Rf value of mesembrine was reported to be 0.6 and the plate was further sprayed with iodoplatonate or Dragendorff's spray reagent for positive identification of alkaloids.

In the present study, TLC was used for qualitative identification of the alkaloids as a primary test. It is very important that the TLC system resolves as many components possible from the extract. Even though the system reported by Smith *et al.* [55], was useful for preliminary identification of the presence of alkaloids, it could not be applied to separate the individual alkaloidal components. Hence, a new TLC system was developed with the objective to use this technique for the purposes of testing for alkaloidal content and also in order to isolate and separate the relevant components whereby higher volumes of 2-3 ml of the extract could be applied to the plates as a preparative procedure. This was a very important and simple procedure to study the alkaloid content irrespective

of the fact that the exact identity of the components could not be established since no reference substances were currently available.

3.4.1 TLC Method Development

The development procedure is shown step-wise below. The spots on the TLC plates were marked after observing them under UV light (UV_{254} and UV_{366}) and then sprayed with Dragendorff reagent which resulted in characteristic orange spots depicting the alkaloids. Figures 3.21a-e below show the various TLC plates used in the development of the separations.



TLC Plate1	*Plate 2*	*Plate 3*	*Plate 4*	*Plate 5*	*Plate 6*	*Plate 7*	*Plate 8*	*Plate 9*
Figures: 3.21a	*3.21b*	*3.21c*	*3.21d*	*3.21e*	*3.21f*	*3.21g*	*3.21h*	*3.21i*

Plate-1: The mobile system was initially 100% DCM showing no appreciable movement of the applied spot; Rf value= 0 (Figure 3.21a).

Plate-2: 95% DCM:5% acetone. No improvement in elution, but a slight stretching of the base spot was observed. Under UV_{366} a distinct fluorescent spot above the primary base spot was observed; Rf value= 0.1 (Figure 3.21b).

Plate-3: 90% DCM:10% acetone. Three spots very close to the base spot were observed, with two fluorescent spots, one above and one below. The resolution was, however, not satisfactory; Rf value= 0.17 (Figure 3.21c).

Plate-4: 80% DCM:20% acetone. The spot was slightly more resolved than that on Plate-3, but the alkaloid spot was not compact. One fluorescent spot appeared above the main spot, which eluted close to the primary spot; Rf value= 0.3 (Figure 3.21d).

Plate-5: 85% DCM:15% methanol. The Dragendorff's spray produced one primary orange spot which appeared to have a 'tail'. The elution was quite fast and the spot was not completely resolved; Rf value= 0.82 (Figure 3.21e).

Plate-6: The methanol concentration was reduced and the system was developed using 95% DCM:5% methanol. The elution of the spot was satisfactory but the alkaloid band was broad and the secondary spots observed under UV were not well resolved; Rf value= 0.75 (Figure 3.21f).

Plate-7: 95% DCM: 5% ethanol (95%). The mobility of the alkaloid spot was satisfactory. When observed under UV light, several secondary spots were apparent. One secondary spot below the primary spot was found to co-elute with the main alkaloid spot; Rf value= 0.5 (Figure 3.21g).

Plate-8: A minor change was made by using a solvent phase comprising of 95% DCM:5% absolute ethanol. This system resulted in the best resolution of the primary alkaloid spot and other secondary spots which were observed under UV light; Rf value= 0.58 (Figure 3.21h).

Plate-9: *Sceletium* extract spotted along with a semi-purified mesembrine for identification purposes (Figure 3.21i) using the system described in Plate-8.

3.5 CHARACTERIZATION OF *SCELETIUM* ALKALOIDS

The isolated and semi-synthesized *Sceletium* alkaloids were subjected to spectral studies in order to establish and confirm structure and purity. The analyses were performed by subjecting the compounds to LCMS, LCMS/MS and NMR. The X-ray crystallographic and Differential Scanning Calorimetry (DSC) analyses were carried out on crystalline mesembrine hydrochloride and mesembranol to determine their configuration and melting points, respectively.

3.5.1 Liquid Chromatograph-Mass Spectroscopy

LCMS analysis typically consists of a liquid chromatograph (LC) connected to a mass spectrometer (MS) as the detector. The sample components that are separated in the LC enter into the MS detector which detects individual components based on their molecular mass. The MS detector used for these analyses consist of an atmospheric pressure ionization (API) source, ion optics, mass analyzer, and ion detection system; the later three components being enclosed in a vacuum manifold. Sample ionization takes place in the ion source. The ions produced in the API source are transmitted by the ion optics into the mass analyzer, which traps the ions by varying the electrical field. The polarity of the potentials applied to the lenses in the API source and ion optics determines which positive or negative charge ions are transmitted into the mass analyzer. The mass-to-charge ratios (*m/z*) of the ions produced in the API source are measured by the mass analyzer and ejected to reach the detection system which produces the signal [62].

3.5.1.1 LCMS Electrospray Ionization Mode

The ESI mode transforms solution into the gaseous phase during which the ions are formed due to application of a strong electric field under atmospheric pressure. The ESI needle sprays the sample solution into an aerosol and the droplets are electrically charged at their surface. This electrical charge density increases as the solvent evaporates from the droplets and reaches a critical point due to accumulation of charge. The droplets divide further into smaller droplets due to the electrostatic repulsion being greater than the surface tension. The droplets undergo repeated divisions yielding very small droplets and the electrostatic repulsion ejects the sample ions into the detector [63]. The ESI mode can produce either positive or negative ions based on the polarity of the sample ion [62]. A schematic diagram of LCMS operation is shown in Figure 3.22.

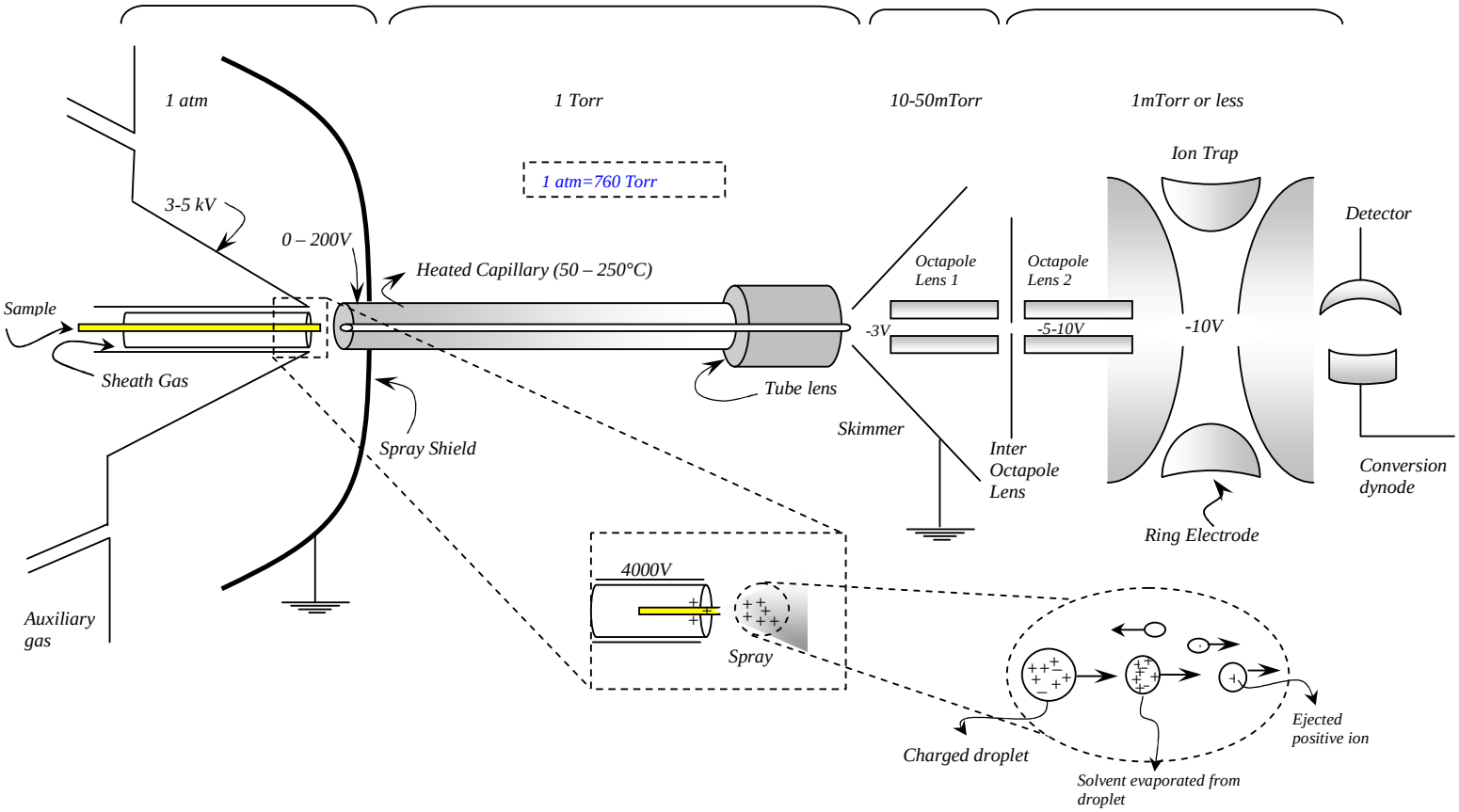


Figure 3.22: Schematic representation of Ion trap- ESI +ve ion Mass Spectroscope

The ESI process can be optimized by using small droplet size, low surface charge, low liquid surface tension, high solvent volatility, strong ion salvation and high surface charge to obtain best results.

Martin *et al.* in 1976, reported MS studies of *Sceletium* alkaloids by an electron impact (EI) method [49]. However, analytical profiles of these alkaloids by LCMS using ESI have not previously been reported.

The conditions for LCMS/MS using the ESI mode were optimized by infusing methanolic solutions at concentrations of 100 μg/ml (methanol) into the MS detector and maintaining the spray voltage at 4.54 kV. The sheath and auxiliary gas were maintained at 80 ml/min and 20 ml/min respectively. The capillary voltage and capillary temperature were maintained at 25 kV and 240°C, respectively. Ionization of the molecules was achieved by simultaneously infusing a solution of water, acetonitrile and ammonium hydroxide mixed in a ratio of 55:45:0.02 (v:v:v), through an LC pump into the MS.

Since the analyzed mesembrine type alkaloids were basic molecules, the ions that formed during the ionization process provided the $[M+H]^+$ ion of the compound as an *m/z* value.

The LCMS/MS analyses of the compounds were carried out by a two stage full scan process. The first stage produced the parent ion which is further fragmented into daughter ions by collision induced dissociation (CID). The collision was induced at different collision energies (CE) which was varied to fragment each individual molecule while partially retaining integrity of the parent ion. The fragmented ions were studied and possible structures of the fragmented ions were proposed.

3.5.1.2 Mass Spectroscopy of Mesembrine

The molecule yielded an ion of *m/z* 290 $[M+H]^+$ (Figure 3.23) which corresponded to the molecular mass of mesembrine (289).

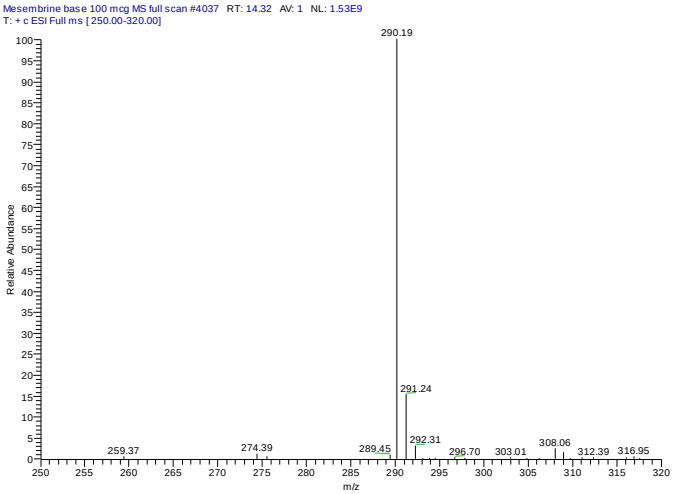


Figure 3.23: LCMS-ESI (+) ion spectrum of mesembrine

The LCMS/MS profile under optimized conditions at CE 37%, mesembrine exhibited major fragmented ions at *m/z* 275, 259, 241, 232 and 219 (Figure 3.24). The fragmented ions were studied by the proposed structures (Figure 3.25) leading to confirmation of the structure of mesembrine.

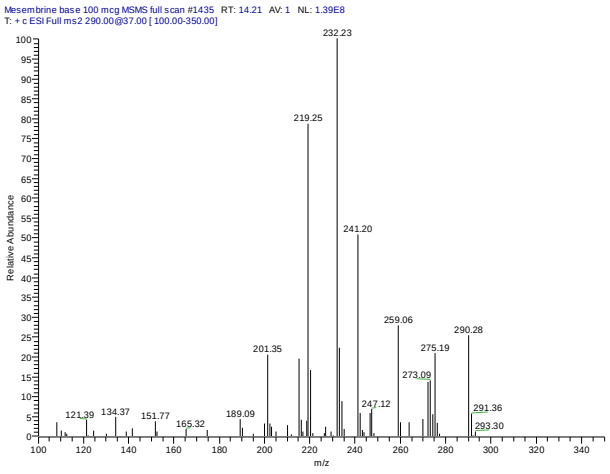


Figure 3.24: LCMS/MS ESI (+) ion spectrum of mesembrine

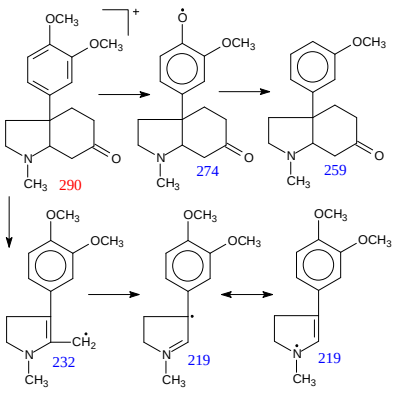


Figure 3.25: LCMS/MS ESI (+) ion fragmentation scheme of mesembrine

3.5.1.3 Mass Spectroscopy of Δ^7Mesembrenone

The Δ^7mesembrenone molecule yielded an *m/z* 288 ion (Figure 3.26) which corresponded to a molecular mass of 287.

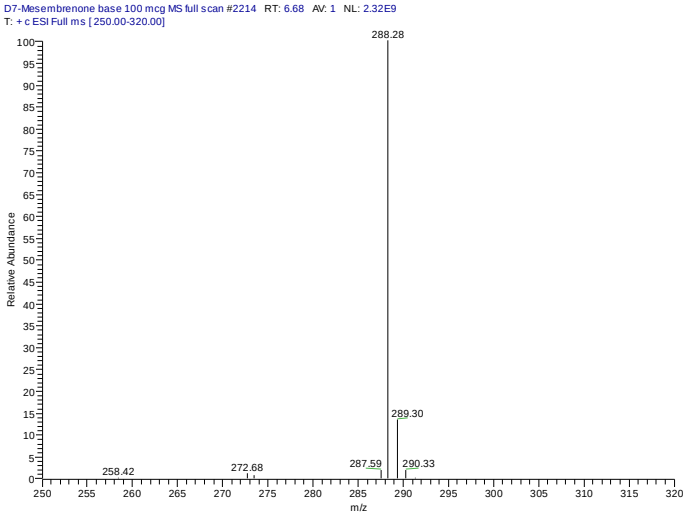


Figure 3.26: LCMS-ESI (+) ion spectrum of Δ^7mesembrenone

Under the optimized conditions at CE 38%, Δ^7mesembrenone exhibited major ions at *m/z* 288, 273, 244, 232 and 149 (Figure 3.27). The fragmented ions were studied by the proposed structures (Figure 3.28) leading to confirmation of the structure of Δ^7mesembrenone.

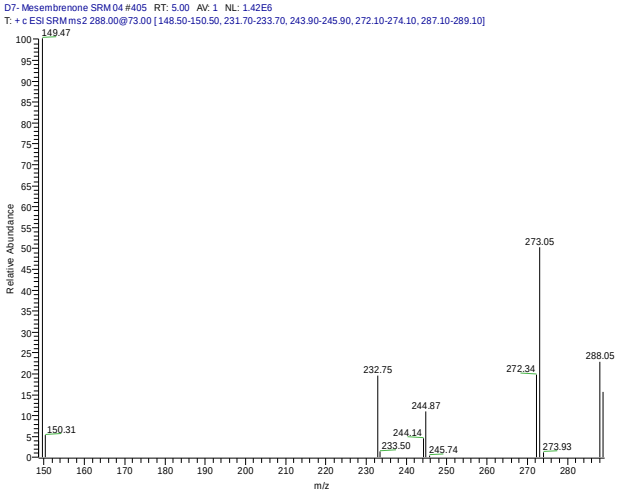


Figure 3.27: LCMS/MS ESI (+) ion spectrum of Δ^7mesembrenone

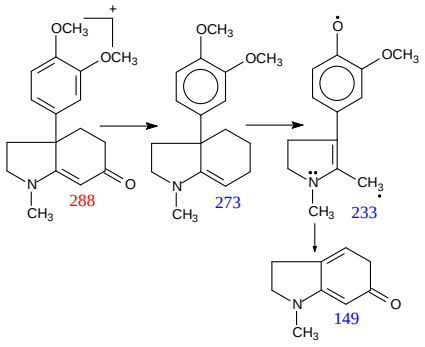


Figure 3.28: LCMS/MS ESI (+) ion fragmentation scheme of Δ^7mesembrenone

3.5.1.4 Mass Spectroscopy of Mesembrenone

The peak corresponding to mesembrenone yielded an *m/z* 288 ion (Figure 3.29) which corresponded to a molecular mass of 287.

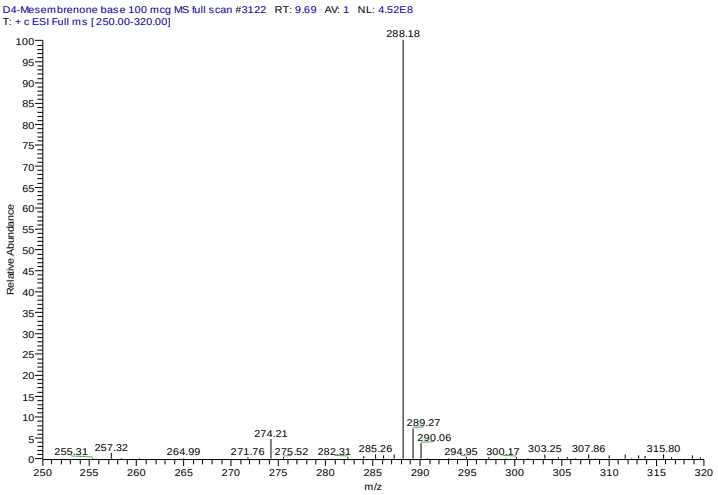


Figure 3.29: LCMS-ESI (+) ion spectrum of mesembrenone

Under the optimized conditions at CE 28%, mesembrenone exhibited major ions at *m/z* 288, 257 and 230 (Figure 3.30). The fragmented ions were studied by the proposed structures (Figure 3.31) leading to confirmation of the structure of mesembrenone.

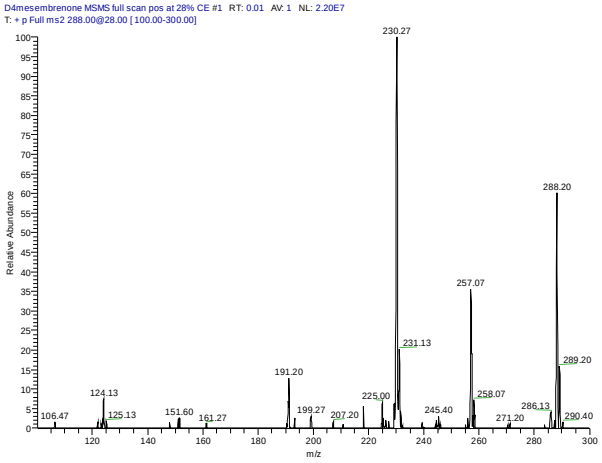


Figure 3.30: LCMS/MS ESI (+) ion spectrum of mesembrenone

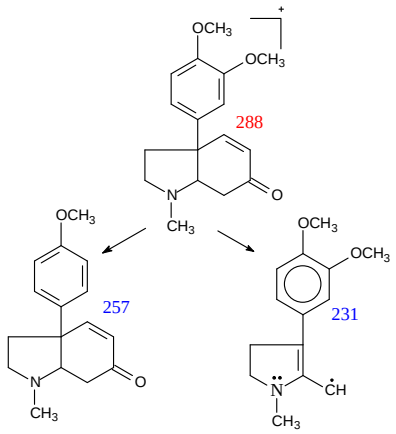


Figure 3.31: LCMS/MS ESI (+) ion fragmentation scheme of mesembrenone

3.5.1.5 Mass Spectroscopy of Mesembranol

The mesembranol molecule yielded an *m/z* 292 ion (Figure 3.32) which corresponded to a molecular mass of 291.

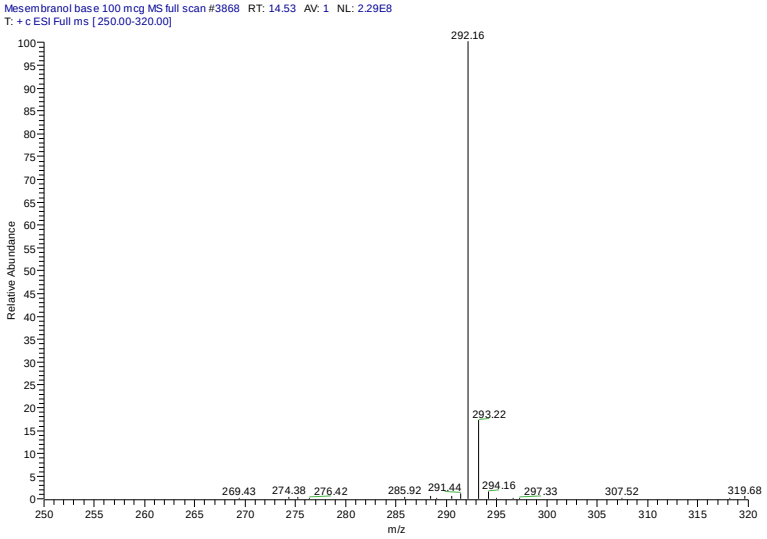


Figure 3.32: LCMS-ESI (+) ion spectrum of mesembranol

Under the optimized conditions at CE 31%, mesembranol exhibited major ions at *m/z* 274 and 243 (Figure 3.33). The fragmented ions were studied by the proposed structures (Figure 3.34) leading to confirmation of the structure of mesembranol.

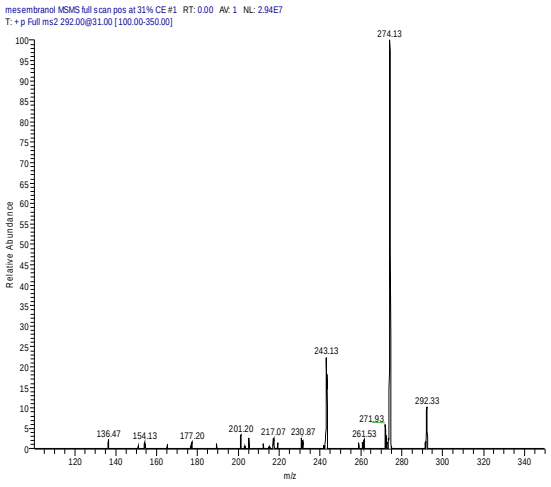


Figure 3.33: LCMS/MS ESI (+) ion spectrum of mesembranol

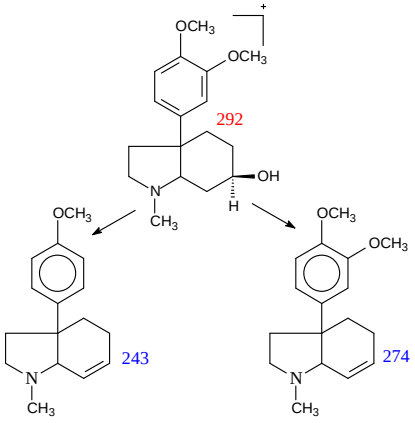


Figure 3.34: LCMS/MS ESI (+) ion fragmentation scheme of mesembranol

3.5.1.6 Mass Spectroscopy of Epimesembranol

The peak corresponding to epimesembranol yielded an *m/z* 292 (Figure 3.35) ion which corresponded to a molecular mass of 291.

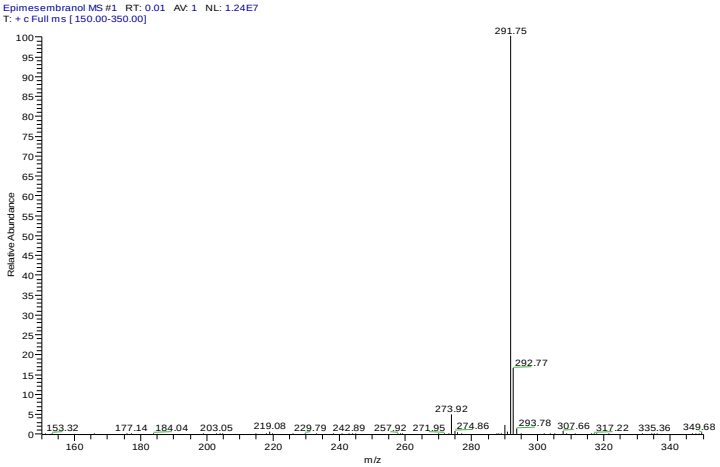


Figure 3.35: LCMS-ESI (+) ion chromatogram of epimesembranol

Under the optimized conditions at CE 31%, epimesembranol exhibited major ions at *m/z* 292, 274 and 243 (Figure 3.36). The fragmented ions were studied by the proposed structures (Figure 3.37) leading to confirmation of its structure.

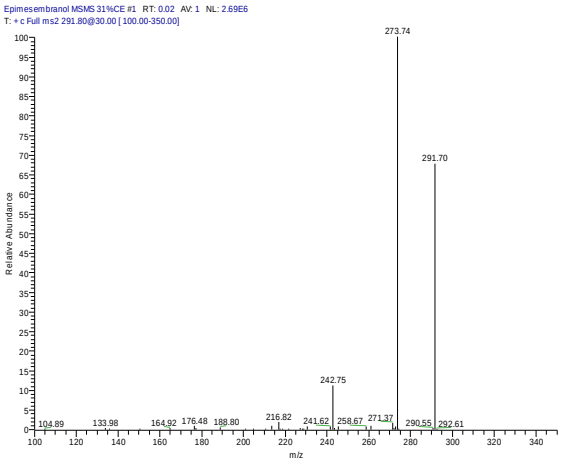


Figure 3.36: LCMS-MS ESI (+) ion spectrum of epimesembranol

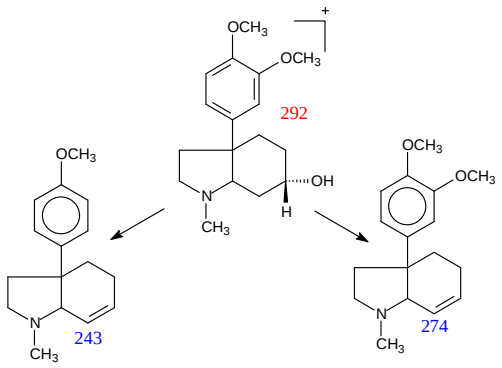


Figure 3.37: LCMS-MS ESI (+) ion fragmentation scheme of epimesembranol

3.5.2 Nuclear Magnetic Resonance Studies

NMR spectroscopy is an important absorption spectroscopic technique to investigate the nuclear structure of compounds where radio frequency waves induce transitions between magnetic energy levels of the nuclei immersed in a strong magnetic field. NMR is considered to be one of the most extensively used techniques for structural elucidation of molecules [64].

NMR analysis was carried out by dissolving each of the compounds, at a concentration of about 10 mg/ml, in deuterated chloroform ($CDCl_3$). The analyses were performed on a Bruker Advance DRX 400 MHz NMR Spectrometer (Rheinstetten, Germany). The carbon-13 NMR (^{13}C) analyses were performed at 100 MHz and the proton NMR (^{1}H) analyses were carried out at 400 MHz. The 2D NMR data were collected in the form of correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) analyses.

3.5.2.1 NMR data of Mesembrine

^{1}H NMR δ (ppm): 6.92 (d, J= 2.2Hz, 1H), 6.95 (dd, 8.3, 2.3, 1H), 6.83 (d, 8.3 , 1H), 3.89 (s, 3H), 3.87 (s, 3H), 3.15 (ddd, 9.4, 7.6, 3.0, 1H), 2.94 (t, 3.6 1H) 2.61 (dd, 3.5,2.0, 2H), 2.46 (mult., 2H), 2.37 (s, 1H), 2.33 (mult., 1H), 2.23 (mult., 2H), 2.13 (mult., 1H.)

^{13}C NMR δ (ppm): 211.4 (C-6), 149.0(C-4´), 147.5, 140.2 (C-3´), 117.9 (C-6´), 111.1 (C-2´), 110.0 (C-5´), 70.4 (C-7a), 56.0 (4´O*C*H_3), 55.9 (3´ O*C*H_3), 54.8 (C-2), 47.5 (C-3a), 40.6 (C-7), 40.1 (N-*C*H_3), 38.9 (C-3), 36.2 (C-5), 35.3 (C-4).

The relevant NMR spectra are included in the digital CD insert: File name: mesembrineNMR.pdf, provided with this thesis.

3.5.2.2 NMR data of Mesembranol

^{1}H NMR δ (ppm): 6.90 (dd, J= 8.3, 2.3 Hz, 1H), 6.86 (d, J=2.2 Hz, 1H), 6.80 (d, J=8.4 Hz, 1H), 3.96 (tt, J=10.9, 4.6 Hz, 1H), 3.87 (s, J=3.87, 3H), 3.19 (td, J=9.1, 4.6), 2.33 (s, 3H) 2.25 (ddd, J=10.9,9.6,6.1 Hz), 2.16 (dt, J=4.3,2.3 Hz), 2.03 (dd J=9.3,3.4 Hz, 2H), 1.89 (ddd, J=12.6,8.8,6.7 Hz), 1.79 (ddd, J=12.6,1.09,4.6 Hz), 1.72 (mult.) 1.19 (mult.).

^{13}C NMR δ (ppm): 148.72 (C-3′) 147.05 (C-4′), 139.24 (C-1′), 118.76 (C-6′), 110.83 (C-5′), 110.58 (C-2′), 69.95 (C-7a), 66.80 (C-6), 55.96 (3′ O*C*H_3), 55.86 (4′ O*C*H_3), 54.33 (C-2), 46.98 (C-3a), 40.50 (N-*C*H_3), 40.22 (C-3), 34.89 (C-4), 33.17 (C-7), 32.86 (C-5). The observed ^{13}C values were concordant with the values reported in literature [65].

The relevant NMR spectra are included in the digital CD insert: File name mesembranolNMR.pdf, provided with this thesis.

3.5.2.3 NMR data of Δ^7 Mesembrenone

^{1}H NMR δ(ppm): 6.78(mult., 1H), 6.76 (mult., 1H), 6.75 (mult, 1H), 5.19 (S, 1H), 3.84 (s, 3H), 3.83 (s, 3H), 3.28 (mult, 1H), 3.26 (mult, 1H), 2.40 (mult, 1H), 2.36 (mult., 1H), 2.25 (dd, J=11.8, 4.7 Hz, 1H), 2.15 (mult., 1H), 2.09 (mult., 1H), 1.92 (t, J= 4.2Hz, 1H).

^{13}C NMR δ (ppm): 196.4 (C-6), 170.8 (C-7a), 149.0 (C-4′), 148.1 (C-3′), 133.6 (C-1′), 119.1 (C-6′), 110.9 (C-2′), 110.1 (C-5′), 93.8 (C-7), 56.0 (C- 4′O*C*H_3), 55.9 (C-3′O*C*H_3), 52.8 (C-2), 52.5 (C-3a), 39.0 (C-3),36.0 (C-4),33.1 (C-5) 32.7 (N-*C*H_3).

The relevant NMR spectra are included in the digital CD insert: File name: Δ^7mesembrenoneNMR.pdf provided with this thesis.

3.5.2.4 NMR data of Mesembrenone

^{1}HNMR δ (ppm): 6.88 (dd, J= 8.4, 2.0 Hz, 1H), 6.86 (mult., 1H), 6.84(mult., 1H), 6.72 (dd, J=10.1, 2.0 Hz, 1H), 3.88 (s, 3H), 3.87 (s, 3H), 3.31 (mult., 1H) 2.65 (mult., 1H), 2.59 (mult, 1H), 2.53 (mult., 1H), 2.48 (mult, 1H), 2.47 (mult., 1H), 2.31 (s, 3H), 2.21(dt, J=12.9, 8.7Hz, 1H)

^{13}C NMR δ (ppm): 197.5 (C-6), 153.8 (C-4), 149.1 (C-3′), 148.1 (C-4′), 135.6 (C-1′), 126.4 (C-5), 119.1 (C-6′), 111.1 (C-5′), 110.1 (C-2′), 73.8 (C-7a), 56.1 (C-2), 56.0 (C-3′ O*C*H_3), 55.9 (C-4′ O*C*H_3), 50.9 (C-3a), 40.1 (N-*C*H_3), 38.6 (C-3), 36.2 (C-7).

The relevant NMR spectra are included in the digital CD insert: File name: mesembranolNMR.pdf provided with this thesis.

3.5.2.5 NMR data of Epimesembranol

^{1}HNMR δ (ppm): 6.88 (dd, J= 8.2, 2.3 Hz, 1H), 6.86 (d, J=2.1 Hz, 1H), 6.80 (d, J=8.2 Hz, 1H), 3.91 (p, J=3.0 Hz, 1H), 3.87 (s, 3H), 3.85 (s, 3H), 3.35 (ddd, J=10.1,8.8,6.6, 1H) 2.45 (s, 3H), 2.34 (mult., 1H) 2.29 (mult., 1H), 2.14 (ddd J=14.7,5.3,2.7 Hz, 1H), 1.92 (mult., 1H), 1.82 (ddd, J=12.4,11.1,6.3Hz, 2H), 1.70 (ddt, J=12.6,6.1,3.2 Hz, 1H.) 1.62 (dt, J=14.9,3.0 Hz 1H), 1.40 (tdd, J=13.9,3.6,2.3 Hz, 1H).

^{13}C NMR δ (ppm): 148.8 (C-4´), 147.2 (C-5´), 138.2 (C-1´), 118.5 (C-2´), 110.8 (C-3´), 110.4 (C-6´), 68.8 (C-7a), 67.0 (C-6), 56.0 (4´ O*C*H$_3$), 55.8 (3´ O*C*H$_3$), 53.3 (C-2), 47.5 (C-3a), 41.2 (N-*C*H$_3$), 40.9 (C-3), 29.8 (C-5), 28.2 (C-4), 28.1 (C-7).

The relevant NMR spectra are included in the digital CD insert: File name: epimesembranolNMR.pdf provided with this thesis.

3.5.3 X-ray Crystallographic Studies

X-ray crystallography provides a detailed 3-dimensional structure of crystalline molecules. The crystal is exposed to X-rays and based upon the internal 3-dimensional arrangement of the atoms in the crystal lattice, a diffraction pattern is generated. The location of this pattern and intensity is used to determine the size and composition of the molecule using mathematical computer software [66].

The crystallized compounds, mesembrine hydrochloride and mesembranol were subjected to X-ray crystallographic studies at the X-ray crystallographic unit, University of Cape Town (Cape Town, South Africa).

3.5.3.1 X-ray Crystallographic Analysis of Mesembrine hydrochloride

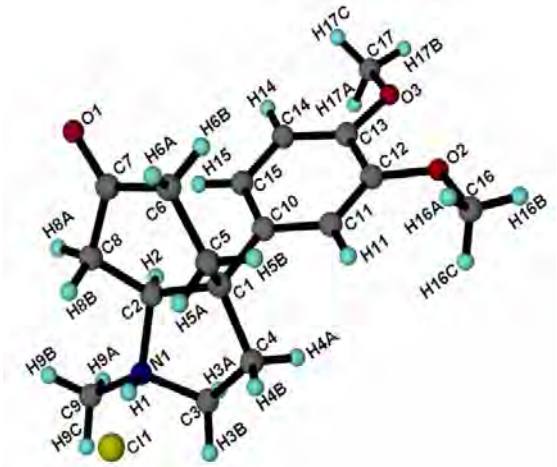


*Figure 3.38: X-ray Crystallographic structure of *M-HCl (LABELCONF.bmp)*

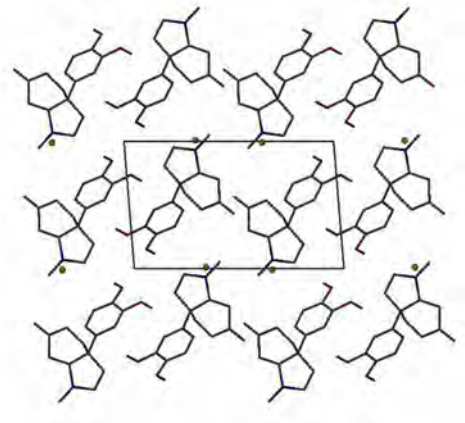


Figure 3.39: Crystal packing diagram of M-HCl (View010.png file)

As quoted by Dr Hong, X-ray crystallographic unit, University of Cape Town. [Personal communication].

"X-ray intensity data were collected on a Nonius Kappa-CCD diffractometer using graphite monochromated Mo Kα radiation. The structure was solved by direct methods using SHELX-86 and refined employing full-matrix least-squares with the program SHELX-97 refining on F^2. MESEMBRINE was crystallized in $P2_1$ with Z = 2. There is one unique MESEMBRINE molecule in the asymmetry unit. The molecular configuration and atomic labeling scheme are shown in the file LABELCONF.bmp (Figure 3.38). *All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed and refined with geometric constraints and isotropic displacement parameters fixed at 1.2 x U_{iso} of the parent carbon atoms with exception for methyl hydrogens which are fixed at 1.5 x U_{iso} of the methyl carbon. Details of data collection and refinement, tables of bond lengths and angles, torsion angles and hydrogen bonds are contained in file MESEMBE.txt. Packing diagram viewed down b axis is shown in View010.png file. Crystallographic information file is MESEMBE.cif* (Figure 3.39)*".*

**Mesembrine hydrochloride*

3.5.3.2 Crystal Data and Structure Refinement for Mesembrine Hydrochloride (Table 3.1)

Table 3.1 Crystal data of Mesembrine hydrochloride

Empirical formula	*C_{17} H_{24} Cl NO_3*
Formula weight	*325.82*
Temperature	*113(2) K*
Wavelength	*0.71073 A*
Crystal system, space group	*Monoclinic, P21*
Unit cell dimensions	*a = 8.1695(2) A alpha = 90 deg.* *b = 7.09020(10) A beta = 94.8540(10) deg.* *c = 14.0688(3) A gamma = 90 deg.*
Volume	*811.99(3) A^3*
Z, Calculated density	*2, 1.333 Mg/m^3*
Absorption coefficient	*0.248 mm^{-1}*
F(000)	*348*
Crystal size	*0.22 x 0.20 x 0.15 mm*
Theta range for data collection	*2.79 to 25.93 deg.*
Limiting indices	*-9<=h<=10, -8<=k<=8, -17<=l<=17*
Reflections collected / unique	*3139 / 3139 [R(int) = 0.0000]*
Completeness to theta= 25.93	*99.4%*
Max. and min. transmission	*0.9638 and 0.9475*
Refinement method	*Full-matrix least-squares on F^2*
Data / restraints / parameters	*3139 / 3 / 206*
Goodness-of-fit on F^2	*1.045*
Final R indices [I>2 σ(I)]	*R1 = 0.0343, wR2 = 0.0695*
R indices (all data)	*R1 = 0.0480, wR2 = 0.0748*
Absolute structure parameter	*-0.02(6)*
Largest diff. Peak and hole	*0.249 and -0.239 $e.A^{-3}$*

3.5.3.3 X-ray Crystallography of Mesembranol

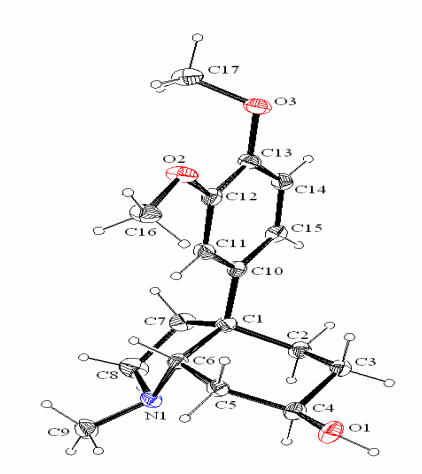


Figure 3.40: ORTEP drawing of the molecular structure with ellipsoidal model at 50% probability level, showing the atomic numbering scheme

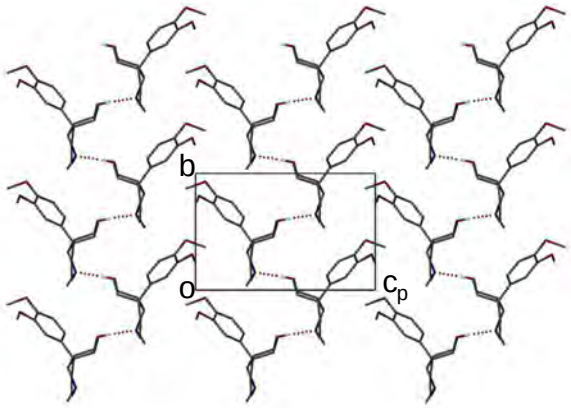


Figure 3.41: Projection viewed down . All hydrogen's except the hydroxyl hydrogen are omitted for clarity The hydrogen bonds O-H···N are shown as dotted lines

As quoted by Dr. Hong , University of Cape Town (UCT), Cape Town, X-ray crystallography unit. [Personal communication]

"X-ray single crystal intensity data were collected on a Nonius Kappa-CCD diffractometer using graphite monochromated MoKα radiation. Temperature was controlled by an Oxford Cryostream cooling system (Oxford Cryostat). The strategy for the data collections was evaluated using the Bruker Nonius "Collect" program. Data were scaled and reduced using DENZO-SMN software (Otwinowski & Minor, 1997). Both structures were solved by direct methods using SHELXS-97 (Sheldrick, 1997) and refined employing full-matrix least-squares with the program SHELXL-97 refining on F^2 (Sheldrick, 1997). Atomic numbering scheme is drawn with ORTEP-III (Farrugia, 1997) (Figure 3.40). *Packing diagrams* (Figure 3.41) *were produced using the program PovRay and graphic interface X-seed (Barbour, 2001)".*

References for X-ray crystallography:

Otwinowski, Z. & Minor, W. (1997). Methods in Enzymology, Macromolecular Crystallography, ed. Carter Jr, C. W. & Sweet, R. M., part A, vol. 276, 307-326, Academic Press.

*Barbour, L. J. (2001). X-Seed: A Software Tool for Supramolecular Crystallography, J. Supramol. Chem., **1**, 189-191.*

Sheldrick, G. M. (1997). SHELXL-97 and SHELXS-97. University of Göttingen, Germany.

Farrugia, L. J. (1997). J. Appl. Cryst. 30, 565.

3.5.3.4 Crystal Data and Structure Refinement for Mesembranol (Table 3.2)

Table 3.2 Crystal data of Mesembranol	
Empirical formula	*C_{17} H_{25} N O_3*
Formula weight	*291.38*
Temperature	*113(2) K*
Wavelength	*0.71073 A*
Crystal system, space group	*Monoclinic, $P2_1$*
Unit cell dimensions	*a = 7.5847(2) A alpha = 90 deg.* *b = 7.7878(2) A beta = 104.7540(10) deg.* *c = 13.3913(4) A gamma = 90 deg.*
Volume	*764.92(4) A^3*
Z, Calculated density	*2, 1.265 Mg/m^3*
Absorption coefficient	*0.086 mm^{-1}*
F(000)	*316*
Crystal size	*0.21 x 0.20 x 0.18 mm*
Theta range for data collection	*3.82 to 25.70 deg*
Limiting indices	*-9<=h<=9, -9<=k<=9, -16<=l<=16*
Reflections collected / unique	*2891 / 2891 [R(int) = 0.0000]*
Completeness to theta= 25.70	*99.3%*
Max. and min. transmission	*0.9847 and 0.9822*
Refinement method	*Full-matrix least-squares on F^2*
Data / restraints / parameters	*2891 / 1 / 197*
Goodness-of-fit on F^2	*1.046*
Final R indices [I>2 sigma(I)]	*R1 = 0.0331, wR2 = 0.0709*
R indices (all data)	*R1 = 0.0469, wR2 = 0.0756*
Absolute structure parameter	*-0.1(9)*
Largest diff. peak and hole	*0.158 and -0.170 $e.A^{-3}$*

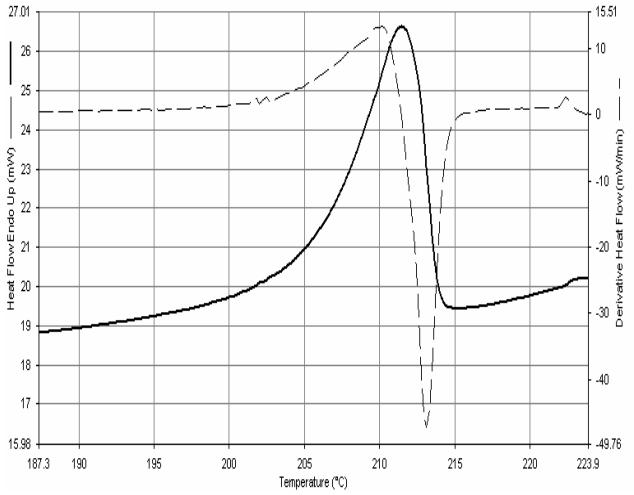
3.5.4 Differential Scanning Calorimetric Analysis

Differential scanning calorimetry (DSC) is a thermal analytical method which records the energy necessary to establish a zero temperature difference between a substance and the reference material during heating or cooling. The temperature difference is recorded as a function of temperature or time when both substance and reference are heated or cooled at a predetermined rate. The analysis

is generally carried out in a gas environment against a reference inert material such as alumina or an empty pan with lid. DSC is useful for analyzing small quantities of sample and its specific heat measurement is accurate [67]. The analysis was carried out on a DSC instrument Model DSC7 (Perkin Elmer Inc., MA, USA). Only mesembrine and mesembranol were subjected to DSC since the other alkaloids Δ^7mesembrenone, mesembrenone and epimesembranol were isolated/synthesized as oils/semisolids. Conversion to their crystalline forms was not attempted due to the unavailability of sufficient quantities of these particular alkaloids.

3.5.4.1 DSC of Mesembrine Hydrochloride

The onset of the melting process was observed at 205°C and the peak melting point was observed at 211°C (Figure 3.42), which is concordant with the reported melting point value of 205-206°C [45].



Sample 1 (Mesembrine hydrochloride) [white needle-like crystals]

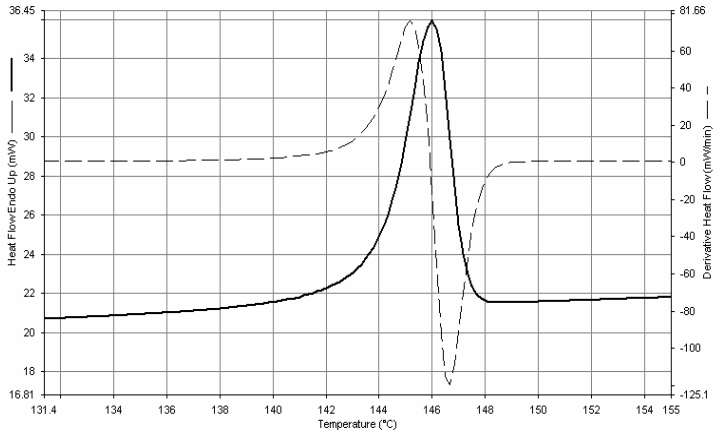
Mass DSC pan empty:	17.777 mg
Mass DSC pan + sample:	19.406 mg (s: 1.629 mg)
Mass pan+sample+lid:	28.378 mg (l: 8.972 mg)
Mass pan+residue+lid:	28.289 mg [yellow-orange]
Onset temperature:	205.898 deg. C (take to 1 decimal place)
Peak temperature:	211.466 deg. C
End temperature:	213.782 deg. C
Delta H:	136.198 J/g (take to 1 decimal place)

Figure 3.42: DSC thermogram and data for mesembrine hydrochloride

3.5.4.2 DSC of Mesembranol

The peak melting point was observed at 146°C (Figure 3.43), which is concordant with the reported melting point value of 146-147°C [65].

Mesembranol



Sample 2 (M-OH) [white crystalline material] (repeat run)

Mass DSC pan empty:	17.803 mg	
Mass DSC pan + sample:	19.379 mg	(s: 1.576 mg)
Mass pan+sample+lid:	28.294 mg	(l: 8.915 mg)
Mass pan+residue+lid:	28.287 mg [white solid]	
Onset temperature:	144.037 deg. C (take to 1 decimal place)	
Peak temperature:	146.000 deg. C	
End temperature:	147.239 deg. C	
Delta H:	122.196 J/g (take to 1 decimal place)	

Figure 3.43: DSC thermogram and data for mesembranol

3.5.5 Ultra Violet Absorption Spectroscopy

The ultra violet (UV) spectral scans of the compounds were obtained from HPLC-PDA analyses conducted by dissolving the samples in methanol and measuring the relevant absorption spectra in the range between 400-200 nm. The UV maxima of the compounds are shown in Table 3.3.

Table 3.3 UV absorption of Mesembrine alkaloids

Figure	Compound	Maxima 1 (nm)	Maxima 2 (nm)
3.44	Mesembrine	279.3	228.5
3.45	Mesembrenone	279.3	229.7
3.46	Mesembranol	278.1	228.5
3.47	Epimesembranol	279.3	225
3.48	Δ^7Mesembrenone	298.2	228

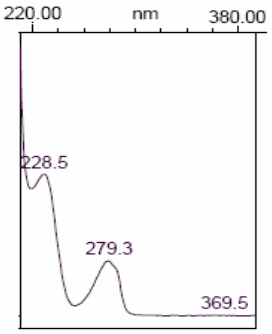


Figure 3.44: Mesembrine

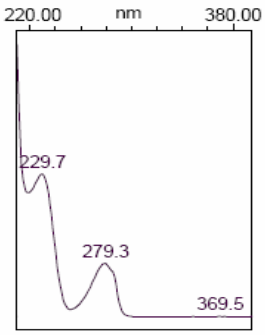


Figure 3.45: Mesembrenone

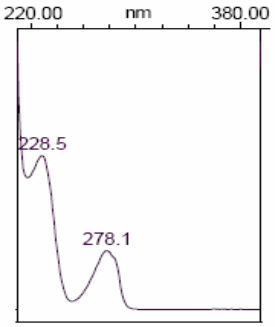


Figure 3.46: Mesembranol

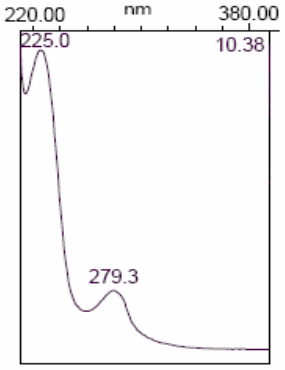


Figure 3.47: Epimesembranol

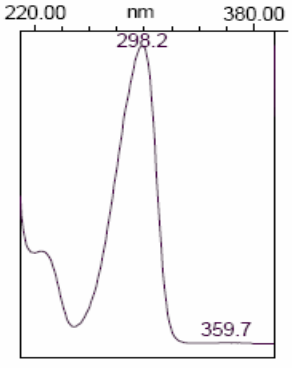


Figure 3.48: Δ^7 Mesembrenone

All compounds, except Δ^7mesembrenone, showed maxima at about 278 and 228 nm. Δ^7 mesembrenone showed maxima at 298.2 and 228 nm.

3.6 CONCLUSIONS

Since the specific *Sceletium* alkaloids were not commercially available, it was necessary to isolate, purify and characterize the relevant alkaloids to qualify as reference substances (analytical markers) for qualitative and quantitative analysis. Hence the objective was to isolate, characterize and qualify these particular alkaloids as reference substances in order to develop analytical methods and recommend specifications for the establishment of monographs for *Sceletium* and its products.

Of the *Sceletium* alkaloids reported in literature, it was observed that only the mesembrine type of alkaloids were more abundant than the other reported compounds. The major alkaloid, mesembrine and the minor alkaloids, Δ^7mesembrenone and mesembrenone were extracted and purified. The methods involved solvent extraction followed by column chromatography, preparative TLC and semi-preparative HPLC used to yield the pure compounds. The process of isolation and synthesis was monitored by TLC, HPLC and LCMS analytical methods.

Isolation of the alkaloids, mesembranol and epimesembranol were not attempted due to small quantities present in the plant material, hence they were synthesized. Mesembranol was successfully synthesized by catalytic reduction of mesembrine base using platinum oxide and addition of hydrogen gas, which yielded a crystalline compound. Epimesembranol was synthesized by treating mesembrine with sodium borohydride which yielded a mixture of epimers with epimesembranol being the major epimer. Epimesembranol was successfully separated and purified by semi-preparative HPLC.

The NMR data collected from the ^{1}H, ^{13}C, COSY, HSQC and HMBC analyses were interpreted to confirm the structures.

The LCMS and MS/MS analyses provided identification of the relevant alkaloids based on the *m/z* values of 288 for mesembrenone (MW: 287) and Δ^7mesembrenone (MW: 287), 290 for mesembrine (MW: 289) and 292 for mesembranol and epimesembranol (MW: 291). Mesembrine was converted successfully to its hydrochloride and its structural configuration was confirmed by X-ray crystallographic analysis. The hydrochloride salt also provided ease of handling compared to having to use its base. The developed ESI technique proved to be a sensitive and precise method to identify the alkaloids in mixtures of components as is generally the case with natural products. The MS/MS fragmentation studies are readily applicable to LCMS scan modes such as selected ion monitoring (SIM) and selected reaction monitoring (SRM) and these techniques are useful for detecting low concentrations of a target compound in a complex mixture when the mass spectrum

of such a compound has been established. The PDA analyses provided the UV absorption characteristics of the alkaloids.

In summary, the alkaloids, mesembrine, mesembrenone, Δ^7mesembrenone, mesembranol and epimesembranol were isolated/synthesized, purified, characterized and qualified for use as analytical markers. These compounds are necessary for use in the development and validation of analytical methods for the assay and QC of *Sceletium* plant material and pharmaceutical dosage forms containing *Sceletium*.

CHAPTER 4

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF *SCELETIUM* ALKALOIDS

4.1 INTRODUCTION

Evaluation of natural products for their chemical components is challenging due to the inherent diversity of their chemical composition. Separation techniques and their application to evaluate specific chemical components of natural products is an important aspect which permits accurate characterization and quantification. It has been reported that almost 80% of known natural substances are non-volatile and thermolabile, which makes High Performance Liquid Chromatography (HPLC) the most commonly used analytical technique for natural products [68]. In general, HPLC is coupled to an ultra violet-visible (UV-Vis) detector which may be a single or a dual wavelength, and in some cases a photo diode array (PDA) detector. The PDA enhances versatility of the analysis by allowing multi-wavelength detection of compounds based on their distinct chromophore active regions. PDA is particularly useful for multi-component sample analysis and one sample can usually provide sufficient data to assess the purity of the individual components [69].

There are many reports on the use of HPLC for qualitative analysis of herbal medicines, more importantly for the fingerprinting of South African traditional medicines [70]. In addition, chromatographic fingerprinting has been widely accepted and recommended by various regulatory authorities such as WHO [1], US-FDA [71], EMEA [36] and MHRA [39] to assess the consistency of herbal components from batch-to-batch of dosage forms and for the harvested herbal plants materials.

Characterization of constituents is an important aspect for the QC of herbal products. The importance of structural information on their chemical components is highly desired for developing efficient isolation and analytical methods [72]. The basic HPLC instrument may easily be integrated with different detection techniques, such as electrochemical, fluorescence, refractive index, MS and more recently, NMR. The LC-MS-NMR combination has been used for online structural identification and for providing preliminary data on the nature of constituents [72].

4.2 BACKGROUND AND OBJECTIVES

In the present international regulatory scenario, qualitative and quantitative analytical methods are considered mandatory. Even though *Sceletium* plants have been relatively well-researched for their chemical constituents, validated methods for quantitative analysis of the various alkaloid components found in *Sceletium* species have not yet been reported in the published literature.

In 1998, Smith *et al.* [55] reported a qualitative method using gas chromatography (GC) - MS attached to a nitrogen-phosphorus specific detector. The GC-MS analysis was carried out on a DB-5 capillary column of 0.25 mm internal diameter (i.d), which was temperature programmed from 230°C to 260°C at 1°C per minute. The detector was maintained at 350°C. The method described the separation of 4′-O-demethylmesembrenol, mesembrine and mesembrenone, and their structures were identified based on comparison of their mass spectral data to the reported values by Martin *et al.* [49].

Gericke *et al.* [52], in their patent application, reported three methods using GC and one using a HPLC method. In all three methods, the samples were prepared by dissolving the extracts in a minimum quantity of methanol.

The first method is mentioned as a “fast system” for “large number of samples”. The method uses DB-1 fused silica capillary column 30 m x 0.25 mm i.d, with helium as carrier gas. The column temperature was maintained isothermally at 200°C for 15 minutes and then programmed at 100°C/min to 300°C. The injector and the flame ionization detector were maintained at 230°C and 300°C respectively [52].

The second method is mentioned as “high resolution analyses” for “selected samples, slow”. The method also uses a DB-1 column and helium as carrier gas, similar to the first method. The column temperature was isothermally maintained at 150°C for 15 minutes and then programmed at 60°C/ min to 320°C. The injector and the NPD were maintained at 230°C and 300°C respectively [52].

The third method mentioned is for GC-MS analysis, with the column, carrier gas and the temperatures maintained similar to the second method. The MS conditions were not described [52].

The HPLC method describes a reverse phase system which used a C_{18} column. The mobile phase comprised 30% trimethylamine solution (1% in water) and 70% acetonitrile solution (60% in water) set at a flow rate of 1 ml per minute. The PDA detector was set for two channels, one at 280±30 nm (Channel A) and the other at 292±10 nm (Channel B). The method suggests that "for concentrations below 0.05 mg per ml, channel A is more accurate" [52].

The South African National Biodiversity Institute (SANBI), South African Medical Research Council (MRC) and the University of Western Cape (UWC) published a collaborative paper describing another HPLC method on the internet as a part of monograph project for herbal plants. It was suggested that the method could be applied to perform fingerprinting analysis of medicinal plant extracts. The HPLC method which was obtained by personal communication from the authors, uses methanol and 1% acetic acid in water mixed over a 25 minute gradient programme, passing through a C_{18} column and dual channel detection at 280 and 325nm. A typical HPLC chromatogram of a methanol extract and the retention times of major compounds purported to elute at 2.38, 3.15, 4.89 and 7.84 was displayed on the chromatogram in the monograph which they compiled [57].

Based on the above information on analytical methods for *Sceletium* and its products, it appears that the reported methods may not have been validated as required by the regulatory guidelines since no validation data were reported. Also, the use of UV wavelengths in the range of 280 nm and 325 nm for detection of *Sceletium* alkaloids are not optimum for most of the *Sceletium* alkaloids. This study shows maximum absorbance at 228±2 nm, except for Δ^7mesembrenone, which had a λ_{max} at 298 nm and secondary maximum at 228nm. Furthermore, from discussions with *Sceletium* cultivators in South Africa, it was evident that no testing facilities are available to monitor the quality of their products.

Hence the main objective was to develop a simple, rapid, accurate, precise and reproducible HPLC method that can be applied for the qualitative and quantitative analysis of *Sceletium* plant material, extracts and their commercial formulations.

4.3 EXPERIMENTAL

4.3.1 Reagents and Materials

Methanol 215 and acetonitrile 200 (HPLC grade) were obtained from Romil Ltd. (Cambridge, Great Britain). Ammonium hydroxide 25% solution was acquired from Associated Chemical Enterprises (Pty) Ltd. (Southdale, South Africa). Water was purified in a Milli-Q® system (Millipore, Bedford, USA) and Millex HV® hydrophilic PVDF 0.45 μm membrane filters were purchased from the same source. *Sceletium* plant material and its products analyzed are those discussed in Chapter 2.

4.3.2 Instrumentation

An Alliance 2690 HPLC connected to a PDA detector 2996 (Waters Corporation, Milford, MA, USA) was used and separation of alkaloids was investigated using 2 different HPLC columns, Luna® C_{18} (2), 5 μm, 250 mm x 10 mm i.d. and Hypersil® 250 x 4.6 mm i.d C_{18} column manufactured by Phenomenex®, Torrence, CA, USA. Analytical balances, Type AG 135 and Electronic Micro Balance MX-5 manufactured by Mettler Toledo, Switzerland were used for weighing standards and samples. An electronic pipette (model 71050XET supplied by Biohit PLC, Helsenki, Finland) was used to transfer standard and sample solutions for dilutions. Method validation was performed on a Finnigan MAT LCQ ion trap mass spectrometer supplied by Finnigan, San Jose, CA, USA coupled to a SpectraSYSTEM P2000 pump connected to an AS1000 auto sampler and UV1000 variable-wavelength UV detector (Thermo Separation Products, Riviera Beach, FL, USA).

4.3.3 Method Development

The qualified reference substances described in Chapter 3, were used for analytical method development and validation studies.

The HPLC method development was carried out initially based on the HPLC profiles obtained from plant extracts. As the development work progressed with the isolation of individual alkaloids, the HPLC peaks were identified and optimized to obtain a suitable analytical method.

Initially, the method described in the patent literature [52] was performed to yield profiles of the *Sceletium* extracts. The analysis was carried out on an Alliance 2690 HPLC connected to a Waters 2996 PDA detector. The profiles that were observed indicated many unresolved peaks (Figure 4.1). However, these peaks could not be identified for any individual alkaloid as reference standards were not available for comparison of retention times of the compounds previously reported in the literature.

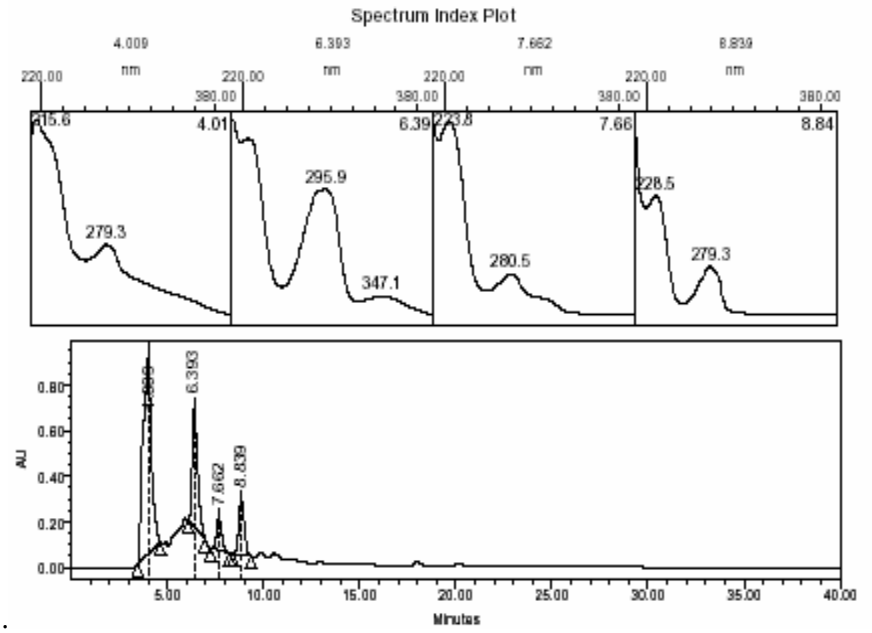


Figure 4.1: HPLC-PDA chromatogram of Sceletium extract using mobile phase described in the patent [52]. The corresponding UV spectra for each relevant peak, obtained on-line using a PDA detector are depicted above the chromatogram

Since the major alkaloids were basic molecules, a mobile phase comprising of 100 mM solution of ammonium acetate in water and ACN 70:30 (v:v) was initially used to investigate the separation of the alkaloid constituents. The mobile phase was pumped at 1ml/min under isocratic conditions through a Hypersil® 250 x 4.6 mm i.d C_{18} column maintained at ambient temperature. The extract showed distinct peaks at RT 5.278, 7.054, 7.668 and 9.543 with UV spectra showing maxima at 229±5 nm, 278±2 nm and 284 nm (Figure 4.2).

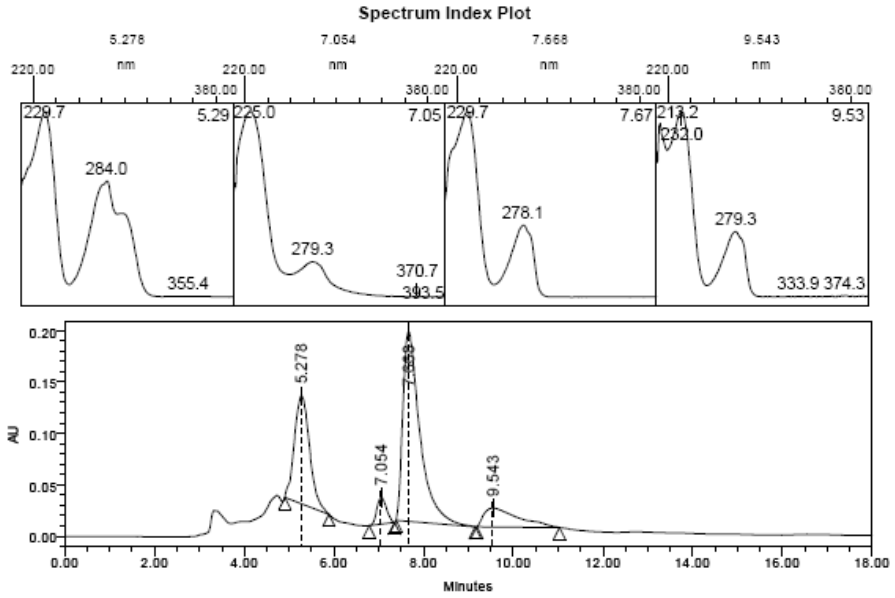


Figure 4.2: HPLC-PDA chromatogram of Sceletium extract using a mobile phase comprising of 100 mM solution of ammonium acetate in water and ACN 70:30 (v:v). UV spectra for each relevant peak obtained on-line using a PDA detector are depicted above the chromatogram

Whereas, using a mobile phase consisting of ammonium acetate (100 mM) facilitated identification of the relevant alkaloid components, the eluted peaks were associated with relatively poor peak shape and use of a relatively high salt concentration. Hence, attempts were made to exclude the use of buffer salts and develop a mobile phase using a higher pH to resolve the various *Sceletium* alkaloids which could subsequently also be used in LCMS investigations.

Meanwhile, the alkaloids, mesembrine, mesembrenone and Δ^7mesembrenone were isolated and characterized for their structures by NMR. The HPLC peaks were identified based on the retention times of the pure compounds.

Subsequently, the continuing method development involved the use of a mobile phase comprising of water:ACN:ammonium hydroxide solution mixed in a ratio of 70:30:0.01 (v:v:v) and tested under isocratic conditions using a Hypersil® 150mm x 4.6mm i.d. C_{18} column at a flow rate of 1ml/minute. The detection was carried out using a PDA detector in the range of 400 to 200 nm. The three alkaloids were well resolved with Δ^7mesembrenone at RT 5.242, mesembrenone at RT 7.579 and mesembrine at RT 10.894 minutes (Figure 4.3).

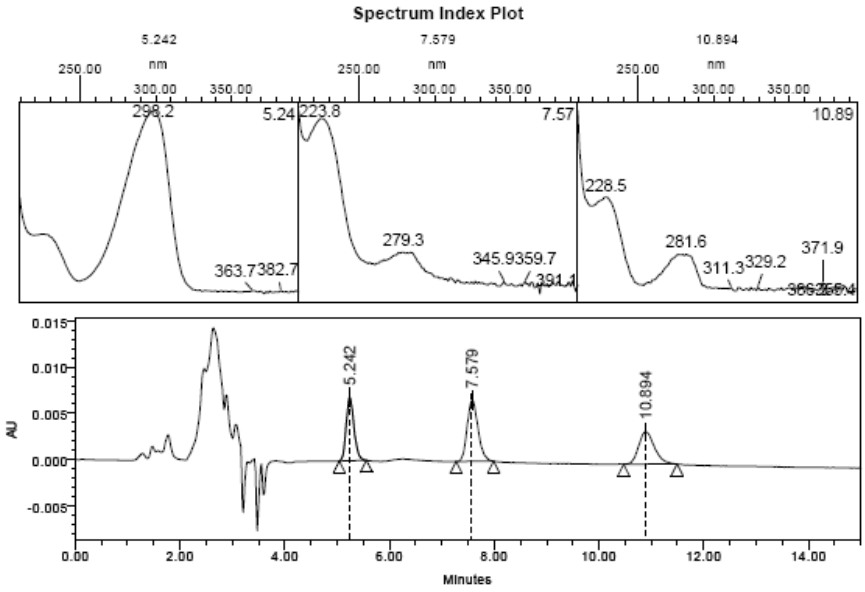


Figure 4.3: HPLC-PDA chromatogram of Sceletium standards, Δ^7mesembrenone, mesembrenone and mesembrine using a mobile phase comprising of water:ACN:ammonium hydroxide solution mixed in a ratio of 70:30:0.01 (v:v:v). UV spectra for each peak obtained on-line using a PDA detector are depicted above the chromatogram

The isocratic conditions when applied to the analysis of mesembranol and epimesembranol, posed two problems. Firstly, the epimesembranol peak was retained on the column for an extended period of 35.646 minutes (Figure 4.4) and secondly, separation between mesembranol and mesembrine could only be achieved with an increase of water content to 72 parts. Even though separation was achieved, the resolution and the peak shape were not satisfactory due to peak tailing (Figure 4.5).

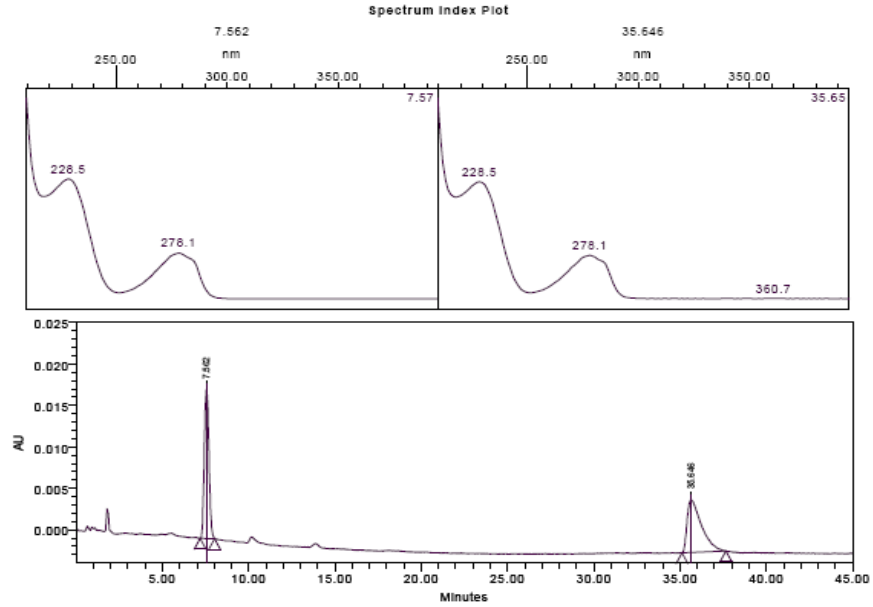


Figure 4.4: HPLC-PDA chromatogram of mesembranol and epimesembranol using a mobile phase comprising of water:ACN:ammonium hydroxide solution mixed in a ratio of 70:30:0.01 (v:v:v). UV spectra for each peak obtained on-line using a PDA detector are depicted above the chromatogram

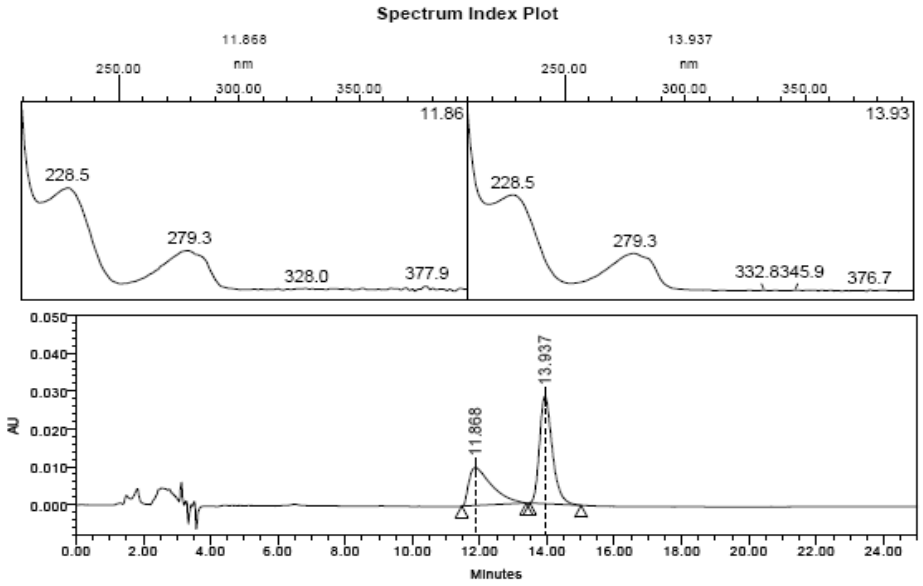


Figure 4.5: HPLC-PDA chromatogram of mesembranol and mesembrine using a mobile phase comprising of water:ACN:ammonium hydroxide solution mixed in a ratio of 72:28:0.01 (v:v:v). UV spectra for each peak obtained on-line using a PDA detector are depicted above the chromatogram

Sceletium plant powder was analyzed using the above mentioned isocratic conditions which separated Δ^7mesembrenone RT 5.981, an unknown compound at RT 6.421, mesembrenone at RT 8.774, mesembranol at RT 10.502 and mesembrine at 12.577 minutes (Figure 4.6).

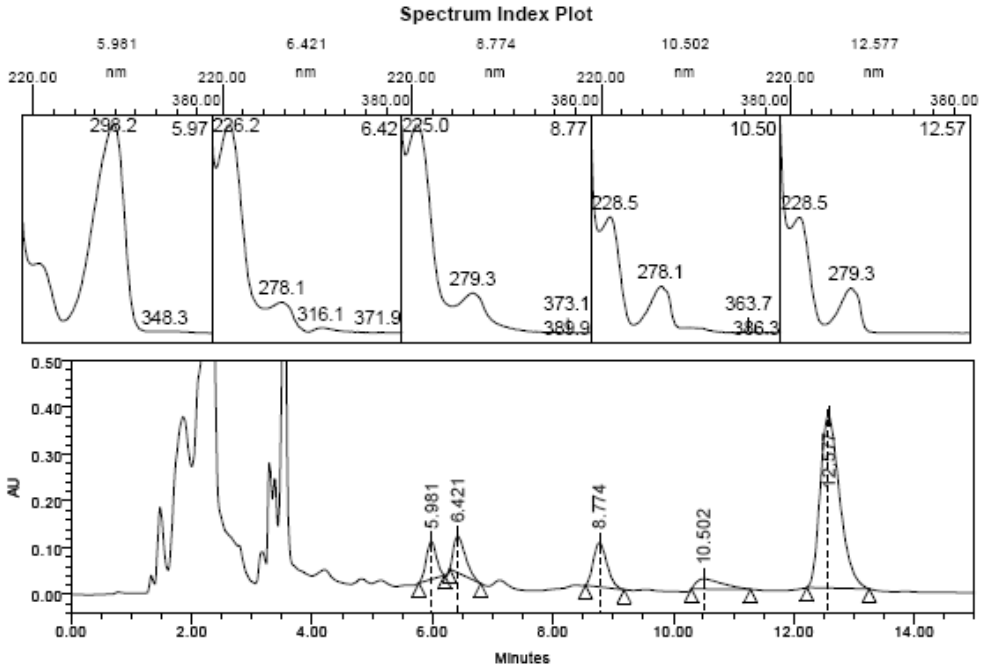


Figure 4.6: HPLC-PDA chromatogram of Sceletium plant extract using a mobile phase comprising of water:ACN:ammonium hydroxide solution mixed in a ratio of 72:28:0.01 (v:v:v). UV spectra for each relevant peak obtained on-line using a PDA detector are depicted above the chromatogram

Even though the developed isocratic system resolved the alkaloidal components, the RTs and the peak shape prompted the mobile system to be changed to a gradient system such that the RTs of the identified alkaloids could be manipulated to optimize the peak shapes and also to reduce the RT of epimesembranol.

A binary gradient elution system made up of 0.1% ammonium hydroxide solution in water (ammonia buffer) mixed with ACN was developed (Table 4.1). The flow rate was maintained at 1.0 ml/min through a Luna® C_{18} (2) 150 x 4.6 mm i.d. HPLC column. The HPLC system constructed of a SpectraSYSTEM P2000 pump connected to an AS1000 auto sampler and a UV1000 variable-wavelength UV detector set at 228 nm was used for further analysis and method validation. The analysis resulted in satisfactory resolution and retention times (Table 4.2) of the identified compounds with a total run time of 16 minutes (Figure 4.7). The method was applied successfully to *Sceletium* tablets (Figure 4.8) and *Sceletium* plant material (Figure 4.9) that showed satisfactory separation of alkaloids from other unidentified components present in such matrices.

Table 4.1
HPLC gradient conditions

Time	Flow (ml/min)	% Ammonia buffer	% ACN
0.0	1.0	80	20
9.0	1.0	50	50
15.0	1.0	50	50
16.0	1.0	80	20

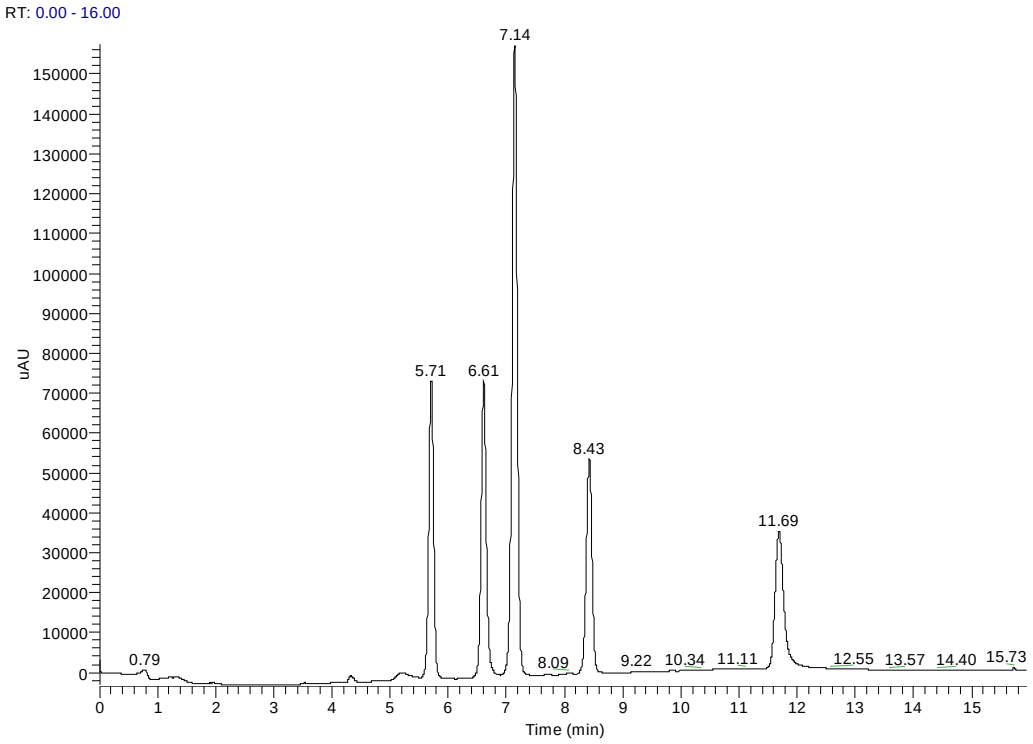


Figure 4.7: HPLC chromatogram of standard Sceletium alkaloids chromatographed using a gradient elution system of ammonia buffer mixed with ACN as show in Table 4.1

Table 4.2
Retention times of standard *Sceletium* alkaloids obtained by gradient method

Compound	Retention time (minutes)
Δ^7Mesembrenone	5.71
Mesembranol	6.61
Mesembrenone	7.14
Mesembrine	8.43
Epimesembranol	11.69

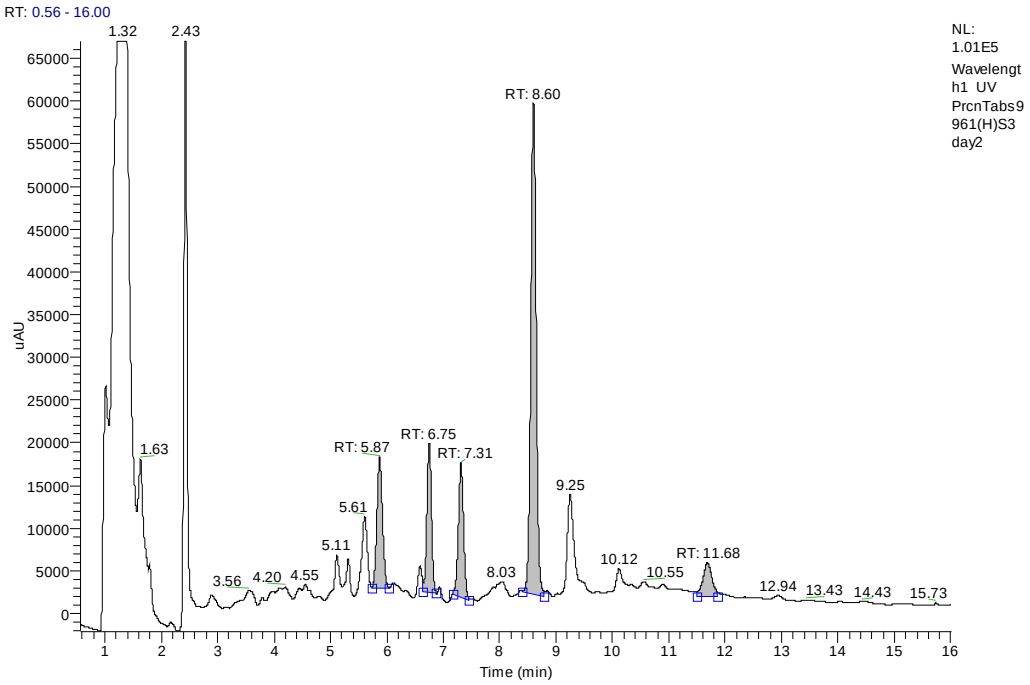


Figure 4.8: HPLC chromatogram of Sceletium tablets chromatographed using a gradient elution system of ammonia buffer mixed with ACN as show in Table 4.1

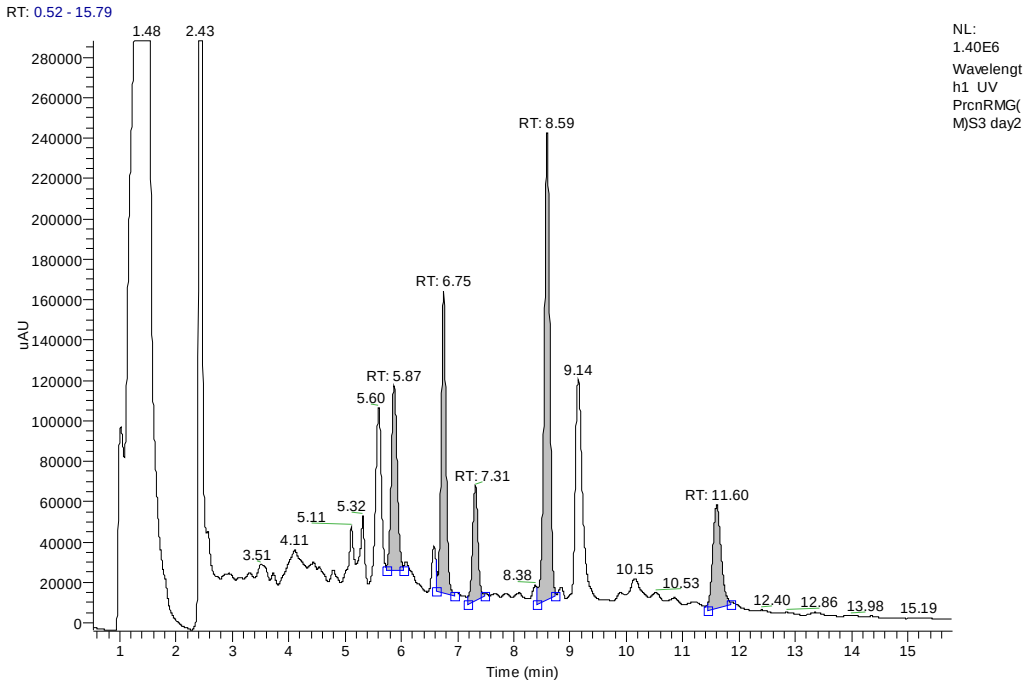


Figure 4.9 HPLC chromatogram of Sceletium plant material chromatographed using a gradient elution of ammonia buffer mixed with ACN as show in Table 4.1

4.3.4 Extraction Efficiency

The extraction efficiency of the solvent used to prepare the sample solution is a critical component of an analytical method. It is important to identify a solvent that is inexpensive and can efficiently extract the sample components from the herbal plant materials and their pharmaceutical dosage forms. Methanol was the solvent of choice for extraction and the extraction method was carried out by sonication of plant material and tablet sample solutions. The samples were prepared by weighing individual masses of 500 mg each of powdered plant material and/or crushed tablet samples to which 8 ml methanol was added, shaken and well-mixed. The samples were systematically sonicated for various lengths of time (10, 15, 20 or 30 minutes) and allowed to cool to ambient temperature prior to any further processing. The samples were filtered through 0.45 μm PVDF membrane filters and made up to volume in volumetric flasks and the content of mesembrine was estimated by HPLC (Table 4.3).

Table 4.3
Extraction efficiency of methanol measured by content of Mesembrine

Time (minutes)	Plant material (%)	Tablets (μg/tablet)
10	0.66	83.22
15	0.65	88.50
20	0.74	92.59
30	0.72	91.70

The 20 and 30 minutes samples showed consistent values for mesembrine content, hence an extraction time of 20 minutes for both plant material and tablet samples using methanol as solvent was subsequently used for all further sample preparations.

4.4 METHOD VALIDATION

4.4.1 Standard and Sample Preparations

Standard solutions were prepared fresh on each of three separate days. Methanolic stock solutions (1mg/ml) of Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine hydrochloride and epimesembranol were prepared. A working stock solution was mixed and diluted appropriately to obtain a concentration of 100 µg/ml of each alkaloid. Standard solutions comprising a set of eleven calibrators in a concentration range of 400-60,000 ng/ml were prepared.

4.4.2 Accuracy and Precision Studies

Accuracy and precision studies were performed by separately preparing standard solutions and appropriate dilutions to obtain final concentrations of 4000, 8000 and 10,000 ng/ml for use as QC standards. The precision studies of a selected *Sceletium* plant powder and a commercially purchased *S. tortuosum* tablet samples (manufactured by Big Tree Health Products, Cape Town, South Africa, Batch number 9961) were prepared in methanol by sonication for 20 minutes and filtered through 0.45 µm PVDF membrane filter.

Sceletium tablet samples of 15 mg/ml, 25 mg/ml and 35 mg/ml and *Sceletium* plant powder of 7.5 mg, 15 mg and 25 mg/ml provided the low, medium and high samples for precision studies, respectively.

4.4.3 Recovery Studies- Tablet, Plant Material and their Matrices

Recovery studies were carried out by preparing in triplicate, three individual concentrations of 25 mg/ml for tablet samples and 10 mg/ml solution for plant material. Standard stock solutions of each of the five alkaloids were added to each of the triplicate sample solutions to result in concentrations of 2000 ng, 4000 ng and 10,000 ng/ml of each of the alkaloids/sample.

Plant matrix was prepared by exhaustive solvent extraction of alkaloidal components from a powdered *Sceletium* plant sample dried at 80°C to remove the solvent. The tablet matrix was prepared by mixing the tablet excipients in the same proportion as that used by the manufacturer as indicated on the product label (Figure 4.10). The matrices were tested and confirmed for absence of the known alkaloidal components by an LCMS method, which was developed to detect very low concentrations of alkaloids *(vide infra)*. Three sets each of tablet and plant matrix samples

were prepared individually by adding stock alkaloid standard mixture to obtain final spiked concentrations of 2000, 4000 and 10,000 ng/ml of each of the alkaloids/sample

Figure 4.10: Sceletium tortuosum tablets label showing the content of excipients used in the formulation http://www.bigtreehealth.com/products/Sceletium-tortuosum.php, date accessed 07-12-2006

4.4.4 Limit of Detection and Limit of Quantitation

Standard stock solutions were diluted appropriately to obtain concentrations for the estimation of the limit of detection (LoD) and limit of quantitation (LoQ) according to a signal to noise (S/N) ratio of 3:1 and 10:1 respectively.

4.4.5 Ruggedness of the Method

Ruggedness of the method was carried out by conducting precision and accuracy studies as described in Section 4.4.2 on a second HPLC system (Waters®-Alliance–PDA system).

4.4.6 Solution Stability

The reference substances in methanol were tested for their stability by analyzing samples which were maintained at room temperature (bench stability) and ~ 4°C (fridge stability).

4.4.7 Results and Discussion

4.4.7.1 Method validation

4.4.7.2 Linearity

Calibration curves were constructed by plotting the peak area of each alkaloid versus the concentration corresponding to that alkaloid on each of three days. The curves obtained were found to be linear with determination coefficients better than 0.99 and in one instance, 0.9858. Details are provided in Table 4.4

Table 4.4
Linear ranges and coefficients of determination (HPLC)

Name of the compound	Day	y = mx + c linear model	Determination coefficient (R^2)
Δ^7Mesembrenone	Day 1	y = 48.968x + 23904	R^2 = 0.9947
	Day 2	y = 49.425x + 25244	R^2 = 0.9961
	Day 3	y = 47.453x + 25688	R^2 = 0.9960
Mesembranol	Day 1	y = 39.347x + 12817	R^2 = 0.9944
	Day 2	y = 39.748x + 9961.1	R^2 = 0.9960
	Day 3	y = 39.120x + 13789	R^2 = 0.9973
Mesembrenone	Day 1	y = 94.156x + 54025	R^2 = 0.9926
	Day 2	y = 93.704x + 50233	R^2 = 0.9974
	Day 3	y = 91.179x + 41683	R^2 = 0.9977
Mesembrine HCl	Day 1	y = 34.676x + 24050	R^2 = 0.9858
	Day 2	y = 33.674x + 23121	R^2 = 0.9956
	Day 3	y = 33.070x + 23720	R^2 = 0.9945
Epimesembranol	Day 1	y = 32.126x + 11333	R^2 = 0.9925
	Day 2	y = 29.952x + 15173	R^2 = 0.9921
	Day 3	y = 32.581x + 13702	R^2 = 0.9964

4.4.7.3 Precision and Accuracy

The studies were performed using QC samples that were prepared separately on each day of the analysis. Precision of the analytical method was performed to assess the ability of the method to produce consistent results. The inter-day relative standard deviation (RSD) values obtained were less than 3% for *Sceletium* alkaloid QC standards. The accuracy of the method was found to be between 96.9%-103% for all five compounds. The results tabulated are shown in Tables 4.5, 4.6, 4.7, 4.8 and 4.9.

Table 4.5
Accuracy of Δ^7Mesembrenone (HPLC)

Δ^7Mesembrenone	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day %RSD
Low spike	1	3520.00	3432.40	97.50	1.71
	2	3360.00	3296.85	98.12	
	3	3840.00	3866.44	100.68	
Medium Spike	1	7040.00	6847.90	97.23	0.30
	2	6720.00	6569.00	97.75	
	3	7680.00	7511.50	97.81	
High spike	1	8800.00	8802.40	100.03	2.17
	2	8400.00	8546.85	101.75	
	3	9600.00	9352.00	97.45	

Table 4.6
Accuracy of Mesembranol (HPLC)

Mesembranol	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day %RSD
Low spike	1	4240.00	4239.11	101.25	0.99
	2	4160.00	4196.00	100.86	
	3	4320.00	4292.60	99.37	
Medium Spike	1	8480.00	8038.42	94.79	1.10
	2	8320.00	8036.60	96.59	
	3	8640.00	8365.60	96.80	
High spike	1	10600.00	10711.20	101.05	1.02
	2	10400.00	10395.51	99.96	
	3	10800.00	10689.50	99.00	

Table 4.7
Accuracy of Mesembrenone (HPLC)

Mesembrenone	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day %RSD
Low spike	1	4320.00	4293.20	99.40	1.64
	2	4160.00	4150.55	99.77	
	3	4400.00	4385.91	99.68	
Medium Spike	1	8640.00	8408.45	97.32	1.57
	2	8320.00	8213.10	98.71	
	3	8800.00	8417.23	95.65	
High spike	1	10800.00	10860.40	100.6	2.30
	2	10400.00	10473.80	100.71	
	3	11000.00	10633.00	96.70	

Table 4 .8
Accuracy of Mesembrine HCl (HPLC)

Mesembrine HCl	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day %RSD
Low spike	1	4240.00	4192.53	98.9	1.60
	2	4080.00	4068.10	99.71	
	3	3840.00	3710.60	96.6	
Medium Spike	1	8480.00	8229.90	97.05	2.30
	2	8160.00	8264.74	101.28	
	3	7680.00	7723.68	100.57	
High spike	1	10600.00	10303.93	97.21	2.80
	2	10200.00	10459.70	102.55	
	3	9600.00	9455.46	98.49	

Table 4.9
Accuracy of Epimesembranol (HPLC)

Epimesembranol	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day %RSD
Low spike	1	4160.00	4109.82	98.79	2.09
	2	3680.00	3777.44	102.64	
	3	4160.00	4129.68	99.27	
Medium Spike	1	8320.00	8382.05	100.75	2.05
	2	7360.00	7545.04	102.51	
	3	8320.00	8185.72	98.39	
High spike	1	10400.00	10715.70	103.04	2.60
	2	9200.00	9535.00	103.64	
	3	10400.00	10264.00	98.70	

4.4.7.4 Precision Studies – Tablet Formulation

Precision studies were performed to assess the ability of the method to produce consistent results for the tablet dosage forms. The studies were carried out by preparing three sets of low, medium and high sample concentrations on each day of analysis. The RSD for inter-day precision data are shown in Table 4.10. The identified *Sceletium* alkaloids were estimated and their contents presented as microgram per tablet are shown in Table 4.11.

Table 4.10

Precision studies of *Sceletium* tablets (HPLC)

Compound	**Content in µg/ tablet (± SD)**			**n=3**
Δ^7Mesembrenone	**Day1**	**Day2**	**Day3**	**Inter-day % RSD**
Low	9.42 (±0.39)	9.89 (±0.64)	8.84 (±0.51)	5.30
Medium	9.57 (±0.14)	9.63 (±0.36)	9.22 (±0.68)	2.30
High	9.96 (±1.27)	10.16 (±0.47)	9.45 (±0.48)	3.70
Mesembranol	**Day1**	**Day2**	**Day3**	**Inter-day % RSD**
Low	29.39 (±2.19)	32.72 (±1.45)	30.02 (±0.74)	5.70
Medium	32.02 (±2.13)	34.10 (±0.94)	32.64 (±1.40)	3.20
High	32.10 (±1.05)	32.75 (±0.37)	31.12 (±0.55)	2.56
Mesembrenone	**Day1**	**Day2**	**Day3**	**Inter-day % RSD**
Low	9.98 (±0.20)	8.07 (±0.70)	9.81 (±0.81)	11.40
Medium	9.10 (±0.28)	9.57 (±0.20)	10.53 (±0.33)	7.40
High	8.78 (±0.19)	8.84 (±0.52)	9.22 (±0.20)	2.70
Mesembrine	**Day1**	**Day2**	**Day3**	**Inter-day % RSD**
Low	120.70 (±1.30)	122.80 (±1.18)	118.90 (±1.7)	1.60
Medium	122.20 (±1.07)	126.40 (±1.60)	119.70 (±4.8)	2.73
High	120.60 (±1.40)	123.70 (±2.37)	121.70 (±2.9)	1.28
Epimesembranol	**Day1**	**Day2**	**Day3**	**Inter-day % RSD**
Low	7.74 (±0.60)	7.37 (±0.25)	6.97 (±0.62)	5.20
Medium	8.14 (±0.87)	7.32 (±0.34)	7.15 (±0.60)	7.02
High	7.93 (±0.60)	7.03 (±0.20)	7.78 (±0.40)	6.40

Average weight of tablet = 497.3mg, SD= Standard deviation
Low = 15 mg/ml (n=3), Medium = 25 mg/ml (n=3), High = 35 mg/ml (n=3); Total samples n=9 each day

Table 4.11

Content of identified *Sceletium* alkaloids per tablet (HPLC)

Compound	*Content in µg/ tablet
Δ^7Mesembrenone	9.6
Mesembranol	31.9
Mesembrenone	9.3
Mesembrine	121.9
Epimesembranol	7.5

*Average values obtained from precision studies

4.4.7.5 Recovery Studies – Tablet Formulation

The recoveries of the spiked *Sceletium* alkaloid standards were evaluated to assess the extraction efficiency of the analytical method. Three sets of *Sceletium* tablet samples were individually prepared and spiked with low, medium and high concentrations of each of the alkaloid standards on each day of analysis. The values obtained for this experiment show good recoveries of all five identified alkaloids in the range of 95%-105% for each of the added compound with RSD of less than 3.2%. The results are shown in Table 4.12a and 4.12b.

In addition, recovery studies were performed to assess the effect of excipients on the extraction of the product by adding of low, medium and high concentrations of *Sceletium* standards to the tablet matrix. The recoveries from the spiked samples of the tablet matrix are depicted in Tables 4.13a and 4.13b. The recoveries ranged between 95%-105% for each of the added alkaloid compounds with inter-day RSDs of less than 3%.

Table 4.12a
Recovery studies of *Sceletium* alkaloids in tablet dosage form (HPLC)

Tablet Sample	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Δ^7**Mesembrenone content** (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	Δ^7**Mesembrenone content** (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	Δ^7**Mesembrenone content** (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	0.49				0.5				0.46				
Low	(+1.76)[1] 2.25[2]	2.26 (±0.06)	100.40	2.60	(+1.84) 2.34	2.33 (±0.07)	99.57	3.30	(+1.92) 2.38	2.42 (±0.01)	101.76	0.70	1.10
Medium	(+3.52) 4.01	4.07 (±0.16)	101.40	3.93	(+3.68) 4.18	4.32 (±0.15)	103.35	3.16	(+3.84) 4.30	4.50 (±0.08)	104.65	1.87	1.60
High	(+8.80) 9.29	9.50 (±0.12)	102.26	1.30	(+9.20) 9.70	10.18 (±0.16)	104.95	1.38	(+9.6) 10.06	10.22 (±0.09)	101.20	0.80	1.90
Tablet Sample	**Day-1**				**Day-2**				**Day-3**				**Inter-day Recovery % RSD (n=3)**
	Mesembranol content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembranol content** (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembranol content** (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	1.57				1.67				1.57				
Low	(+2.12) 3.69	3.60 (±0.12)	97.65	3.19	(+2.08) 3.75	3.65 (±0.07)	97.33	2.05	(+2.08) 3.65	3.60 (±0.10)	98.74	2.70	0.76
Medium	(+4.24) 5.81	5.59 (±0.1)	96.27	1.8	(+4.16) 6.24	5.95 (±0.08)	95.41	1.34	(+4.16) 6.24	6.14 (±0.09)	98.44	1.73	1.61
High	(+10.6) 12.17	11.99 (±0.27)	98.54	2.22	(+10.4) 12.48	11.95 (±0.26)	95.75	2.27	(+10.4) 12.48	11.58 (±0.24)	95.79	2.1	1.65
Tablet Sample	**Day-1**				**Day-2**				**Day-3**				**Inter-day Recovery % RSD (n=3)**
	Mesembrenone content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrenone content** (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrenone content** (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	0.47				0.487				0.50				
Low	(+2.16) 2.63	2.54 (±0.05)	96.57	2.04	(+2.08) 2.57	2.49 (±0.08)	96.90	3.03	(+2.04) 2.54	2.43(±0.06)	95.66	2.47	0.70
Medium	(+4.32) 4.79	4.73 (±0.11)	97.53	2.24	(+4.16) 4.65	4.73 (±0.05)	101.72	1.10	(+4.16) 4.66	4.54 (±0.09)	97.42	2.00	2.48
High	(+10.8) 11.27	11.35 (±0.27)	99.45	2.42	(+10.4)10.89	10.52 (±0.46)	96.60	4.35	(+10.2) 10.70	10.35 (±0.25)	96.73	2.42	1.65

* Actual content [1]; spiked amount [2]; represents the total content, i.e. spiked plus original content (follows throughout the table), (±SD), Average weight = 497.30 mg/ tablet

Table 4.12b
Recovery studies of *Sceletium* alkaloids in tablet dosage form (HPLC)

Sample	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrine content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrine** content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrine** content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	6.09				6.25				6.03				
Low	(+1.88)[1] 7.97[2]	7.94 (±0.08)	99.62	0.94	(+1.81) 8.06	8.10 (±0.04)	100.50	0.50	(+1.85) 7.88	7.82 (±0.05)	99.24	0.64	0.65
Medium	(+3.77) 9.86	9.91 (±0.03)	100.51	0.31	(+3.63) 9.88	9.91 (±0.14)	100.30	1.47	(+3.70) 9.73	9.64 (±0.06)	99.10	0.63	0.76
High	(+9.42) 15.51	15.52 (±0.03)	100.10	0.21	(+9.07) 15.32	15.48 (±0.17)	101.04	1.14	(+9.25) 15.28	14.92 (±0.17)	97.64	1.14	1.76
Sample	**Day-1**				**Day-2**				**Day-3**				**Inter-day Recovery % RSD (n=3)**
	Epimesembranol content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** content (µg/25 mg dosage/ml)	Observed content (µg/25 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	0.39				0.36				0.37				
Low	(+2.08) 2.47	2.45 (±0.05)	99.20	2.24	(+1.84) 2.20	2.25 (±0.04)	102.30	1.60	(+2.08) 2.45	2.34 (±0.04)	95.51	1.53	3.43
Medium	(+4.16) 4.55	4.49 (±0.10)	98.68	2.30	(+3.68) 4.04	4.05 (±0.14)	100.10	3.50	(+4.16) 4.53	4.46 (±0.07)	98.44	1.73	0.90
High	(+10.40) 10.79	10.97 (±0.29)	101.67	2.61	(+9.20) 9.56	9.62 (±0.36)	100.62	3.80	(+10.40) 10.77	10.59 (±0.29)	95.80	2.80	3.16

* Actual content [1]; spiked amount [2]; represents the total content, i.e. spiked plus original content (follows throughout the table), (±SD), Average weight = 497.30 mg/ tablet

Table 4.13a
Recovery studies of *Sceletium* alkaloids from tablet matrix (HPLC)

	Day 1			**Day 2**			**Day 3**			
Tablet Matrix Sample	Δ^7**Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	Δ^7**Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Δ7Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	1760.00	1725.90	98.06	1680.00	1622.00	96.60	1920.00	1930.90	100.60	2.46
Medium	3520.00	3387.13	98.27	3360.00	3301.90	98.27	3840.00	3707.60	96.60	1.13
High	8800.00	8577.93	97.48	8400.00	8596.22	102.34	9600.00	9475.33	98.70	2.54
	Day 1			**Day 2**			**Day 3**			
Tablet Matrix Sample	**Mesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	2120.00	2026.00	95.56	2080.00	2056.33	98.96	2160.00	2087.50	96.60	1.73
Medium	4240.00	4066.80	95.90	4160.00	4128.70	99.25	4320.00	4303.50	99.62	2.08
High	10600.00	10381.40	97.90	10400.00	10568.8	101.60	10800.00	10572.00	97.90	2.15
	Day 1			**Day 2**			**Day 3**			
Tablet Matrix Sample	**Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	2160.00	2211.50	102.40	2080.00	2058.40	99.00	2080.00	2058.40	99.90	1.76
Medium	4320.00	4225.70	97.90	4160.00	4068.80	97.81	4080.00	4160.70	102.00	2.42
High	10800.00	10386.00	96.20	10400.00	10466.60	100.70	10200.00	10179.20	99.80	2.40

Table 4.13b
Recovery studies of *Sceletium* alkaloids from tablet matrix (HPLC)

	Day 1			**Day 2**			**Day 3**			
Tablet Matrix Sample	**Mesembrine** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine HCl** content in matrix (ng/ml)	Observed content (µg/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	1884.70	1843.24	97.80	1813.56	1809.90	99.80	1884.68	1818.72	96.50	1.72
Medium	3769.40	3705.30	98.30	3627.10	3703.27	102.10	3769.36	3603.51	95.60	2.90
High	9423.40	9338.60	99.10	9067.80	9076.90	100.10	9423.40	9451.67	100.30	0.70
	Day 1			**Day 2**			**Day 3**			
Tablet Matrix Sample	**Epimesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	2120.00	2203.00	103.90	1840.00	1896.00	103.10	2080.00	2079.00	100.00	2.22
Medium	4240.00	4237.40	99.90	3680.00	3586.70	97.50	4160.00	4012.70	96.50	1.83
High	10600.00	10711.30	101.00	9200.00	9556.20	103.90	10400.00	10408.40	100.10	1.94

4.4.7.6 Precision studies – Sceletium Plant Material

Studies were carried out on *Sceletium* plant material (Sample SRM02) by preparing three sets of low, medium and high sample concentrations on each day of analysis. The RSD for inter-day precision was found to be less than 6% from the results obtained, which are shown in Table 4.14. The content of the identified *Sceletium* alkaloids in the plant material are presented in Table 4.15.

Table 4.14
Precision studies on *Sceletium* plant material (HPLC)

Compound	**Content in µg/100mg of plant material**			
Δ^7Mesembrenone	**Day1**	**Day2**	**Day3**	**Inter-day % RSD (n=3)**
Low	20.00 (±1.00)	20.33 (±0.58)	21.33 (±0.58)	3.37
Medium	23.67 (±0.58)	23.00 (±1.00)	24.33 (±1.15)	2.81
High	23.00 (±1.73)	23.67 (±1.53)	23.00 (±2.00)	1.67
Mesembranol	**Day1**	**Day2**	**Day3**	**Inter-day % RSD (n=3)**
Low	91.33 (±7.50)	94.67 (±9.30)	97.30 (±2.52)	3.17
Medium	97.67 (±3.22)	96.33 (±5.70)	91.30 (±5.03)	3.53
High	88.67 (±1.12)	93.00 (±2.65)	88.67 (±5.13)	2.77
Mesembrenone	**Day1**	**Day2**	**Day3**	**Inter-day % RSD (n=3)**
Low	12.10 (±1.87)	12.87 (±0.84)	12.67 (±1.40)	3.20
Medium	12.87 (±0.38)	13.20 (±0.38)	13.03 (±1.72)	1.27
High	13.37 (±0.71)	13.43 (±0.64)	13.83 (±0.47)	1.85
Mesembrine	**Day1**	**Day2**	**Day3**	**Inter-day % RSD (n=3)**
Low	144.50 (±0.12)	147.90 (±1.10)	149.40 (±2.51)	1.70
Medium	165.40(±1.63)	166.03(±5.65)	163.30 (±4.54)	0.87
High	165.30 (±3.15)	167.24 (±10.16)	162.50 (±5.91)	1.44
Epimesembranol	**Day1**	**Day2**	**Day3**	**Inter-day % RSD (n=3)**
Low	55.00 (±2.56)	60.56 (±7.51)	56.03 (±3.47)	5.17
Medium	61.13(±3.75)	64.80 (±5.60)	64.00(±3.80)	3.05
High	62.25 (±2.74)	68.43 (±2.92)	62.14 (±0.86)	5.60

(±SD) = Standard deviation
Low = 7.5 mg/ml (n=3), Medium = 15 mg/ml (n=3), High = 25 mg/ml (n=3); Total samples n=9 each day

Table 4.15

Content of identified *Sceletium* alkaloids in plant material

Compound	*Content in %
Δ^7Mesembrenone	0.022
Mesembranol	0.095
Mesembrenone	0.013
Mesembrine	0.16
Epimesembranol	0.061

*Average values obtained from precision studies

4.4.7.7 Recovery Studies – Sceletium Plant Material

The values obtained for this experiment which involved spiking plant material with the relevant alkaloids, are shown in Tables 4.16a and 4.16b where the recoveries of all five identified alkaloids were seen to be in the range of 95%-105% for each of the added compounds with inter-day RSDs of less than 5%.

The recoveries of the alkaloids spiked in plant matrix samples are shown in Tables 4.17a and 4.17b and the results were found to range between 95%-105% for each of the added compound with inter-day RSDs of less than 5%.

4.4.7.8 Limit of Detection and Limit of Quantitation

The LoD and LoQ of each of the 5 alkaloids were found to be 100 and 200 ng/ml respectively using the respective S/N ratios of 3 and 10.

4.4.7.9 Ruggedness

The accuracy of the standard alkaloids were found to range between 97.4%-102.7%. The precision studies for tablets showed RSDs of less than 5% and the precision studies for plant material was found to be less than 7% and the results obtained are shown in Table 4.18.

4.4.7.10 Solution Stability

The reference substances in methanol were tested for their stability by analyzing samples which were maintained at room temperature 22 ±2°C (bench stability) (Figure 4.11) and ~ 4°C (fridge stability) (Figure 4.12). The results showed no significant change or degradation of the alkaloids.

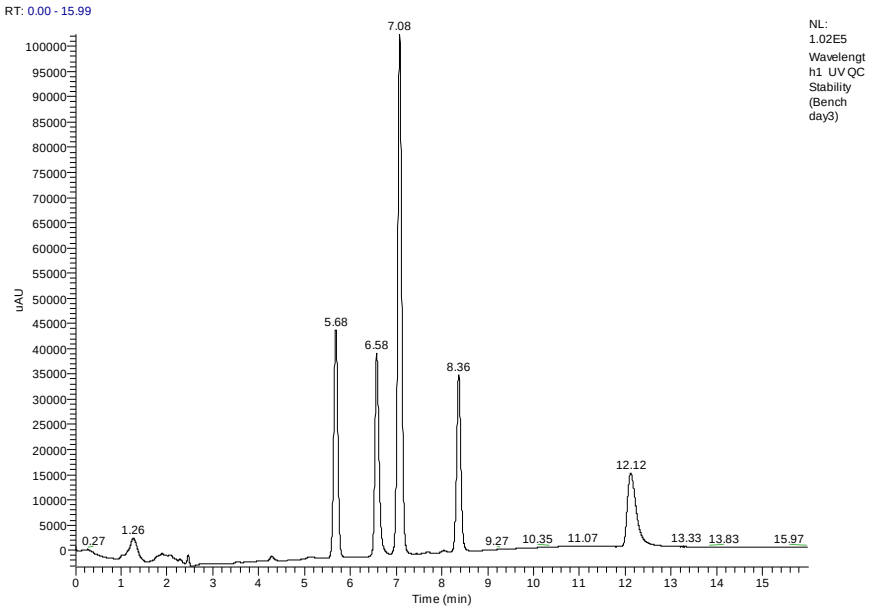


Figure 4.11: HPLC chromatogram of Sceletium standards in methanol maintained at room temperature

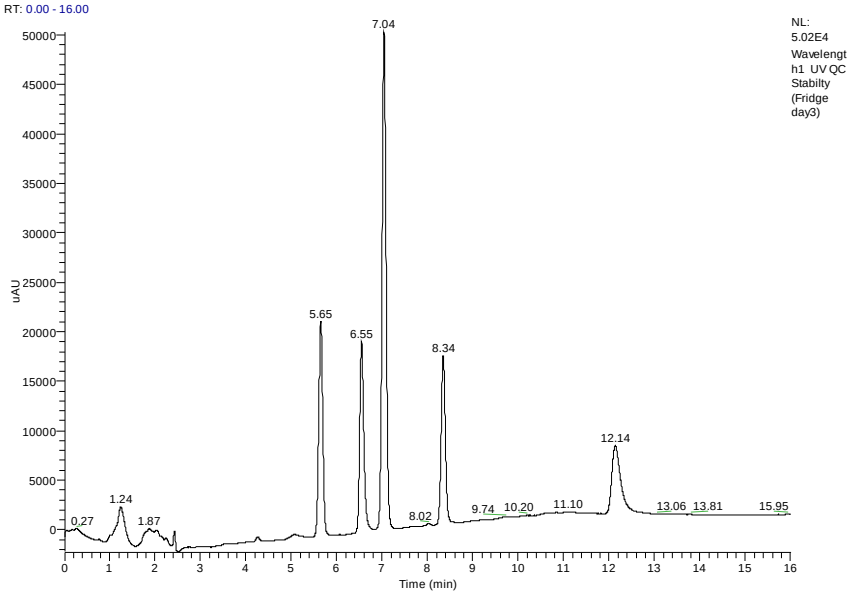


Figure 4.12: HPLC chromatogram of Sceletium standards in methanol maintained at ~ 4°C

Table 4.16 a
Recovery studies of *Sceletium* alkaloids in plant material (HPLC)

Plant RM	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Δ^7**Mesembrenone** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	Δ^7**Mesembrenone content** (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	Δ^7**Mesembrenone** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	
*Content	2.2				2.2				2.3				
Low	(+1.76)[1] 3.96[2]	3.89 (±0.05)	96.20	2.96	(+1.84) 4.04	4.00 (±0.07)	99.00	1.75	(+1.92) 4.22	4.31 (±0.05)	102.13	1.22	3.00
Medium	(+3.52) 5.72	5.83 (±0.10)	101.90	2.85	(+3.68) 5.88	5.72 (±0.10)	97.30	1.83	(+3.84) 6.14	6.17 (±0.11)	100.50	1.82	2.36
High	(+8.80) 11.00	10.73 (±0.13)	97.50	1.50	(+9.20) 11.40	10.92 (±0.24)	95.80	2.20	(+9.60) 11.90	11.69 (±0.12)	98.70	1.10	1.50
Plant RM	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembranol content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	**Mesembranol** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	**Mesembranol** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	
*Content	9.2				9.5				9.2				
Low	(+2.12) 11.32	11.25 (±0.04)	99.38	2.05	(+2.08) 11.58	11.58 (±0.06)	100.00	0.51	(+2.08) 11.28	11.38 (±0.05)	100.90	0.50	0.76
Medium	(+4.24) 13.44	13.14 (±0.16)	97.16	3.63	(+4.16) 13.66	13.65 (±0.15)	99.90	1.10	(+4.16) 13.36	13.46 (±0.08)	100.70	0.60	1.9
High	(+10.60) 19.80	19.87 (±0.50)	100.40	4.50	(+10.40) 19.90	19.81 (±0.34)	99.50	1.72	(+10.40) 19.60	19.95 (±0.30)	101.80	1.47	1.15
Plant RM	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrenone content (µg/ml)	Observed Content (µg/ml)	% Recovery	% RSD (n=3)	**Mesembrenone** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	**Mesembrenone** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	
*Content	1.30				1.30				1.30				
Low	(+2.16) 3.46	3.33 (±0.90)	95.40	4.25	(+2.08) 3.38	3.32 (±0.07)	98.20	2.10	(+2.2) 3.50	3.32 (±0.05)	95.00	1.44	1.80
Medium	(+4.32) 5.62	5.37 (± 0.60)	95.60	1.53	(+4.16) 5.46	5.31 (±0.09)	97.30	1.60	(+4.4) 5.70	5.45 (±0.04)	95.60	0.73	1.02
High	(+10.80) 12.10	12.12 (±0.18)	100.16	1.70	(+10.40) 11.70	11.44 (±0.42)	97.80	3.71	(+11.00) 12.30	11.70 (±0.22)	95.10	1.87	2.60

* Actual content [1]; spiked amount [2]; represents the total content, i.e. spiked plus original content (follows throughout the table), (±SD)

Table 4.16b
Recovery studies of *Sceletium* alkaloids in plant material (HPLC)

Plant RM	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrine content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	**Mesembrine** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	**Mesembrine** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	
*Content	15.82				16.00				15.80				
Low	(+1.88)[1] 17.7[2]	17.64 (±0.09)	99.65	4.65	(+1.81) 17.81	17.88 (±0.30)	100.40	1.62	(+1.85) 17.65	18.23 (±0.08)	103.30	0.42	1.91
Medium	(+3.77) 19.52	19.58 (±0.09)	100.30	2.33	(+3.63) 19.63	19.75 (±0.12)	100.60	0.63	(+3.70) 19.50	20.12 (±0.11)	103.10	0.53	1.51
High	(+9.42) 25.24	25.21 (±0.17)	99.90	1.78	(+9.07) 25.07	25.45 (±0.18)	101.50	0.72	(+9.24) 25.04	25.62 (±0.14)	102.30	0.55	1.21
Plant RM	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Epimesembranol content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** content (µg/ml)	Observed content (µg/ml)	% Recovery	% RSD (n=3)	
*Content	5.90				6.20				6.10				
Low	(+2.08) 7.98	8.04 (±0.06)	100.80	3.04	(+1.84) 8.04	7.75 (±0.08)	96.40	1.00	(+2.08) 8.18	8.14 (±0.04)	99.50	3.50	2.30
Medium	(+4.16) 10.06	10.26 (±0.06)	102.00	1.31	(+3.68) 9.88	9.54 (±0.08)	96.50	0.86	(+4.16) 10.26	10.30 (±0.03)	100.40	0.30	2.80
High	(+10.40) 16.30	17.01 (±0.48)	104.40	4.30	(+9.2) 15.40	15.13 (±0.21)	98.20	1.36	(+10.40) 16.50	16.53 (±0.26)	100.60	1.58	0.45

* Actual content [1]; spiked amount [2]; represents the total content, i.e. spiked plus original content (follows through the table), (±SD)

Table 4.17a
Recovery studies of *Sceletium* alkaloids from plant matrix (HPLC)

	Day 1			**Day 2**			**Day 3**			
Plant matrix	Δ^7**Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	Δ^7**Mesembrenone** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	Δ^7**Mesembrenone** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD
Low	1760.00	1752.20	99.60	1680.00	1708.90	101.70	1920.00	1870.10	97.40	2.15
Medium	3520.00	3409.00	96.80	3360.00	3351.30	99.74	3840.00	3707.60	96.60	1.51
High	8800.00	8541.00	97.06	8400.00	8435.80	100.40	9600.00	9475.30	98.70	1.51
	Day 1			**Day 2**			**Day 3**			
Plant matrix	**Mesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD
Low	2120.00	2061.20	97.23	2080.00	212.20	101.60	2160.00	2078.00	96.21	2.91
Medium	4240.00	4342.40	102.40	4160.00	4294.90	103.24	4320.00	4356.00	100.83	1.20
High	10600.00	11069.20	104.40	10400.00	10526.20	101.20	10800.00	10657.40	98.70	2.80
	Day 1			**Day 2**			**Day 3**			
Plant matrix	**Mesembrenone** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD
Low	2160.00	2100.60	97.54	2080.00	2040.70	98.10	2080.00	2083.30	102.10	2.50
Medium	4320.00	4393.70	101.70	4160.00	3953.80	95.00	4080.00	4153.70	101.80	3.90
High	10800.00	10699.20	99.07	10400.00	10364.40	99.70	10200.00	10314.70	101.10	1.04

Table 4.17b
Recovery studies of *Sceletium* alkaloids from plant matrix (HPLC)

	Day 1			**Day 2**			**Day 3**			
Tablet matrix	**Mesembrine** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine** content Matrix (ng/ml)	Observed content (µg/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	1884.68	1817.77	96.45	1813.56	1824.40	100.60	1884.68	1822.49	96.70	2.40
Medium	3769.36	3833.44	101.70	3627.12	3772.20	104.00	3769.36	3701.50	98.20	2.90
High	9423.40	9288.65	98.57	9067.80	9231.00	101.80	9423.40	9385.70	99.60	1.65
	Day 1			**Day 2**			**Day 3**			
Tablet matrix	**Epimesembranol** content in matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** content Matrix (ng/ml)	Observed content (ng/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	2120.00	2043.30	96.40	1840.00	1903.10	103.40	2080.00	2009.40	96.60	4.06
Medium	4240.00	4207.80	99.24	3680.00	3717.00	101.00	4160.00	4167.40	100.20	0.90
High	10600.00	10719.40	101.13	9200.00	9566.40	104.00	10400.00	10678.00	102.60	1.40

Table 4.18

Ruggedness studies of HPLC method

Compound (determination coefficient R^2)	QC	Actual weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Precision (Tablets) Content in µg/ tablet	RSD (%)	Precision (Plant material) Content in µg/100mg	%RSD (n=3)
Δ^7Mesembrenone (R^2 = 0.9966)	Low	3680.00	3612.50	98.20	9.90 (± 0.128)	1.30	24.70 (±1.10)	4.45
	Medium	7360.00	7559.90	102.70	8.48 (± 0.25)	3.00	26.00 (±1.00)	3.85
	High	9200.00	9211.60	100.10	8.93 (± 0.33)	3.70	21.70 (±1.53)	7.05
Mesembranol (R^2 = 0.9985)	Low	4080.00	4076.90	99.90	29 (± 1.03)	3.55	94.70 (±3.21)	3.40
	Medium	8160.00	8261.80	101.20	30.8 (± 1.2)	3.90	92.33 (±1.52)	1.65
	High	10200.00	10191.70	99.90	30.2 (± 1.26)	4.17	82.67 (±1.52)	1.84
Mesembrenone (R^2 = 0.9979)	Low	3920.00	3924.00	100.10	9.74 (± 0.44)	4.50	10.33 (±0.06)	0.60
	Medium	7840.00	7715.50	98.40	8.70 (± 0.4)	4.60	12.27 (±0.51)	4.16
	High	9800.00	9548.80	97.40	9.53 (± 0.25)	2.62	12.73 (±0.80)	6.30
Mesembrine HCl (R^2 = 0.9986)	Low	3698.24	3614.32	98.90	*121.2 (± 4.3)	3.55	*147.77 (±5.00)	3.40
	Medium	7396.48	7488.50	101.20	*127.3 (± 6.45)	5.07	*160.91 (±2.67)	1.66
	High	9245.60	9215.00	99.70	*122.8 (± 0.98)	0.80	*162.74 (±5.11)	3.14
Epimesembranol (R^2 = 0.9956)	Low	3840.00	3755.90	97.80	7.27 (± 1.00)	1.35	44.72 (±2.13)	4.76
	Medium	7680.00	7649.60	99.60	8.30 (±0.24)	2.91	56.60 (±0.90)	1.60
	High	9600.00	9562.70	99.60	8.10 (± 0.14)	1.80	59.45 (±1.90)	3.20

* Mesembrine

4.5 CONCLUSIONS

Validated analytical methods to assay *Sceletium* plant material and dosage forms for relevant alkaloidal content have not hitherto been reported in the published scientific literature. Published analytical procedures have focused only on qualitative determinations.

Since the identified markers have closely related structures, of which two alkaloids, mesembranol and epimesembranol are epimers, the main objective of this investigation was to develop an efficient HPLC method for the separation and quantitative analysis of relevant alkaloid components in *Sceletium*, and also to reduce, in particular the RT of epimesembranol, which is unacceptably long (> 30 min) under isocratic conditions. Use of a PDA detector was extremely valuable for peak identification and homogeneity testing during the initial method development.

A simple, accurate, precise, rapid and reproducible HPLC method was developed for the identification and quantitative analysis of 5 relevant *Sceletium* alkaloids which has been successfully applied for the assay and QC of *Sceletium* plant material and its dosage forms. Furthermore, this method was found to efficiently separate the alkaloidal markers from complex components present in plant material as well as from excipients used in the tablet dosage form using a simple methanol sample extraction procedure.

Various species of *Sceletium* plants were subsequently provided by *Sceletium* plant cultivators and this method was successfully used for chemo-taxonomy of some *Sceletium* species (*vide infra* – Chapter 6) and has provided impetus for the future development of quality monographs for plant and dosage forms containing *Sceletium*.

CHAPTER 5

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY OF *SCELETIUM* ALKALOIDS

5.1 INTRODUCTION

Plants and plant products used for their medicinal properties have generated popular interest in their use and also commercial activities related to their cultivation and sale. QC of medicinal plants is more complex than usually understood and this could be due to a number of variables including species differences, harvesting time, growing conditions, storage and processing. These are just some of the factors that may influence the nature and composition of active ingredients occurring in a particular plant species. In light of the foregoing, the development of QC methods is an important aspect of the quality which will subsequently reflect on the safety and efficacy of herbal products [73]. Due to current quality issues of herbal products, enormous interest has been generated in the use of HPLC coupled with mass spectrometry (LCMS), which is considered a valuable technique to study phytochemical constituents in plants and their products.

Sceletium alkaloids have been subjected to MS analysis by electron impact (EI) to study the structural characteristics of the molecules [49]. Qualitative analysis, using GCMS [55] has been reported to monitor *Sceletium* alkaloids during the processing of *Sceletium* plant material. However, there are no published methods on the use of LCMS techniques and their application for the assay and QC of *Sceletium* plant material and their products.

Electrospray ionization mass spectrometry (ESI-MS) has been successfully applied to the analysis of plant products due to the provision of a soft ionization process of the molecules and its suitability to characterize multiple components that are usually present in plant products [73]. The LCMS/MS method is reported to be more emphatic when used to generate characteristic fingerprinting in conditions where there is uncertainty in the identification of compounds characterized by HPLC. It is suggested that LCMS is extremely beneficial in cases when RTs vary during HPLC analysis and also in situations where reference standards are unavailable [74].

5.2 OBJECTIVES

Since analytical methods for *Sceletium* alkaloids using LCMS are conspicuously absent from the scientific literature, the main objective was to develop a simple, rapid, precise and reproducible LCMS method that can be specifically applied for chromatographic fingerprinting, quantitative assessment and assay as well as for the identification of *Sceletium* alkaloids in plant material and its commercial products.

5.3 EXPERIMENTAL

5.3.1 Reagents and Materials

HPLC grade methanol 215 and acetonitrile 200 were obtained from Romil Ltd. (Cambridge, Great Britain). Ammonium hydroxide 25% solution was acquired from Associated Chemical Enterprises (Pty) Ltd. (Southdale, South Africa). Water was purified in a Milli-Q® system Millipore (Bedford, USA) and Millex HV® hydrophilic PVDF 0.45 μm membrane filters were purchased from the same source. The source of *Sceletium* reference compounds and plant material and its products have previously been discussed in Chapters 2 and 3, respectively.

5.3.2 Instrumentation

A Cole-Parmer ultrasonic bath, Model 8845-30 (ColeParmer Instrument Company, Chicago, Illinois, USA) was used for the sonication procedures for solvent extraction of samples. The LCMS analyses were carried out using a Finnigan MAT LCQ ion trap mass spectrometer (Finnigan, San Jose, CA, USA) coupled to a SpectraSYSTEM P2000 pump connected to an AS1000 auto sampler and UV1000 variable-wavelength UV detector (Thermo Separation Products, Riviera Beach, FL, USA). The separation of alkaloids was achieved on an HPLC column (Luna® C_{18} (2) 5 μm, 250 mm x 4.6 mm i.d.) manufactured by Phenomenex® (Torrence, CA, USA). Type AG 135 analytical balance and Electronic Micro Balance MX-5 (Mettler Toledo, Switzerland) were used for samples and weighing standards respectively. An electronic pipette 71050XET (Biohit PLC, Helsenki, Finland) was used to transfer standard and sample solutions for dilutions.

5.3.3 Method Development

The qualified reference substances described in Chapter 3 were used for the development of the analytical method and validation studies.

Method development was carried out by adapting the HPLC technique for the separation of identified *Sceletium* alkaloids previously described in this thesis (Chapter 4) and based on those HPLC profiles. Mesembrine alkaloids, being basic in character, their respective ions that formed during the ionization process were positive, which provided the $[M+H]^+$ molecular ion of the relevant compound as an *m/z* value. The system was tuned using the optimized LCMS conditions previously discussed in Chapter 3, (Section 3.5.1.1). A binary gradient elution system made up of 0.1% ammonium hydroxide solution in water (ammonia buffer) mixed with ACN, adapted from the HPLC analysis (Section 4.3.3, Table 4.1) was used to ionize the molecules. The flow rate was maintained at 1.0 ml/min through a Luna® C_{18} (2) 150 x 4.6 mm i.d. HPLC column. The analysis resulted in satisfactory chromatographic separation and ionization of the identified alkaloids. The full scan ESI mode provided the TIC chromatogram (Figures 5.1a and 5.1b) for the masses identified by their *m/z* ratios as follows:

- *m/z* 288 for Δ^7mesembrenone and mesembrenone at RT 5.66 and 7.09 minutes respectively
- *m/z* 290 for mesembrine at RT 8.38 minutes
- *m/z* 292 for mesembranol and epimesembranol at RT 6.54 and 11.67 minutes respectively

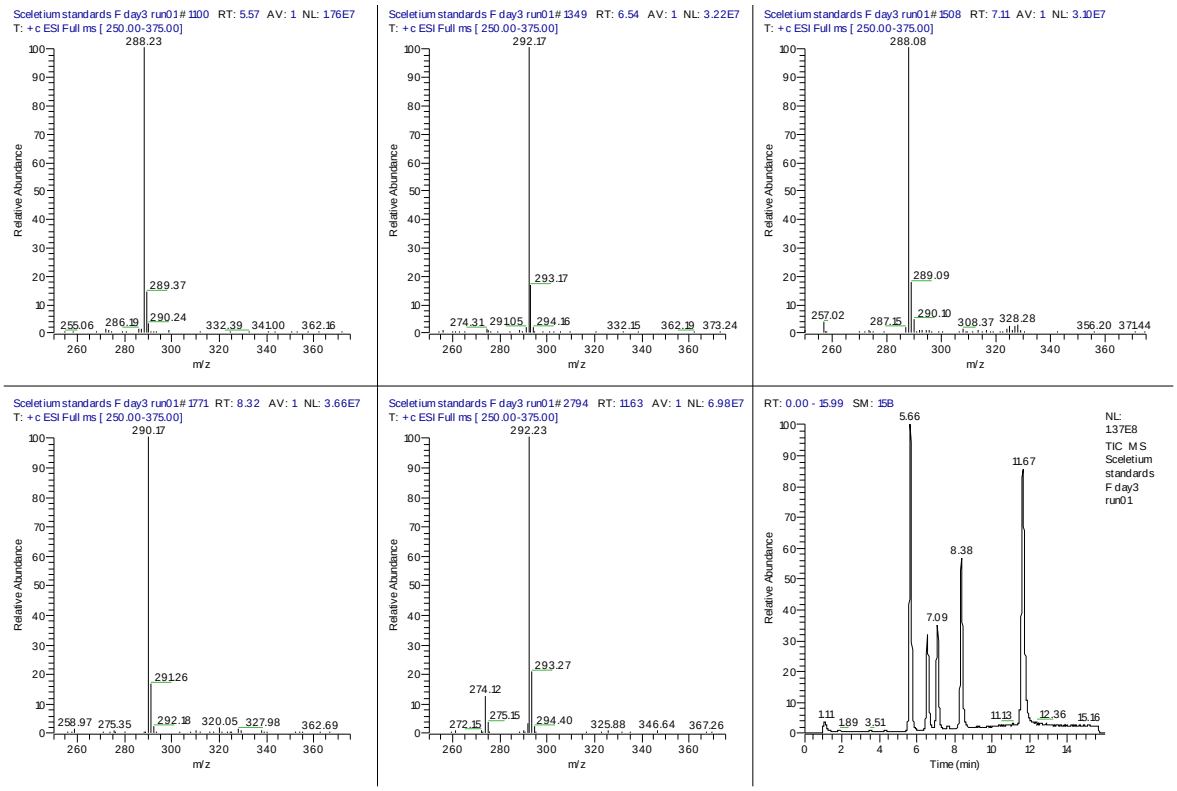


Figure 5.1a: Bottom right-TIC chromatogram of standard Sceletium alkaloids. Ion chromatograms- top left- Δ7mesembrenone, top middle- mesembranol, top right- mesembrenone, bottom left- mesembrine and bottom middle- epimesembranol

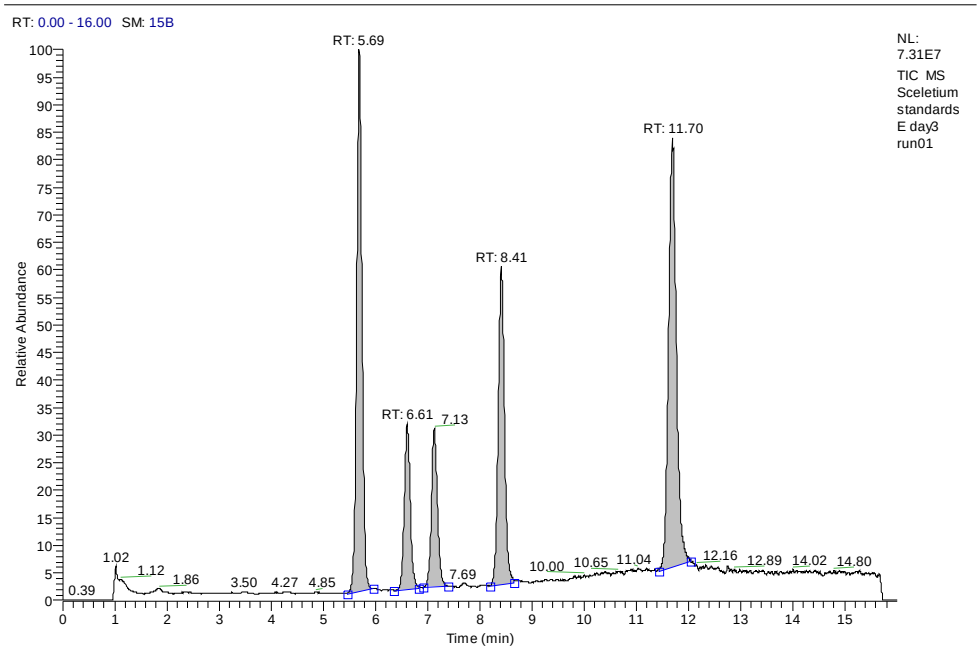


Figure 5.1b: TIC chromatogram of standard Sceletium alkaloids

The method was successfully applied to *Sceletium* tablets (Figure 5.2) and *Sceletium* plant material (Figure 5.3) and showed satisfactory ionization of the identified alkaloids from other unidentified components present in such matrices.

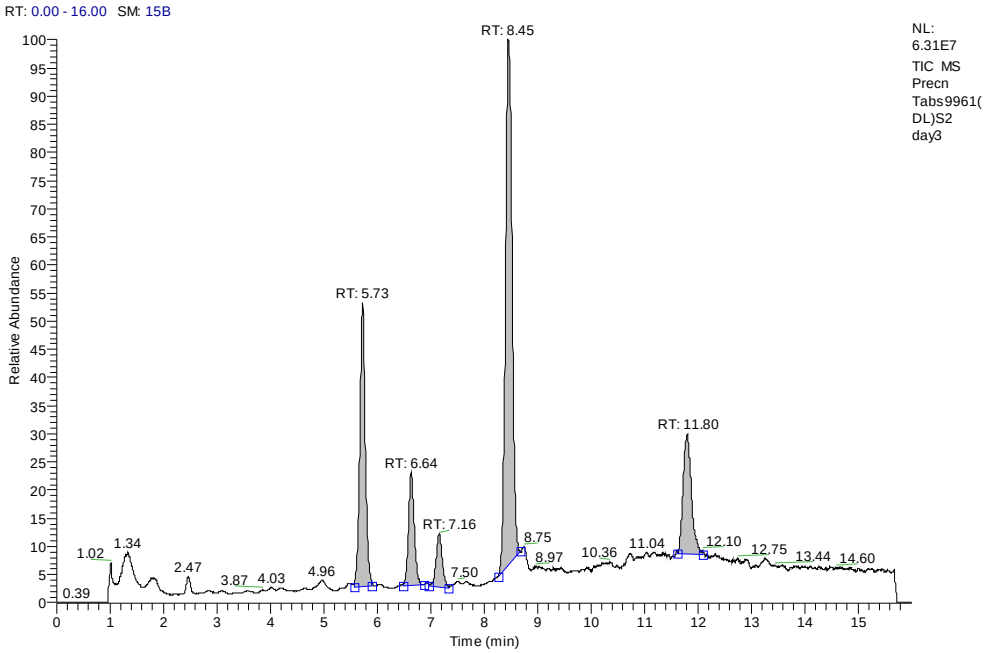


Figure 5.2: TIC chromatogram of Sceletium tablets (Big Tree Health Products)

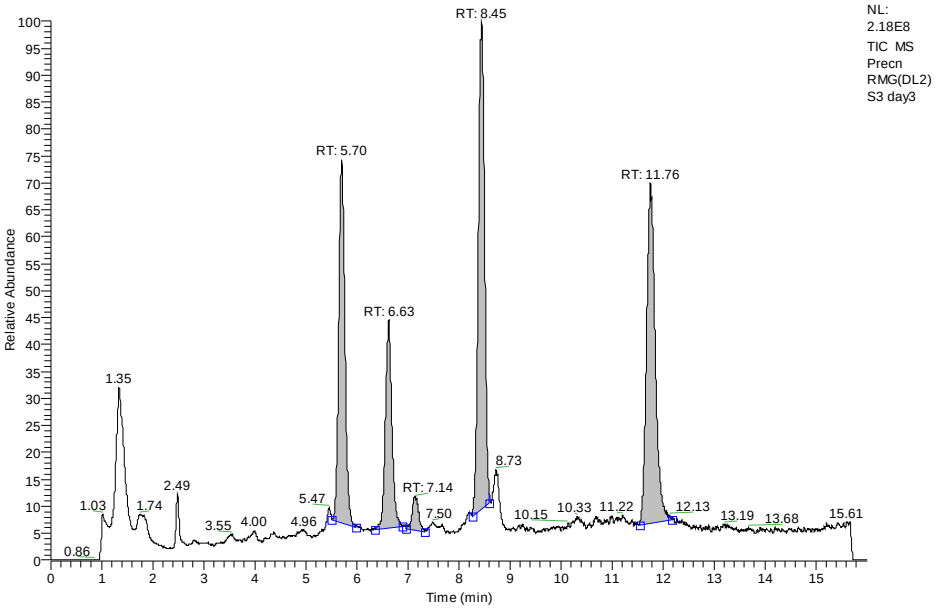


Figure 5.3: TIC chromatogram of Sceletium plant material

During this study, there were many instances where the LCMS method was applied to confirm the identity of the alkaloids since they eluted very close to other unidentified peaks observed during HPLC analysis. A typical situation may be explained by referring to the chromatograms shown in Figure 5.4.

A sample of *Sceletium tortuosum* tablets (Big Tree Health Products, Cape Town, South Africa, Batch number 9332) when analyzed by HPLC, displayed the characteristic peaks for *Sceletium* alkaloids and were well resolved. However, the peak appearing at RT 5.50 minutes was not Δ^7mesembrenone but one of the related alkaloids with an *m/z* value of 273.96 as shown in the TIC chromatogram at RT 5.56 minutes. The peak at RT of 5.72 by UV detection and 5.79 minutes by MS detection, displayed the *m/z* 288.2, which corresponds to Δ^7mesembrenone. Application of the LCMS method to *Sceletium* products is discussed further in Chapter 6.

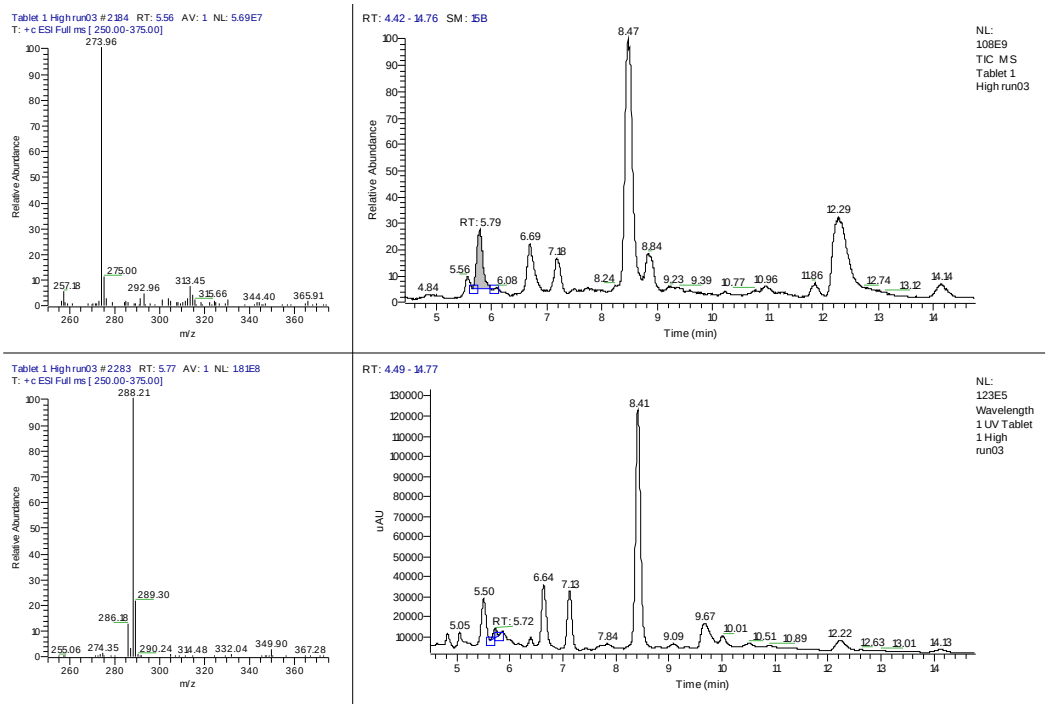


Figure 5.4: Fingerprinting of Sceletium tablets (Big Tree Health Products) showing improved detection of Δ^7mesembrenone by MS compared to UV detection. Top right - TIC chromatogram. Bottom right - HPLC-UV chromatogram. Top left - ion chromatogram showing m/z 273.96 (related alkaloid), bottom left - ion chromatogram showing m/z 288.21 (Δ^7mesmbrenone)

In another instance, a sample of *Sceletium emarcidum* (Figure 5.5) when analyzed by HPLC/UV showed peaks eluting close to the RTs of the expected reference alkaloids. However, the TIC showed only one peak at RT 13.53 min with an *m/z* of 362.04 confirming the absence of the relevant alkaloids in the sample. This emphasizes the need to use appropriate methods such as

HPLC/PDA and importantly, MS detection for qualitative QC of herbal products and its dosage forms.

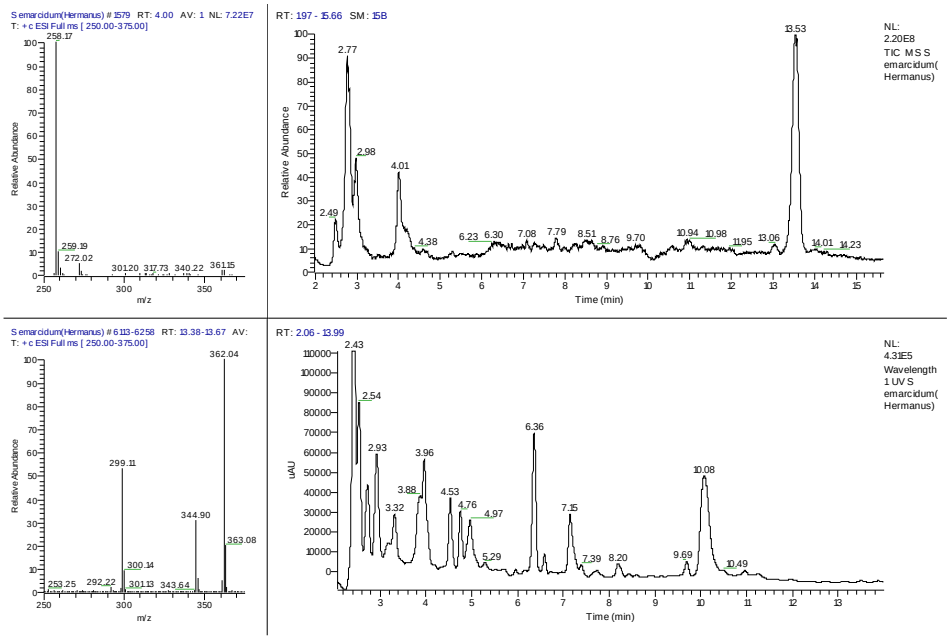


Figure 5.5: Fingerprinting of Sceletium emarcidum. Top right - TIC chromatogram. Bottom right- HPLC-UV chromatogram. Top left - ion chromatogram showing m/z 258.17 (unidentified compound), bottom left - ion chromatogram showing m/z 362.04 (unidentified compound)

5.4 METHOD VALIDATION

5.4.1 Preparation of Standards and Samples

Standard solutions were prepared fresh on each of three separate days. Methanolic stock solutions (1mg/ml) of Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine hydrochloride and epimesembranol were prepared. A working stock solution was prepared and diluted to obtain a concentration of 100 µg/ml of each alkaloid. Standard solutions comprising a set of nine calibrators in the concentration range of 25-2000 ng/ml were prepared.

5.4.2 Accuracy and Precision Studies

Accuracy and precision studies were performed by separately preparing standard solutions and appropriate dilutions to obtain final concentrations of 200, 400 and 1,000 ng/ml for use as QC standards. The precision studies of a selected *Sceletium* plant powder and a commercially purchased lot of *S. tortuosum* tablets from Big Tree Health Products (Cape Town, South Africa, Batch number 9961) were prepared in methanol by sonication for 20 minutes and filtered through 0.45 µm PVDF membrane filters.

Preparations of *Sceletium* tablets containing 1.5, 2.5 and 3.5 mg/ml of crushed tablets in methanol and also preparations of *Sceletium* plant powder containing 0.75, 1.5 and 2.5 mg/ml of the powder in methanol provided the low, medium and high samples for precision studies, respectively.

5.4.3 Recovery Studies- Tablet, Plant Material and their Matrices

Recovery studies were carried out by preparing three individual concentrations of 2.5 mg/ml of crushed tablet samples in triplicate and 1 mg/ml preparations of the plant material. Standard stock solutions of each of the five alkaloids were added to each of the triplicate sample solutions to result in concentrations of 200 ng, 400 ng and 1,000 ng/ml of each of the alkaloids/sample.

Plant and tablet matrices (as placebo samples) were confirmed for absence of the known alkaloidal components by LCMS analysis. Tablet and plant matrix samples were prepared individually by adding stock alkaloid standard mixture to obtain final spiked concentrations of 200, 400 and 1,000 ng/ml of each of the alkaloids/sample.

5.4.4 Limit of Quantitation and Limit of Detection

Standard stock solutions were diluted appropriately to obtain concentrations for the estimation of the limit of detection (LoD) and limit of quantitation (LoQ) according to a signal to noise (S/N) ratio of 3:1 and 10:1, respectively.

5.4.5 Solution Stability

The reference substances in methanol were tested for their stability by analyzing samples that were maintained at room temperature 22 ±2°C (bench stability) and ~ 4°C (fridge stability).

5.4.6 Results and Discussion

5.4.6.1 Method Validation

5.4.6.1.1 Linearity

Calibration curves were constructed by plotting the peak area of each alkaloid versus the concentration corresponding to that alkaloid on each of three days. The curves obtained were found to be linear with determination coefficients better than 0.99. Details are provided in Table 5.1.

Table 5.1

Linear ranges and coefficients of determination (LCMS)

Name of the compound	Day	y = mx + c linear model	Determination coefficient (R^2)
Δ^7Mesembrenone	Day 1	y = 2056.4x + 127880	R^2 = 0.9958
	Day 2	y = 2151.9x + 128087	R^2 = 0.9913
	Day 3	y = 2055.6x + 133190	R^2 = 0.9925
Mesembranol	Day 1	y = 599.58x + 48816	R^2 = 0.9934
	Day 2	y = 570.64x + 52306	R^2 = 0.9903
	Day 3	y = 597.90x + 51522	R^2 = 0.9920
Mesembrenone	Day 1	y = 675.33x + 31024	R^2 = 0.9958
	Day 2	y = 694.67x + 29422	R^2 = 0.9966
	Day 3	y = 697.84x + 29766	R^2 = 0.9957
Mesembrine HCl	Day 1	y = 1357.4x + 54759	R^2 = 0.9975
	Day 2	y = 1388.8x + 47784	R^2 = 0.9975
	Day 3	y = 1373.6x + 49343	R^2 = 0.9972
Epimesembranol	Day 1	y = 2415.3x + 135995	R^2 = 0.9922
	Day 2	y = 2533.5x + 94820	R^2 = 0.9952
	Day 3	y = 2456.6x + 99137	R^2 = 0.9928

5.4.6.1.2 Precision and Accuracy

The studies were performed using QC samples that were prepared separately on each day of the analysis. Precision of the analytical method was performed to assess the ability of the method to produce consistent results. The inter-day RSD values obtained were less than 5% for *Sceletium* alkaloid QC standards. The accuracy of the method was found to be between 95.2%-104.7% for all five compounds. The results were tabulated and are shown in Tables 5.2, 5.3, 5.4, 5.5 and 5.6.

Table 5.2
Accuracy: Δ^7Mesembrenone (LCMS)

Δ^7Mesembrenone	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day % RSD
Low spike	1	176.00	184.20	104.70	4.00
	2	184.00	176.70	100.50	
	3	192.00	185.50	96.60	
Medium Spike	1	352.00	345.30	98.10	1.60
	2	368.00	353.10	95.90	
	3	384.00	365.50	95.20	
High spike	1	880.00	863.40	98.50	0.50
	2	920.00	918.00	99.80	
	3	960.00	954.10	99.40	

Table 5.3
Accuracy: Mesembranol (LCMS)

Mesembranol	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day % RSD
Low spike	1	212.00	207.00	97.60	2.30
	2	208.00	211.60	99.30	
	3	212.00	207.20	102.20	
Medium Spike	1	424.00	427.10	103.00	0.53
	2	416.00	419.70	103.90	
	3	424.00	438.60	104.50	
High spike	1	1080.00	1097.10	101.60	1.05
	2	1040.00	1113.50	103.10	
	3	1060.00	1065.90	103.70	

Table 5.4
Accuracy: Mesembrenone (LCMS)

Mesembrenone	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day % RSD
Low spike	1	216.00	213.90	99.00	0.70
	2	216.00	210.30	97.35	
	3	220.00	217.00	98.80	
Medium Spike	1	432.00	427.10	98.90	1.70
	2	432.00	417.50	96.64	
	3	440.00	432.03	98.20	
High spike	1	1080.00	1097.10	101.60	0.50
	2	1080.00	1068.90	98.97	
	3	1100.00	1074.90	97.70	

Table 5.5
Accuracy: Mesembrine HCl (LCMS)

Mesembrine HCl	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day % RSD
Low spike	1	212.00	218.40	103.00	1.90
	2	204.00	211.20	103.50	
	3	192.00	191.90	100.00	
Medium Spike	1	424.00	427.70	100.90	2.10
	2	408.00	423.00	104.60	
	3	384.00	404.10	105.00	
High spike	1	1060.00	1033.20	97.50	1.56
	2	1020.00	1014.90	99.50	
	3	960.00	926.60	96.50	

Table 5.6
Accuracy: Epimesembranol (LCMS)

Epimesembranol	Day	Actual Weight (ng/ml)	Calculated Weight (ng/ml)	% Accuracy	Inter-day % RSD
Low spike	1	208.00	212.00	101.90	2.70
	2	184.00	191.60	104.10	
	3	208.00	221.00	104.80	
Medium Spike	1	416.00	410.20	98.60	4.80
	2	368.00	348.10	94.60	
	3	416.00	425.00	98.70	
High spike	1	1040.00	1082.30	104.00	4.20
	2	920.00	906.00	98.50	
	3	1040.00	1004.40	96.60	

5.4.6.1.3 Precision Studies – Tablet Formulation

Precision studies were performed to assess the ability of the method to produce consistent results for the tablet dosage forms. The studies were carried out by preparing three sets of low, medium and high sample concentrations on each day of analysis. The RSD for inter-day precision data are shown in Table 5.7. The identified *Sceletium* alkaloids were estimated and their contents presented as microgram per tablet are shown in Table 5.8.

Table 5.7

Precision studies of *Sceletium* tablets (LCMS)

Compound	Content in µg/tablet (± SD)			
Δ^7Mesembrenone	Day1	Day2	Day3	Inter-day % RSD (n=3)
Low	13.06 (±13.06)	12.62 (±1.20)	11.33 (±1.00)	7.20
Medium	13.41 (±1.64)	11.87 (±0.70)	12.44 (±0.80)	6.20
High	13.26 (±1.00)	12.02 (±0.60)	12.1 (±0.42)	5.60
Mesembranol	Day1	Day2	Day3	Inter-day % RSD (n=3)
Low	32.07 (±2.50)	33.3 (±2.40)	31.74 (±2.60)	2.50
Medium	33.15 (±0.70)	34 (±0.60)	33.21 (±1.15)	1.40
High	36.31 (±1.70)	35.6 (±2.30)	36 (±0.80)	1.00
Mesembrenone	Day1	Day2	Day3	Inter-day % RSD (n=3)
Low	8.50 (±1.00)	9.40 (±0.40)	9.33 (±0.34)	5.60
Medium	8.57 (±0.30)	9.23 (±0.15)	9.03 (±0.56)	3.80
High	9.40 (±0.70)	9.20 (±0.90)	9.73 (±0.70)	2.80
Mesembrine	Day1	Day2	Day3	Inter-day % RSD (n=3)
Low	117.60 (±3.80)	115.70 (±2.20)	118.00 (±4.80)	1.10
Medium	123.10 (±4.10)	121.80 (±0.80)	120.10 (±4.40)	1.20
High	113.00 (±4.21)	114.10 (±1.80)	116.70 (±3.30)	1.60
Epimesembranol	Day1	Day2	Day3	Inter-day % RSD (n=3)
Low	8.80 (±0.80)	10.81 (±0.90)	10.50 (±0.70)	10.60
Medium	9.50 (±1.26)	10.35 (±0.65)	11.70 (±0.80)	10.30
High	10.00 (±0.75)	10.60 (±1)	10.60 (±1.00)	3.50

Average weight of tablet = 497.3 mg, SD = Standard deviation
Low = 1.5 mg/ml (n=3), Medium = 2.5 mg/ml (n=3), High = 3.5 mg/ml (n=3); Total n=9 per day

Table 5.8

Content of identified *Sceletium* alkaloids per tablet (LCMS)

Compound	*Content in µg/tablet
Δ^7Mesembrenone	12.5
Mesembranol	33.9
Mesembrenone	9.15
Mesembrine	117.8
Epimesembranol	10.3

*Average values obtained from precision studies

5.4.6.1.4 Recovery Studies – Tablet Formulation

The recoveries of the spiked *Sceletium* alkaloid standards were evaluated to assess the extraction efficiency of the analytical method. The values obtained for this experiment show good recoveries of all five identified alkaloids in the range of 89%-108% for each of the added compound with RSD values of less than 10%. The results are shown in Tables 5.9a and 5.9b.

In addition, recovery studies were performed to assess the effect of excipients on the extraction of the product by adding low, medium and high concentrations of *Sceletium* standards to the tablet matrix.

The recoveries from the spiked samples of the tablet matrix are depicted in Tables 5.10a & 5.10b and ranged between 94%-108% for each of the added alkaloid compounds with inter-day RSDs of less than 7%.

Table 5.9a
Recovery studies of *Sceletium* alkaloids in tablet dosage form (LCMS)

Tablet Sample	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Δ^7Mesembrenone (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Δ^7Mesembrenone** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Δ^7Mesembrenone** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	63.00				63.80				60.30				
Low	(+176.00)[1] 239.00[2]	247.46 (±9.30)	103.50	3.74	(+208.00) 271.80	264.40 (±6.5)	97.30	2.45	(+192.00) 252.3	266.84 (±4.5)	105.70	1.70	4.20
Medium	(+352.00) 415.00	414.4 (±27.40)	99.90	6.61	(+416.00) 479.80	473.00 (±28.2)	98.60	5.90	(+384.00) 444.3	474.40 (±27.4)	106.80	5.80	4.30
High	(+880.00) 943.00	1011.00 (±44.10)	107.20	4.36	(+1040.00) 1103.80	1082.00 (±62.8)	98.00	4.30	(+960.00) 1020.3	1078.60 (±60.10)	105.70	5.50	4.80
Tablet Sample	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembranol (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembranol** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembranol** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	170.40				170.90				169.10				
Low	(+212.00) 382.40	370.63 (±7.4)	96.90	2.00	(+208.00) 378.9	386.90 (±9.1)	102.10	8.90	(+212.00) 381.10	378.50 (±11.90)	99.30	3.10	4.20
Medium	(+424.00) 594.40	615.61 (±10.10)	103.60	1.63	(+416.00) 586.9	609.10 (±21.4)	103.80	3.50	(+424.00) 593.10	603.70 (±18.20)	101.80	3.00	1.07
High	(+1060.00) 1230.40	1245.90 (±20.80)	101.30	1.67	(+1060.00) 1230.9	1282.50 (±50.2)	104.20	3.90	(+1060.00) 1229.10	1204.50 (±46.2)	98.00	3.80	1.90
Tablet Sample	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrenone (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrenone** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrenone** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	46.00				46.60				47.10				
Low	(+212.00) 258.00	263.80 (±17.00)	102.30	6.10	(+208.00) 254.60	263.50 (±14.60)	103.30	5.60	(+220.00) 267.10	255.20 (±17.10)	95.60	6.70	4.17
Medium	(+424.00) 470.00	503.40 (±15.10)	107.10	2.90	(+416.00) 462.60	462.50 (±7.20)	100.00	1.70	(+440.00) 487.10	472.80 (±33.10)	97.10	7.00	5.00
High	(+1060.00) 1106.00	1247.90 (±15.10)	112.80	2.60	(+1060.00) 1106.6	1138.20 (±46.10)	102.90	4.10	(+1100.00) 1147.10	1170.10(±75.80)	102.00	6.50	5.65

* Actual content [1]; spiked amount [2]; represents the total content, i.e. spiked plus original content (follows through the table), (±SD), Average weight = 497.30 mg/ tablet

Table 5.9b
Recovery studies of *Sceletium* alkaloids in tablet dosage form (LCMS)

Tablet Sample	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrine (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrine** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrine** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	592.20				589.30				594.40				
Low	(+184.90)[1] 777.10[2]	783.13 (± 2.82)	100.80	0.36	(+181.40) 770.70	760.00 (±6.60)	98.60	0.90	(+170) 764.4	785.4 (±10.70)	102.80	1.40	2.10
Medium	(+369.80) 962.00	972.60 (±10.60)	101.10	1.10	(+ 362.70) 952.00	938.50 (±48.7)	98.60	5.20	(+320) 914.4	946.5 (±32.40)	103.50	3.40	2.40
High	(+924.60) 1516.80	1610.20 (±65.1)	106.16	4.10	(+924.60) 1513.90	1526.40 (±66.3)	100.80	4.30	(+853.4) 1706.8	1544.2 (±65.40)	90.40	4.20	8.00
Tablet Sample	**Day-1**				**Day-2**				**Day-3**				Inter-day Recovery % RSD (n=3)
	Epimesembranol (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** (ng/2.5 mg dosage/ml)	Observed content (ng/2.5 mg dosage/ml)	% Recovery	% RSD (n=3)	
Content	51.70				53.20				55.30				
Low	(+208.00) 259.70	252.70 (±8.90)	97.30	3.00	(+184.00) 237.20	257.20 (±8.50)	108.00	3.30	(+208.00) 263.30	235.9 (±10.80)	89.60	4.60	9.40
Medium	(+416.00) 467.70	448.40 (±13.70)	95.90	3.20	(+368.00) 421.20	443.70 (±13.00)	105.30	2.90	(+416.00) 471.30	448.6 (±21.70)	95.20	4.80	5.50
High	(+1040.00) 1091.70	1159.70 (±45.40)	106.20	3.90	(+920.00) 973.20	1042.90 (±47.80)	107.20	4.60	(+1040.00) 1095.30	1128.7 (±53.70)	103.00	4.70	2.00

* Actual content [1]; spiked amount [2]; represents the total content, i.e. spiked plus original content (follows through the table), (±SD), Average weight = 497.30 mg/ tablet

Table 5.10a
Recovery studies of *Sceletium* alkaloids from tablet matrix (LCMS)

	Day 1			**Day 2**			**Day 3**			
Tablet matrix	Δ^7**Mesembrenone** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	Δ^7**Mesembrenone** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Δ7Mesembrenone** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	176.00	186.50	106	192.00	182.80	95.20	192.00	180.70	94.10	6.70
Medium	352.00	351.00	99.70	384.00	349.30	91.00	384.00	367.40	95.70	4.60
High	880.00	854.00	97.00	960.00	955.40	99.50	960.00	964.40	100.50	1.80
	Day 1			**Day 2**			**Day 3**			
Tablet matrix	**Mesembranol** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	212.00	213.20	100.10	208.00	217.90	104.70	212.00	219.00	103.30	2.30
Medium	424.00	454.20	107.00	416.00	418.60	100.60	424.00	434.80	102.60	3.20
High	1080.00	1123.00	104.00	1040.00	1121.30	107.80	1060.00	1071.60	101.00	3.30
	Day 1			**Day 2**			**Day 3**			
Tablet matrix	**Mesembrenone** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	212.00	200.80	94.70	208.00	197.50	95.00	220.00	218.50	99.30	2.70
Medium	424.00	429.60	101.30	416.00	419.90	100.90	440.00	457.20	103.90	1.60
High	1080.00	1082.60	100.20	1040.00	997.20	95.60	1100.00	1083.10	98.50	2.40

Table 5.10b
Recovery studies of *Sceletium* alkaloids from tablet matrix (LCMS)

	Day 1			**Day 2**			**Day 3**			
Tablet matrix	**Mesembrine** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine** (ng/2.5mg atrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine** (ng/2.5mg matrix/ml)	Observed content (µg/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	188.50	191.50	101.40	181.40	191.30	105.50	170.70	183.30	107.40	2.90
Medium	376.90	371.00	98.40	362.70	367.10	101.20	341.40	357.20	104.60	3.10
High	960.10	987.20	102.80	906.80	899.00	99.10	853.40	886.30	103.90	2.50
	Day 1			**Day 2**			**Day 3**			
Tablet matrix	**Epimesembranol** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** (ng/2.5mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	208.00	211.40	101.60	184.00	198.10	107.60	208.00	216.60	104.10	2.90
Medium	416.00	407.90	98.10	368.00	349.80	95.10	416.00	448.70	107.90	1.80
High	1060.00	1025.20	96.70	920.00	875.20	95.10	1040.00	1010.80	97.20	1.10

5.4.6.1.5 Precision studies – Sceletium Plant Material

The RSD for inter-day precision was found to be less than 6% from the results obtained, which are shown in Table 5.11. The content of the identified *Sceletium* alkaloids in the plant material are presented in Table 5.12.

Table 5.11
Precision studies on *Sceletium* plant material (LCMS)

Compound	**Content in µg/100mg (± SD)**			
Δ^7Mesembrenone	Day1	Day2	Day3	Inter-day % RSD
Low	54.4 3(±3.90)	52.12 (±2.78)	57.44 (±7.96)	4.90
Medium	63.15 (±1.71)	56.75 (±2.57)	59.18 (±1.80)	5.41
High	55.40 (±3.52)	50.50 (±0.73)	55.72 (±3.87)	5.43
Mesembranol	Day1	Day2	Day3	Inter-day % RSD
Low	111.67 (±5.86)	106.54 (±2.84)	112.74 (±6.00)	2.90
Medium	129.43 (±.8.62)	118.57 (±6.91)	134.30 (±7.81)	6.32
High	112.31 (±6.35)	118.53 (±6.90)	119.75 (±10.21)	3.41
Mesembrenone	Day1	Day2	Day3	Inter day % RSD
Low	15.10 (±0.70)	15.00 (±1.37)	14.95 (±2.10)	0.50
Medium	17.72 (±0.51)	17.40 (±0.36)	18.00 (±1.63)	1.70
High	18.40 (±1.73)	17.35 (±1.67)	17.20 (±1.00)	3.30
Mesembrine	Day1	Day2	Day3	Inter day % RSD
Low	138.90 (±6.87)	129.32 (±7.78)	130.15 (±8.20)	4.00
Medium	142.75 (±2.87)	142.37 (±2.90)	132.27 (±4.10)	4.20
High	124.38 (±3.65)	121.15 (±2.85)	119.84 (±4.70)	1.68
Epimesembranol	Day1	Day2	Day3	Inter day % RSD
Low	63.48 (±1.48)	66.81 (±2.90)	69.37 (±5.2)	4.44
Medium	76.44 (±3.95)	76.64 (±3.51)	78.00 (±2.90)	1.10
High	73.06 (±4.57)	71.61 (±4.90)	77.00 (±6.00)	3.77

SD= Standard deviation
Low = 0.75 mg/ml (n=3), Medium = 1.5 mg/ml (n=3), High = 2.5 mg/ml (n=3); Total samples n=9 each day

Table 5.12 Content of identified *Sceletium* alkaloids in plant material (LCMS)	
Compound	Content in %
Δ^7Mesembrenone	0.056
Mesembranol	0.120
Mesembrenone	0.017
Mesembrine	0.130
Epimesembranol	0.073

5.4.6.1.6 Recovery Studies – Sceletium Plant Material

The values obtained for this experiment which involved spiking plant material with the relevant alkaloids, are shown in Tables 5.13a and 5.13b where the recoveries of all five identified alkaloids were seen to be in the range of 85%-115% for each of the added compounds with inter-day RSD values of less than 10%.

The recoveries of the alkaloids spiked in plant matrix samples are shown in Tables 5.14a and 5.14b and the results were found to range between 85%-115% for each added compound with inter-day RSDs of less than 10%.

5.4.6.1.7 Limit of Detection and Limit of Quantification

The LoD and LoQ of mesembranol, mesembrenone, mesembrine and epimesembranol were found to be 25 and 50 ng/ml respectively. The LoD and LoQ of Δ^7mesembrenone were found to be 10 and 20 ng/ml. The values were calculated using the respective S/N ratios of 3 and 10.

5.4.6.1.8 Solution stability

The reference substances in methanol were tested for their stability by analyzing samples that were maintained at room temperature 22 ± 2°C (bench stability) (Figure 5.6) and ~ 4°C (fridge stability) (Figure 5.7). The results showed no significant change in content or degradation of the alkaloids.

Table 5.13 a
Recovery studies of *Sceletium* alkaloids in plant material (LCMS)

Plant material	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Δ^7**Mesembrenone** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	Δ^7**Mesembrenone** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	Δ^7**Mesembrenone** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	
Content	580.00				530.00				580.00				
Low	(+176.00) 756.00	755.20 (±4.50)	99.90	0.60	(+184.00) 714.00	699.10 (±15.8)	97.90	2.30	(+176.00) 756.00	753.10 (±17.00)	99.90	2.30	1.20
Medium	(+352.00) 932.00	934.60 (±150)	100.30	1.60	(+368.00) 898.00	883.00 (±6.75)	98.30	0.80	(+352.00) 932.00	953.30 (±15.40)	102.30	1.60	2.00
High	(+880.00) 1460.00	1351.40 (±10.10)	92.60	0.70	(+920.00) 1450.00	1367.90(±31.20)	94.30	2.30	(+880.00) 1460.00	1482.60 (±93.60)	92.60	6.30	1.10
Plant material	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembranol (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Mesembranol** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Mesembranol** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	
Content	1200.00				1200.0				1210.0				
Low	(+212.00) 1412.00	1410.20 (±20.40)	99.90	1.40	(+208.00) 1408.00	1404.40 (±13.70)	99.70	1.00	(+212.00) 1422.00	1407.60 (±16.50)	98.90	1.20	0.50
Medium	(+424.00) 1624.00	1650.80 (±40.50)	101.70	2.40	(+416.00) 1616.00	1591.50 (±36.70)	98.50	2.30	(+424.00) 1634.00	1636.00 (±54.90)	100.10	3.40	1.60
High	(+1020.00) 2220.00	2251.10 (±83.60)	101.40	3.70	(+1040.00) 2240.00	2237.30 (±134.00)	99.90	6.00	(+1020.00) 2230.00	2373.50 (±70.20)	106.40	3.00	3.30
Plant material	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrenone (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Mesembrenone** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Mesembrenone** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	
Content	190.00				170.00				170.00				
Low	(+216.00) 406.00	431.00 (±19.5)	106.20	4.50	(+208.00) 378.00	389.70 (±22.50)	103.10	5.80	(+216.00) 386.00	393.70 (±10.10)	102.00	2.60	2.10
Medium	(+432.00) 622.00	627.30 (±16.00)	100.90	2.60	(+416.00) 586.00	629.00 (±15.70)	107.30	2.50	(+432.00) 602.00	633.50 (±19.90)	105.20	3.10	3.10
High	(+1080.00) 1270.00	1386.70 (±98.00)	109.20	7.10	(+1040.00) 1210.00	1321.20 (±61.50)	109.20	4.70	(+1080.00) 1250.00	1282.40 (±92.30)	102.60	7.20	3.60

* Actual content[1]; spiked amount[2]; represents the total content, i.e. spiked plus original content (follows through the table), (±SD)

Table 5.13b
Recovery studies of *Sceletium* alkaloids in plant material (LCMS)

Plant Material	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrine (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Mesembrine** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Mesembrine** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	
*Content	1289.00				1306.80				1271.30				
Low	(+188.50)[1] 1477.50[2]	1469.7 (±12.20)	99.50	0.80	(+188.50) 1495.30	1507.00 (±12.00)	100.80	0.80	(+188.50) 1459.80	1451.4 0(±21.10)	99.40	1.50	0.80
Medium	(+376.90) 1665.90	1691.9 (±41.40)	101.60	2.40	(+376.90) 1683.70	1694.50 (±52.80)	100.60	3.10	(+376.90) 1648.20	1674.70 (±6.80)	101.60	1.60	0.60
High	(+942.30) 2231.30	2261.70 (±58.30)	101.40	2.60	(+942.30) 2249.1	2255.80 (±89.00)	100.30	3.90	(+942.30)2213.60	2146.30 (±87.00)	97.00	4.10	2.30
Plant Material	**Day-1**				**Day-2**				**Day-3**				Inter-day Recovery % RSD (n=3)
	Epimesembranol (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	**Epimesembranol** (ng/1mg/ml)	Observed content (ng/ml)	% Recovery	% RSD (n=3)	
Content	710.00				710.0				750.0				
Low	(+208.00) 918.00	908.20 (±13.80)	98.90	1.50	(+208.00) 990.00	915.8 (±33.20)	92.50	3.6.00	(+208) 958.00	970.80 (±25.10)	101.30	2.60	4.70
Medium	(+416.00) 1126.00	1161.00 (±17.40)	103.10	1.50	(+416.00) 1126.00	1129 (±23.70)	100.30	2.10	(+416) 1166.00	1175.70 (±12.40)	100.80	1.10	4.50
High	(+1040.00) 1750.00	1864.00 (±37.50)	106.50	2.00	(+1040.00) 1750.00	1725.20 (±129.00)	98.60	7.50	(+1040) 1790.00	1862.80 (±18.60)	104.10	1.00	3.90

* Actual content[1]; spiked amount[2]; represents the total content, i.e. spiked plus original content (follows through the table), (±SD)

Table 5.14a
Recovery studies of *Sceletium* alkaloids from plant matrix (LCMS)

	Day 1			**Day 2**			**Day 3**			
Plant Matrix Sample	Δ^7**Mesembrenone** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	Δ^7**Mesembrenone** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	Δ^7**Mesembrenone** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	176.00	173.70	98.70	184.00	179.80	97.80	176.00	179.50	102.00	2.20
Medium	352.00	355.60	101.00	368.00	349.00	94.80	352.00	383.30	108.90	7.00
High	880.00	916.30	104.10	920.00	875.60	95.20	880.00	915.70	104.10	5.10
	Day 1			**Day 2**			**Day 3**			
Plant Matrix Sample	**Mesembranol** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembranol** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	212.00	200.70	94.70	208.00	208.80	100.40	212.00	212.50	100.20	3.30
Medium	424.00	439.90	103.80	416.00	459.40	110.40	424.00	451.00	106.40	3.10
High	1060.00	1069.60	100.90	1040.000	1121.40	107.80	1060.00	1105.10	104.30	3.30
	Day 1			**Day 2**			**Day 3**			
Plant Matrix Sample	**Mesembrenone** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrenone** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	216.00	204.50	94.70	208.00	220.00	105.80	216.00	209.10	96.80	6.00
Medium	432.00	431.70	100.00	416.00	420.40	101.10	432.0	446.70	103.40	2.00
High	1080.00	1027.80	95.20	1040.00	1010.90	97.20	1080.00	1064.40	98.60	4.20

Table 5.14b
Recovery studies of *Sceletium* alkaloids from plant matrix (LCMS)

	Day 1			**Day 2**			**Day 3**			
Plant Matrix Sample	**Mesembrine** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Mesembrine** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	188.50	192.80	102.30	188.50	194.20	103.10	188.50	195.60	103.70	0.70
Medium	376.90	371.90	98.70	376.90	373.50	99.10	376.90	385.90	102.40	2.80
High	960.10	943.30	98.30	960.10	937.60	97.70	960.10	953.20	99.30	2.20
	Day 1			**Day 2**			**Day 3**			
Plant Matrix Sample	**Epimesembranol** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	**Epimesembranol** (ng/1mg matrix/ml)	Observed content (ng/ml)	% Recovery	Inter-day Recovery % RSD (n=3)
Low	208.00	212.50	102.20	208.00	219.70	105.60	208.00	210.30	101.10	2.30
Medium	416.00	408.50	98.20	416.00	442.30	106.30	416.00	412.50	99.20	4.40
High	1040.00	1088.50	104.70	1040.00	1111.80	106.90	1040.00	1081.40	104.00	1.40

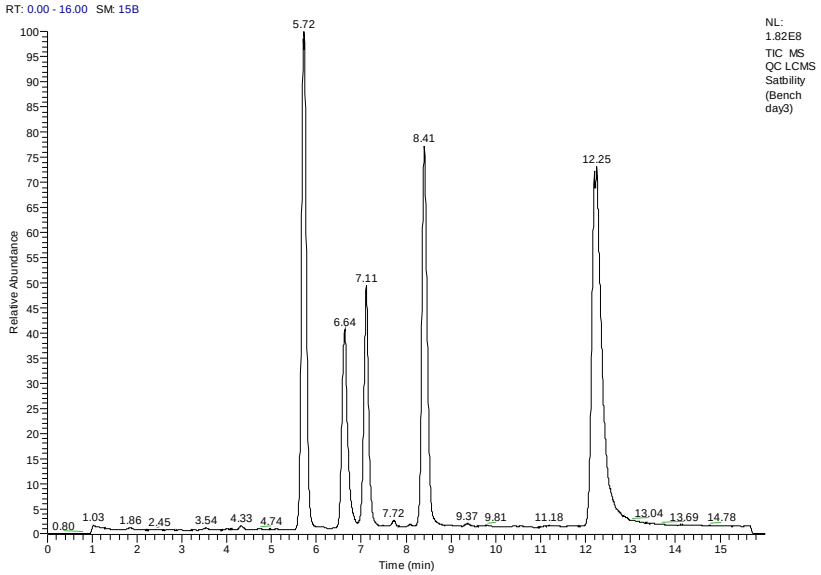


Figure 5.6: TIC chromatogram of Sceletium standards in methanol maintained at room temperature

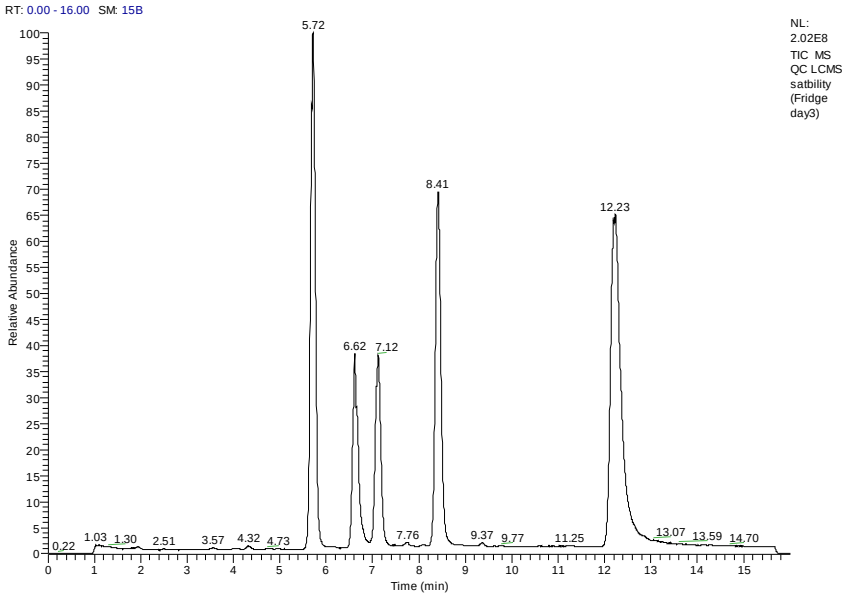


Figure 5.7: TIC chromatogram of Sceletium standards in methanol maintained at ~ 4°C

5.5 CONCLUSIONS

LCMS is fast becoming the method of choice for the analysis of natural compounds due to its unique ability to identify the compounds by their molecular masses and characteristic fragmentation pattern. The application of this technique to the analysis of *Sceletium* plant material and dosage forms for relevant alkaloidal components have not hitherto been reported in the published scientific literature.

The efficiency of the LCMS method was enhanced by development of a satisfactory HPLC method for the chromatographic separation and quantitative analysis of relevant alkaloids which was discussed in Chapter 4. The ionization method used also enhanced the sensitivity, which was found to be selective for *Sceletium* alkaloids. Since the identified markers have closely related structures, the chromatographic separation of these related alkaloids provided specificity for the unequivocal identification of the alkaloids based on their *m/z* values.

The main objective of this investigation was to develop an analytical method that has the following properties:

- Accuracy.
- Specificity.
- Sensitivity.
- Use for chromatographic fingerprinting and precise identification of *Sceletium* alkaloids.
- Assay of alkaloidal components in *Sceletium* plant material and its dosage forms.
- Use in QC of *Sceletium* plant material and dosage forms containing *Sceletium*.

In light of the above, a simple, accurate, precise, rapid and reproducible LCMS method was developed for the identification and quantitative analysis of five relevant *Sceletium* alkaloids which has been successfully applied for the identification and QC of *Sceletium* plant material and its dosage forms. Furthermore, this method was found to efficiently ionize the alkaloidal markers amongst the complex components present in plant material and in tablet dosage form without any interference from excipients present in those formulations.

Various species of *Sceletium* plants were subsequently provided by *Sceletium* plant cultivators and this LCMS method was successfully used for chemo-taxonomy of some *Sceletium* species and commercially available *Sceletium* dosage forms (*vide infra* Chapter 6). More importantly this method proved valuable to monitor the fermentation process of *Sceletium* plant (*vide infra*-

Chapter 8). Hence, the method constitutes a versatile analytical technique for fingerprinting and quantitative analysis of *Sceletium* plant material or dosage forms containing *Sceletium* and can readily be applied for the QC of those compounds and products. Furthermore, the method is capable of detecting nanogram levels of the identified alkaloids and it can probably be used for bioanalytical applications as well. The sensitivity of MS detection can be further enhanced by adopting specific scanning modes such as single ion monitoring (SIM), single reaction monitoring (SRM) and multiple reaction monitoring (MRM). These modes facilitate the specific detection and quantification of co-eluting or unresolved peaks, which are often observed in natural products [75].

CHAPTER 6

SCELETIUM PRODUCT ANALYSIS

6.1 INTRODUCTION

It has become evident that phyto-pharmaceutical therapy has gained immense popularity and as a consequence thereof, commercial growth of plant material and the production of their dosage forms are undergoing great expansion. This is associated with increased concerns relating to issues of quality. Standardized/constant composition of the components in herbal preparations is a pre-requisite [76]. However due to the complexity of endogenous compounds in plant material and products, standardization of active components is generally not achieved.

It is reported that 70% South Africans use traditional medicines and hence well known plants in South Africa have been studied for their pharmacognosy. Fingerprinting of these plants are being processed to develop monographs. Plant species recognized for their traditional use are being commercially propagated, which require application of standard pharmaceutical methods to improve the QSE of these products [77].

Sceletium and its products are gaining popularity in South Africa and also world-wide, which is evidenced by the volume of plant material grown by cultivators as well as internet advertisements promoting their sales. The purported activity of *Sceletium* products has been described on websites (Chapter 2) and often misleading claims related to anti-anxiety/ antidepressant activity are being made.

6.2 OBJECTIVES

The main objective of this study was to collect *Sceletium* plant material and also, where available, its products and to analyze them by HPLC and LCMS methods, specifically developed to identify their alkaloidal content and assess the quality of such products. The *Sceletium* products selected for this study were purchased from health shops in South Africa. One sample of *Sceletium emarcidum* was purchased over the internet from an Australian company Herbalistics (www.herbalistics.com.au). The plant material and live plants were obtained as gift samples from *Sceletium* cultivators in South Africa.

6.3 EXPERIMENTAL

6.3.1 Reagents and Materials

All reagents and materials used have previously been described and detailed in Chapters 4 and 5 whereas *Sceletium* reference compounds and plant material and its products have been described and detailed in Chapters 2 and 3, respectively.

6.3.2 Instrumentation

Previously described in Chapters 4 and 5.

6.3.3 Preparation of standard solutions and samples

Standard methanolic stock solutions (1mg/ml) of Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine hydrochloride and epimesembranol were prepared. A working stock solution was prepared and diluted to obtain a concentration of 100 µg/ml of each alkaloid. Standard solutions comprising a set of nine calibrators in the concentration range of 400–30,000 ng/ml were prepared for HPLC-UV analysis.

The *Sceletium* plant material and product sample solutions were prepared to contain 50 mg/ml of the respective material and fresh plant was extracted with methanol to obtain a final concentration of 150 mg/ml plant material in methanol. The extraction of alkaloids was carried out by sonication and the solutions were filtered through 0.45 µm PVDF membrane filters prior to chromatography.

6.4 *SCELETIUM* PRODUCT PROFILES

6.4.1 *Sceletium emarcidum* Plant (SP01, GRA)

6.4.1.1 Locality

Collected from a greenhouse located at Hermanus, South Africa, Specimen number (SP01, GRA).

6.4.1.2 HPLC - UV profile

The sample showed a complex profile with three peaks observed at RTs 6.36, 7.15, and 10.08 minutes. These observed peaks were not comparable to any of the identified alkaloids with respect to (w.r.t) the RTs of standard alkaloid peaks (Figure 6.1a).

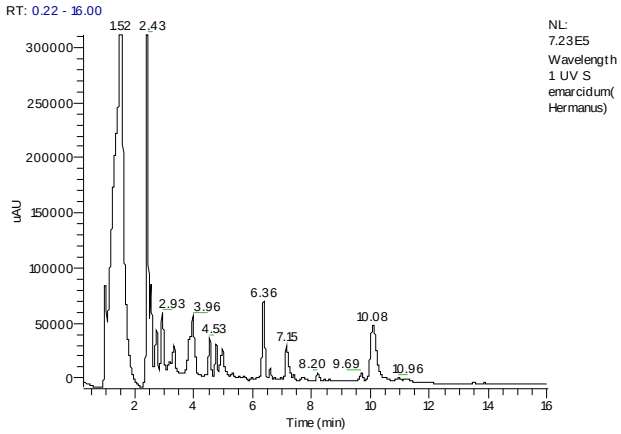


Figure 6.1a: HPLC chromatogram of Sceletium emarcidum plant sample (SP01, GRA)

6.4.1.3 ESI-MS profile

The MS profile of the sample showed one ionized peak at 13.53 minutes (Figure 6.1b) that corresponded to *m/z* 360, which does not correspond to any of the previously identified *Sceletium* alkaloids.

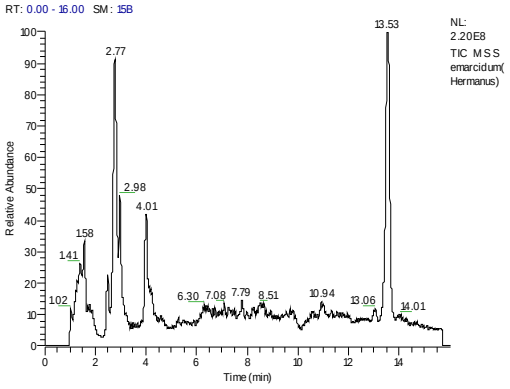


Figure 6.1b: TIC chromatogram of Sceletium emarcidum plant sample (SP01, GRA)

6.4.1.4 Inference

This sample did not contain any of the following alkaloids: Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol.

6.4.2 *Sceletium rigidum* Plant sample (SP02, GRA)

6.4.2.1Locality

Sample provided from a greenhouse located in the Vanwyksdorp, Western Cape Province, South Africa, Specimen number (SP02, GRA).

6.4.2.2 HPLC-UV profile

The HPLC profile showed no peaks corresponding to any of the relevant alkaloids (Figure 6.2a).

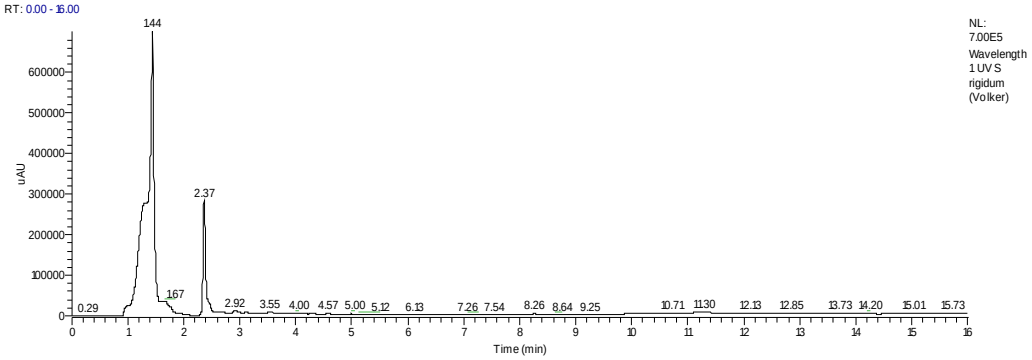


Figure 6.2a: HPLC-UV chromatogram of Sceletium rigidum plant sample (SP02, GRA)

6.4.2.3 ESI-MS profile

The MS profile of the sample showed no ionized peaks (Figure 6.2b).

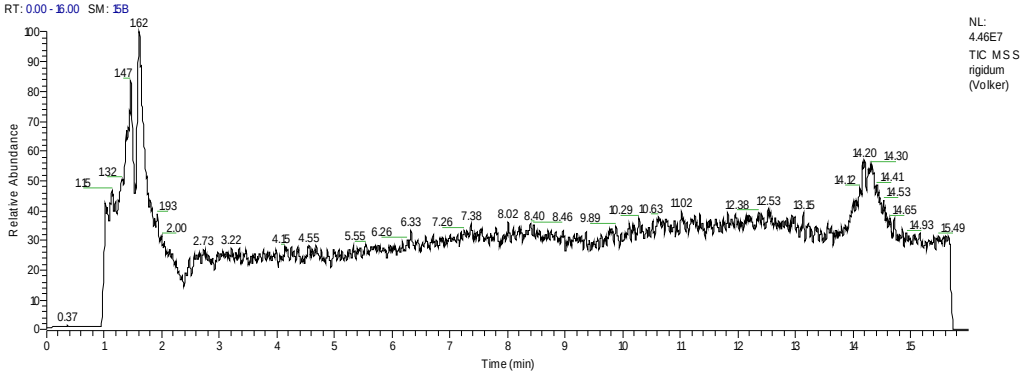


Figure 6.2b: TIC chromatogram of Sceletium rigidum plant sample (SP02, GRA.)

6.4.2.4 Inference

This sample did not contain any of the following alkaloids: Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol.

6.4.3 *Sceletium exalatum* Plant sample (SP03, GRA)

6.4.3.1 Locality

Sample submitted for identification from a greenhouse located at Somerset West, Western Cape Province, South Africa, Specimen number (SP03, GRA).

6.4.3.2 HPLC-UV profile

The sample showed a complex profile with peaks observed at RTs 5.04, 5.52, 8.30 and 9.29 minutes. These observed peaks did not correspond to any of the previously identified alkaloids with respect to the RTs of the standard alkaloid peaks (Figure 6.3a).

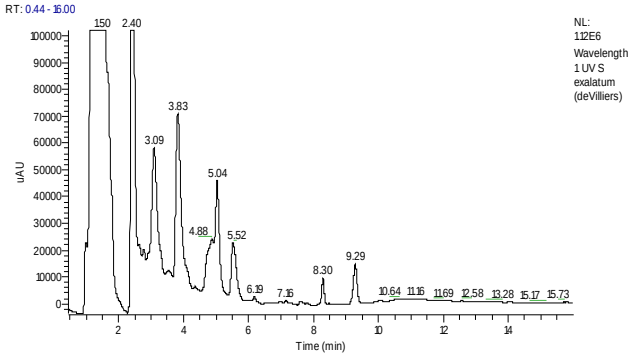
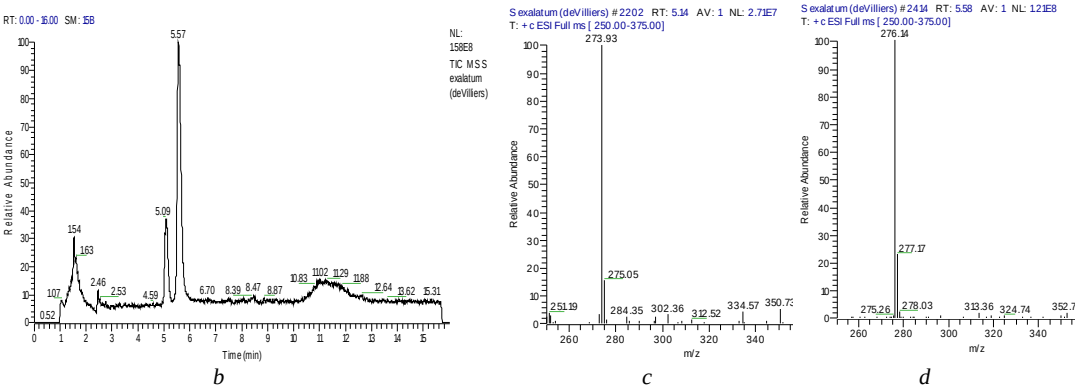


Figure 6.3a: HPLC-UV chromatogram of Sceletium exalatum plant sample (SP03, GRA)

6.4.3.3 ESI-MS profile

The MS profile of the sample showed two prominent ionized peaks at 5.09 and 5.57 (Figure 6.3b) with *m/z* values of 274 (Figure 6.3c) and 276 (Figure 6.3d), which do not correspond to the previously identified *Sceletium* alkaloids.



b *c* *d*

Figure 6.3b: TIC chromatogram of Sceletium exalatum plant sample (SP03, GRA). Figure 6.3c & d: Ion spectra

6.4.3.4 Inference

This sample did not contain any of the following alkaloids: Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol. The obtained *m/z* values suggest that the compounds may be demethyl mesembranol (MW 276) and mesembrane (MW 275).

6.4.4 *Sceletium tortuosum* Plant sample (SP04, GRA)

6.4.4.1 Locality

This sample was provided by Sheilam Cactus and Succulent Garden; a greenhouse located in Robertson, Western Cape, South Africa, Specimen number (SP04, GRA).

6.4.4.2 HPLC-UV profile

The sample profile showed peaks at RTs 5.48, 6.05, 6.64, 7.12, 8.39, 11.82 and 12.09 minutes. The peaks at RT 6.64, 7.12, 8.39, and 12.09 minutes correspond to mesembranol, mesembrenone, mesembrine and epimesembranol (Figure 6.4a).

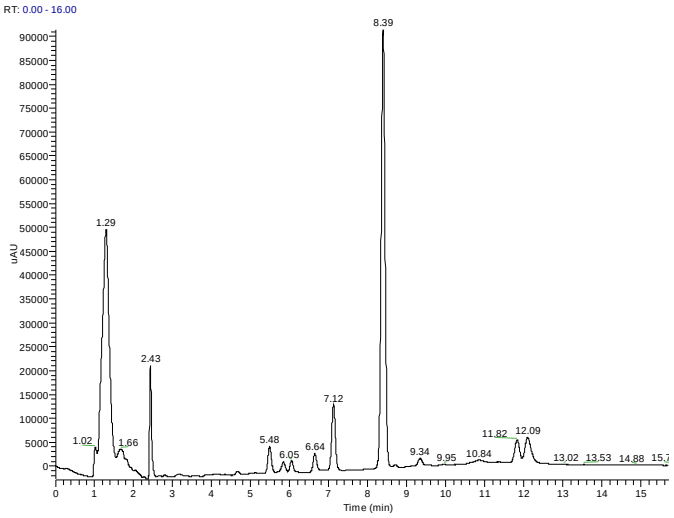


Figure 6.4a: HPLC-UV chromatogram of Sceletium tortuosum plant sample (SP04, GRA)

6.4.4.3 ESI-MS profile

The MS profile of the sample showed ionized peaks at 6.69, 7.16, 8.45, 11.88 and 12.16 minutes which corresponded to *m/z* 292, 288, 290 and 292. The *m/z* ratios correspond to mesembrenone, mesembranol, mesembrine and epimesembranol (Figure 6.4b). The peak at RT 11.88 minutes with *m/z* 334, did not correspond to the identified alkaloids (Figure 6.4c).

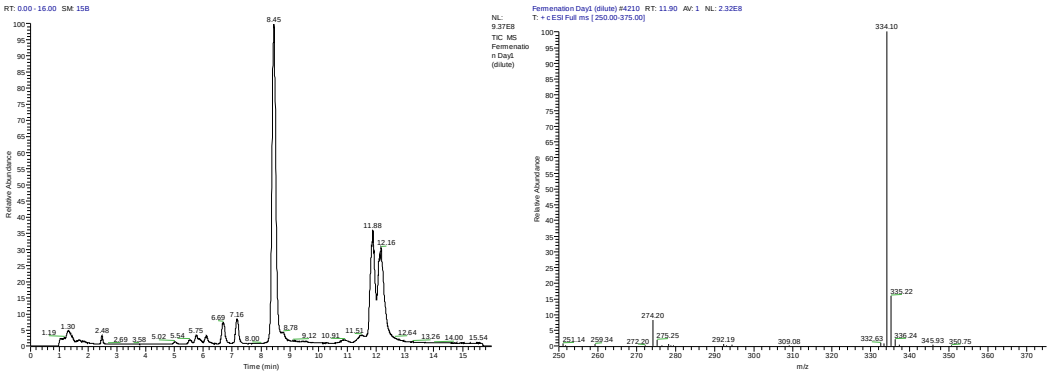


Figure 6.4b: TIC chromatogram of Sceletium tortuosum plant sample (SP04, GRA).Figure 6.4c: Ion spectrum of peak at 11.88 minutes

6.4.4.4 Inference

The alkaloids mesembranol, mesembrenone, mesembrine and epimesembranol were assayed by HPLC-UV and found to be present at 0.18%, 0.16%, 2.20% and 0.41% respectively. Δ^7Mesembrenone was below LoD by HPLC-UV and detected by MS appearing at RT 5.75 minutes.

6.4.5 *Sceletium expansum* Plant sample (SP05, GRA)

6.4.5.1 Locality

This sample was provided by a greenhouse located in the North-West Province, South Africa, Specimen number (SP05, GRA).

6.4.5.2 HPLC-UV profile

The sample profile showed three peaks at RTs 5.44, 7.07 and 8.34 minutes. The peaks at RT 7.07 and 8.34 minutes correspond to mesembrenone and mesembrine (Figure 6.5a).

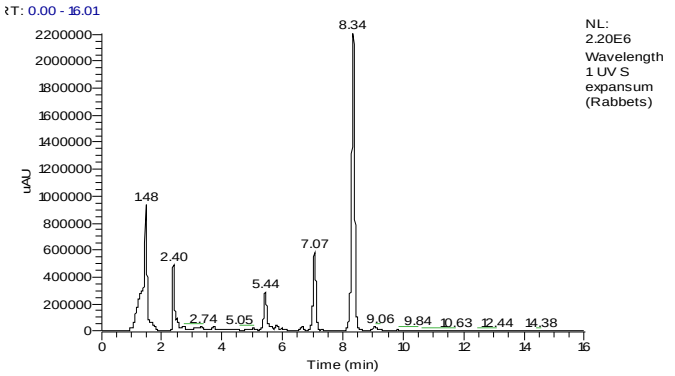
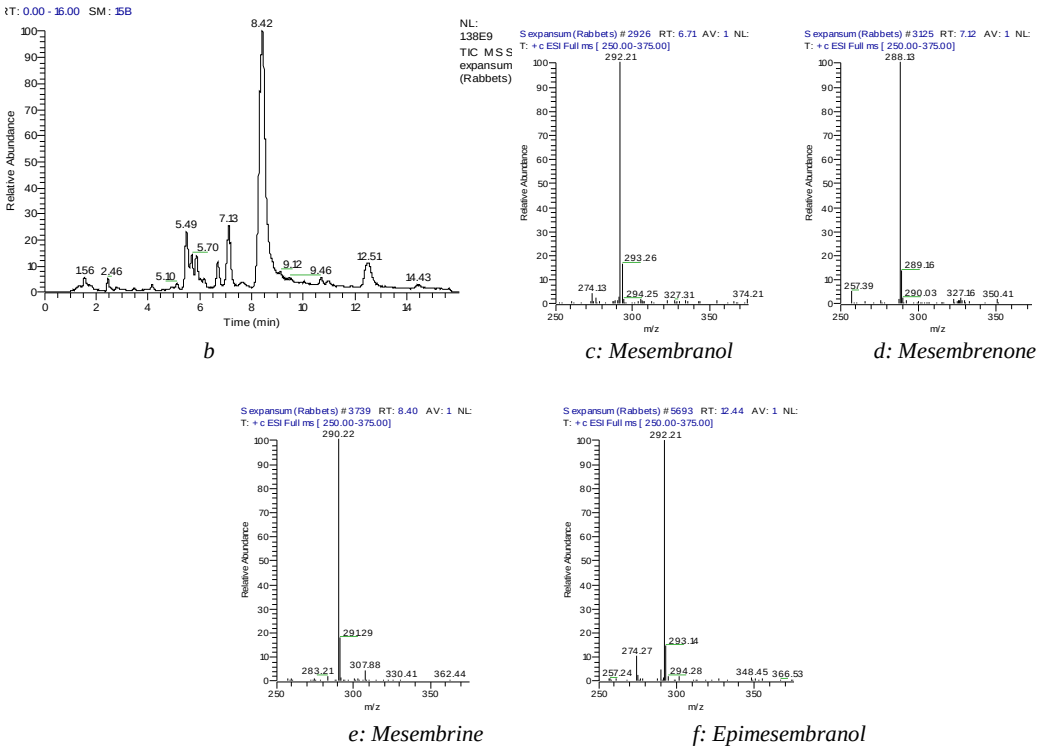


Figure 6.5a: HPLC-UV chromatogram of Sceletium expansum plant sample (SP05, GRA)

6.4.5.3 ESI-MS profile

The MS profile of the sample showed ionized peaks at 5.49, 6.71, 7.13, 8.42 and 12.51 minutes (Figure 6.5b) which corresponded to *m/z* 274, 292, 288, 290 and 292. The latter four *m/z* ratios (Figure 6.5c-f) correspond to mesembrenone, mesembranol, mesembrine and epimesembranol.



b *c: Mesembranol* *d: Mesembrenone*

e: Mesembrine *f: Epimesembranol*

Figure 6.5b TIC chromatogram Sceletium expansum plant sample (SP05, GRA). Figure 6.5c-f -Ion spectra of the identified alkaloids.

6.4.5.4 Inference

The alkaloids mesembrenone and mesembrine were assayed by HPLC-UV and found to be present at 0.03% and 0.30% respectively. Mesembranol and epimesembranol were below LoD by HPLC-UV and the presence of these alkaloids was confirmed by MS detection.

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6.4.6 *Sceletium strictum* Plant Sample (SP06, GRA)

6.4.6.1 Locality

This sample was obtained from a greenhouse located in the Vanwyksdorp, Western Cape Province, South Africa, Specimen number (SP06, GRA).

6.4.6.2 HPLC-UV profile

The HPLC profile showed three peaks at RTs 5.44, 7.08 and 8.37 minutes. The peaks at RT 7.08 and 8.37 minutes corresponded to mesembrenone and mesembrine (Figure 6.6a).

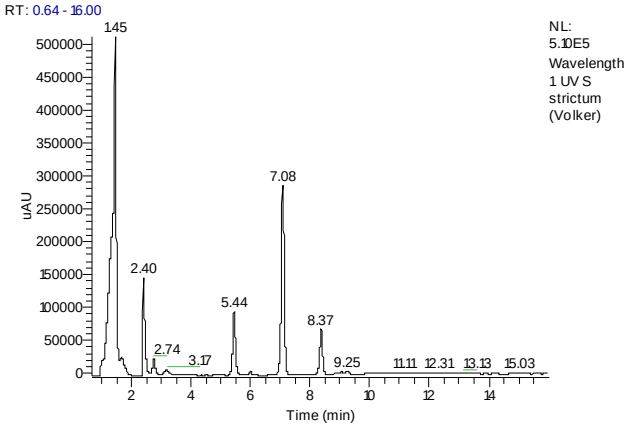


Figure 6.6a: HPLC-UV chromatogram of Sceletium strictum plant sample (SP06, GRA)

6.4.6.3 ESI-MS profile

The MS profile showed ionized peaks at 5.48, 7.12, and 8.41 minutes (Figure 6.6b) which corresponded to *m/z* 274, 288 and 290 (Figure 6.6c–e). The later two *m/z* ratios correspond to mesembrenone and mesembrine.

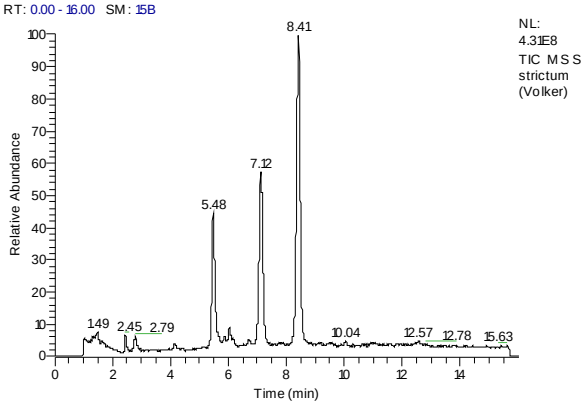
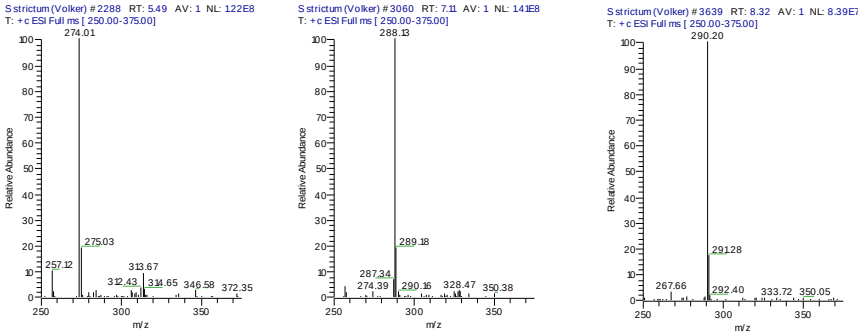


Figure 6.6b: TIC chromatogram Sceletium strictum plant sample (SP06, GRA)



c *d* *e*

Figure 6.6c-e: Ion spectra of the identified alkaloids (SP06, GRA)

6.4.6.4 Inference

The alkaloids mesembrenone and mesembrine assayed by HPLC-UV detection were found to be present in the plant at percentages of 0.76 and 0.68, respectively. The peak at RT 5.44 minutes with *m/z* of 274 (Figure 6.6c) corresponds to (+)-N-demethylmesembrenol (MW 275), which has been reported to be present in *S. strictum* [42]. The compounds mesembranol and epimesembranol could not be detected by either method.

6.4.7 *Sceletium tortuosum* Powder Sample (SRM02)

6.4.7.1 Description

Brown colored dried plant powder having characteristic odour.

6.4.7.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 5.67, 6.67, 7.11, 8.40 and 12.42 minutes, which correspond to Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol (Figure 6.7a).

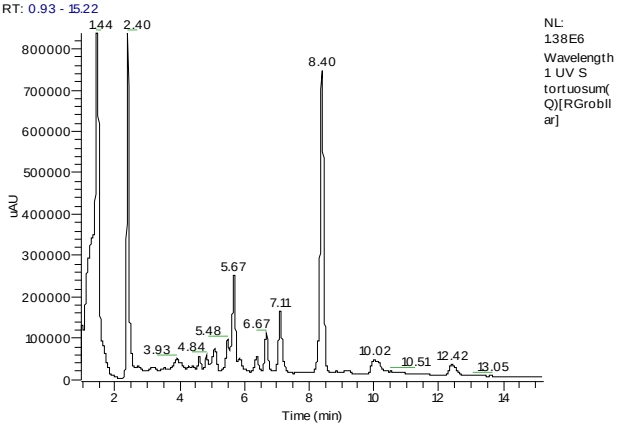


Figure 6.7a: HPLC-UV chromatogram of Sceletium tortuosum powder sample (SRM02)

6.4.7.3 ESI-MS profile

The MS profile showed ionized peaks at 5.74, 6.72, 7.17, 8.43 and 12.44 minutes (Figure 6.7b) which corresponded to *m/z* 288, 292, 288, 290 and 292 respectively (Figures 6.7c-g) . The *m/z* ratios corresponded to the alkaloids, Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol (Figure 6.7).

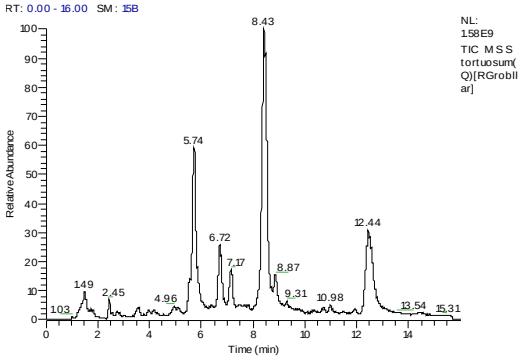
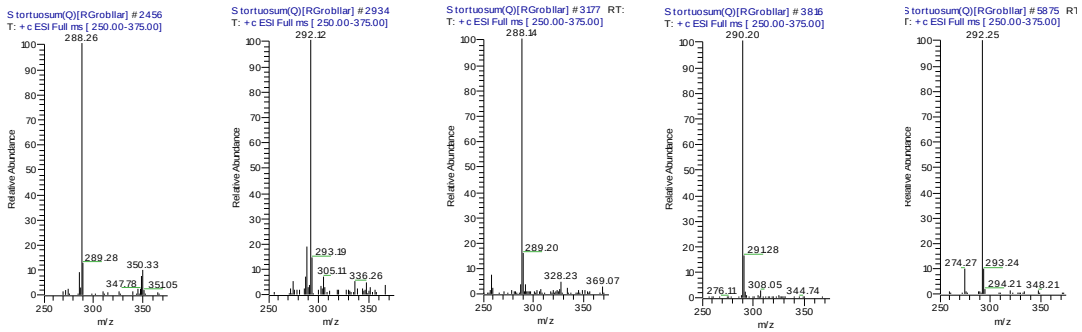


Figure 6.7b: TIC chromatogram of Sceletium tortuosum powder (SRM02)



c *d* *e* *f* *g*

Figure 6.7c-g: Ion spectra of the identified alkaloids (SRM02)

6.4.7.4 Inference

All the relevant alkaloids were assayed by HPLC and were found to be present at concentrations of 0.08, 0.5, 0.04, 0.6, and 0.05% for alkaloids Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol, respectively. The presence of these alkaloids was confirmed by LCMS.

6.4.8 *Sceletium tortuosum* Powder Sample (SRM03)

6.4.8.1 Description

Brown colored dried plant powder having characteristic odour.

6.4.8.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 5.82, 6.66, and 7.14 and a relatively small peak at 8.37 minutes. The peaks at RT 6.66, 7.14 and 8.37 minutes were identified as mesembranol, mesembrenone and mesembrine (Figure 6.8a).

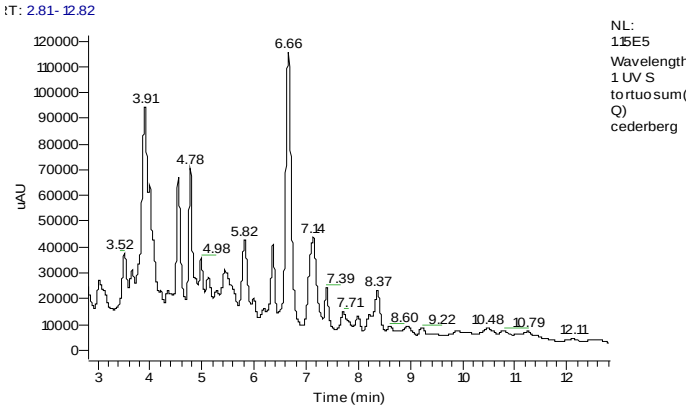


Figure 6.8a: HPLC-UV chromatogram of Sceletium tortuosum powder sample (SRM03)

6.4.8.3 ESI-MS profile

The MS profile showed ionized peaks at 5.87, 6.71, 7.14 and 8.42 minutes (Figure 6.8b) which corresponded to *m/z* 274 (Figure 6.8c), 292, 288 and 290 respectively (Figure 6.8 d-f). The latter three *m/z* ratios correspond to the alkaloids, mesembranol, mesembrenone and mesembrine.

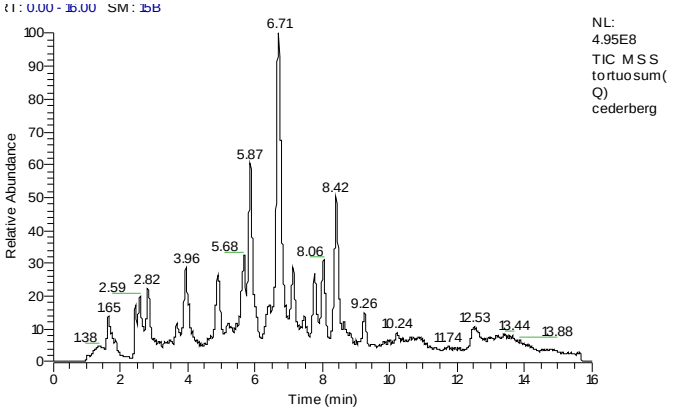


Figure 6.8b: TIC chromatogram of Sceletium tortuosum powder sample (SRM03)

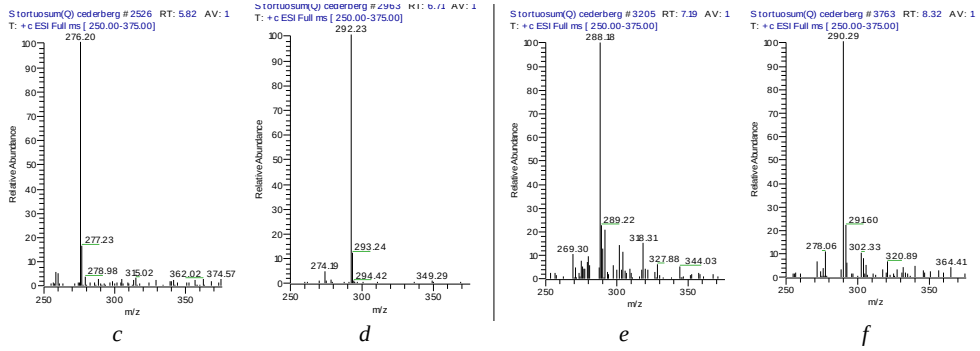


Figure 6.8c-f: Ion spectra of the identified alkaloids (SRM03)

6.4.8.4 Inference

The alkaloids, mesembranol and mesembrenone and mesembrine were detected by HPLC-UV but only mesembranol was quantifiable as 0.03%. The content of alkaloids mesembrenone and mesembrine were below their respective LoQs. The compound, Δ^7mesembrenone, was not detected and the presence of the other relevant alkaloids was confirmed by peaks at 6.71, 7.19 and 8.42 minutes. A very faint response for epimesembranol was observed at 12.53 minutes by MS.

6.4.9 *Sceletium emarcidum* Powder Sample (SRM04)

6.4.9.1 Description

A dark brown processed plant powder having characteristic strong fermented organic odour.

6.4.9.2 HPLC-UV profile

The sample showed a complex profile with a few prominent peaks at RTs 6.95, 8.26, and 9.77. These observed peaks did not correspond to any of the previously identified alkaloids with respect to the RTs of the standard alkaloid peaks (Figure 6.9a).

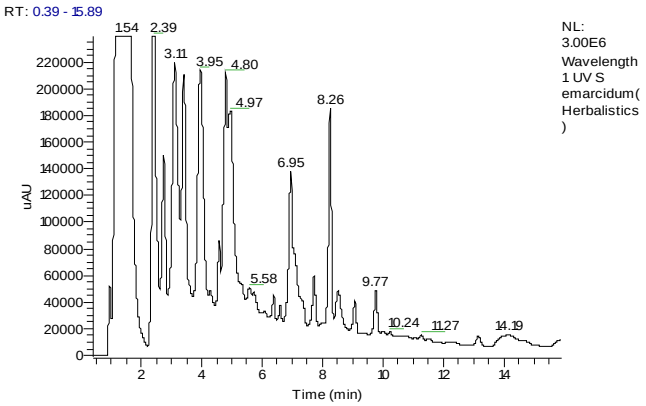


Figure 6.9a: HPLC-UV chromatogram of Sceletium emarcidum powder sample (SRM04)

6.4.9.3 ESI-MS profile

The MS profile of the sample showed ionized peaks at 5.72, 7.79, 8.61 and 9.12 minutes (Figure 6.9b) which corresponded to *m/z* values of 276, 260, 262 and 258 (Figure 6.9c–f) respectively. However, these *m/z* values do not correspond to any of the identified *Sceletium* alkaloids (Figure 6.8). Furthermore, treatment with Dragendorff's reagent indicated that no alkaloids (Spot 1 and 2) were present in this particular plant (Figure 6.9g)

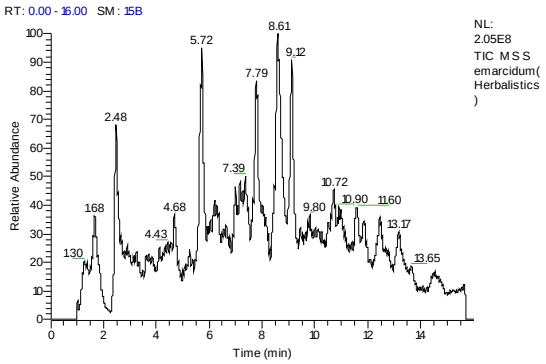


Figure 6.9b: TIC chromatogram of Sceletium emarcidum powder sample (SRM04)

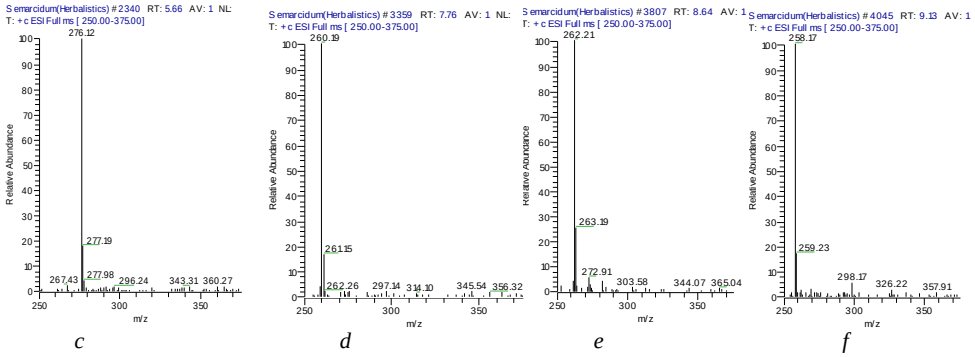


Figure 6.9c-f: Ion spectra of the unidentified alkaloids in Sceletium emarcidum powder sample (SRM04)

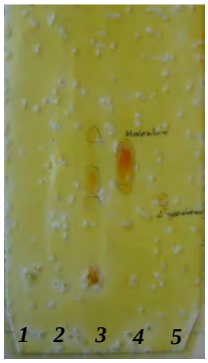


Figure 6.9g: TLC of Sceletium emarcidum powder sample (SRM04)

1. *S emarcidum (low concentration)*
2. *S emarcidum (high concentration)*
3. *Sceletium tortuosum sample*
4. *Mesembrine + Mesembrenone*
5. *Δ^7Mesembrenone*

6.4.9.4 Inference

This sample did not contain any of the following alkaloids: Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol.

6.4.10 *Sceletium tortuosum* Powder Sample (SRM05)

6.4.10.1 Description

Brown colored dried plant powder having characteristic odour.

6.4.10.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 5.79 and 6.62 minutes and relatively small peaks at 7.07 and 8.26 minutes. The peaks at RT 6.62, 7.07 and 8.26 minutes were identified as mesembranol, mesembrenone and mesembrine (Figure 6.10a).

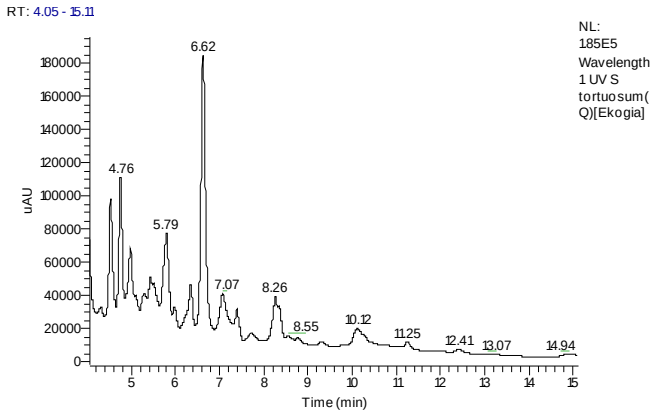


Figure 6.10a: HPLC-UV chromatogram of Sceletium tortuosum powder sample (SRM05)

6.4.10.3 ESI-MS profile

The MS profile showed ionized peaks at 5.85, 6.68, 8.40 and 12.46 minutes (Figure 6.10b) which corresponded to *m/z* 274, 292, 288 and 290 respectively. The later three *m/z* ratios correspond to alkaloids mesembranol, mesembrenone and mesembrine (Figure 6.10c-e).

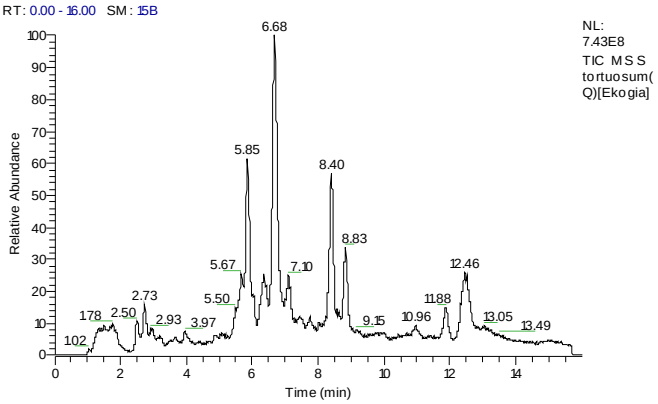
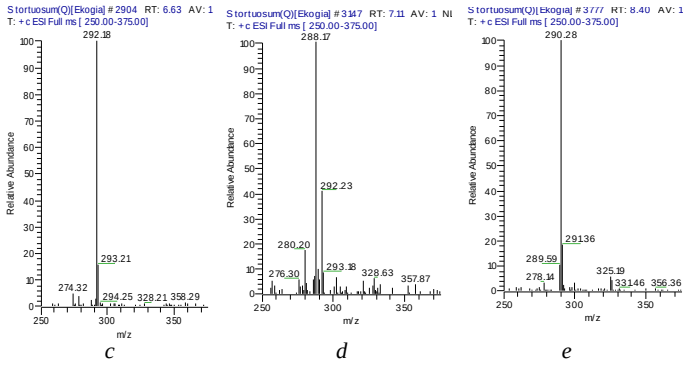


Figure 6.10b: TIC chromatogram of Sceletium tortuosum powder sample (SRM05)



c *d* *e*

Figure 6.10c-e: Ion spectra of the identified alkaloids in Sceletium tortuosum powder (SRM05)

6.4.10.4 Inference

The alkaloids, mesembranol and mesembrenone and mesembrine, were detected but only mesembranol was quantifiable as 0.03%. The alkaloids mesembrenone and mesembrine were below their respective LoQs. The compound, Δ^7mesembrenone, was not detected and the presence of other previously identified alkaloids was confirmed by peaks at 6.58, 7.10 and 8.40. A very minor response for epimesembranol was observed at 12.46 minutes by MS.

6.4.11 *Sceletium tortuosum* Powder Sample (SRM07)

6.4.11.1 Description

Brown colored dried plant powder having characteristic odour.

6.4.11.2 HPLC UV profile

The HPLC profile showed peaks at RTs 6.64, 7.08 and 8.28 minutes. The peaks corresponded to mesembranol, mesembrenone and mesembrine (Figure 6.11a).

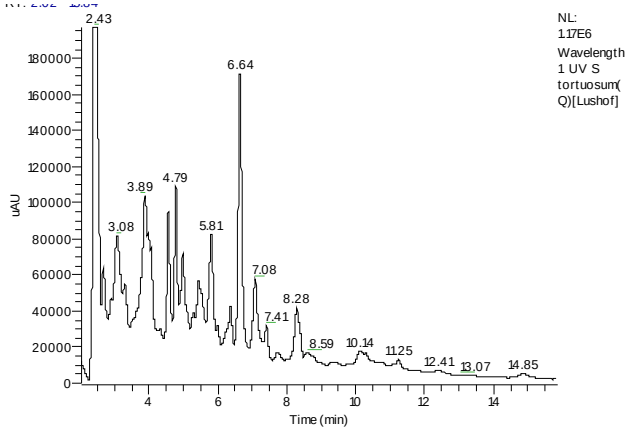


Figure 6.11a: HPLC-UV chromatogram of Sceletium tortuosum powder sample (SRM07)

6.4.11.3 ESI-MS profile

The MS profile showed ionized peaks at 5.85, 6.69, 7.10, 8.41 and 12.48 minutes (Figure 6.11b) which corresponded to *m/z* 274, 292, 288 290 and 292 respectively. The latter three *m/z* ratios correspond to the alkaloids, mesembranol, mesembrenone, mesembrine (Figure 6.11c–e) and epimesembranol respectively.

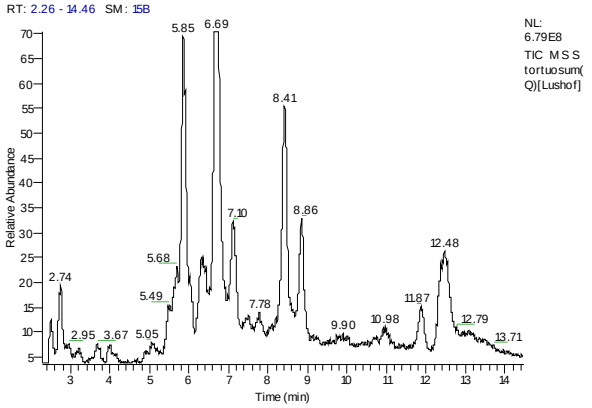


Figure 6.11b: TIC chromatogram of Sceletium tortuosum sample (SRM07)

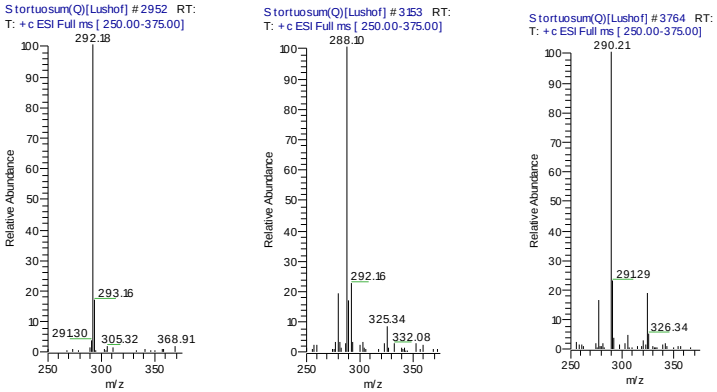


Figure 6.11c-e: Ion spectra of the identified alkaloids in Sceletium tortuosum powder sample (SRM07)

6.4.11.4 Inference

The alkaloids, mesembranol, mesembrenone and mesembrine were detected but only mesembranol was quantifiable as 0.03%. The alkaloids mesembrenone and mesembrine were below their respective LoQs. The compound, Δ^7mesembrenone, was not detected and the presence of other previously identified alkaloids was confirmed by peaks at 6.69, 7.10 and 8.41. A minor response for epimesembranol was observed at 12.48 minutes by MS.

6.4.12 *Sceletium Capsules* (Manufactured by: Herbal Care -Product B)

6.4.12.1 Description

Brown colored dried plant powder enclosed in a '0' size transparent hard gelatin capsule. The average weight was found to be 250 mg per capsule.

6.4.12.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 9.73 and 16.69 minutes that did not correspond to the RTs of the previously identified alkaloids (Figure 6.12a).

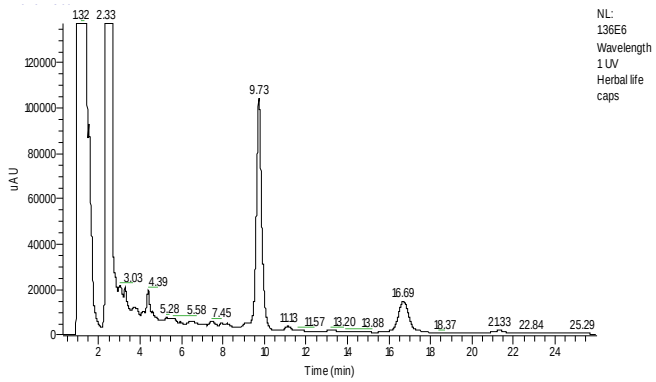


Figure 6.12a: HPLC-UV chromatogram of Sceletium tortuosum capsules- (Herbal Care - Product B)

6.4.12.3 ESI-MS profile

The MS profile showed ionized peaks at 3.92, 4.43 and 7.69 minutes which do not correspond to the previously identified alkaloids (Figure 6.12b).

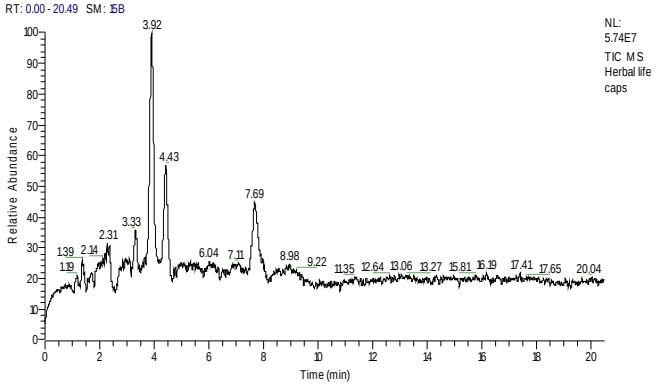


Figure 6.12b: TIC chromatogram of Sceletium capsules (Herbal Care - Product B)

6.4.12.4 Inference

None of the previously identified alkaloids were observed in the sample in spite of a label claim of 25 mg *Sceletium* per capsule.

6.4.13 *Sceletium tortuosum Capsules* (Manufactured by: Essential Source - Product C)

6.4.13.1 Description

Brown colored dried plant powder enclosed in a '0' size transparent hard gelatin capsule. The average weight was found to be 250 mg per capsule.

6.4.13.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 5.81, 6.64, 7.08 and a very small peak at 8.38 minutes. The peaks at RT 6.64, 7.08 and 8.38 minutes were identified as mesembranol, mesembrenone and mesembrine (Figure 6.13a).

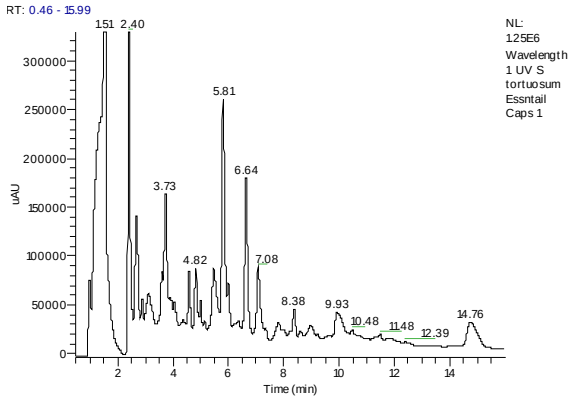


Figure 6.13a: HPLC-UV chromatogram of Sceletium tortuosum capsules (Essential Source - Product C)

6.4.13.3 ESI-MS profile

The MS profile showed ionized peaks at 5.86, 6.69, 7.14 and 8.43 minutes (Figure 6.13b) with *m/z* values of 274, 292, 288 and 290 respectively. The latter three *m/z* ratios correspond to the alkaloids, mesembranol, mesembrenone and mesembrine (Figure 6.13c-e).

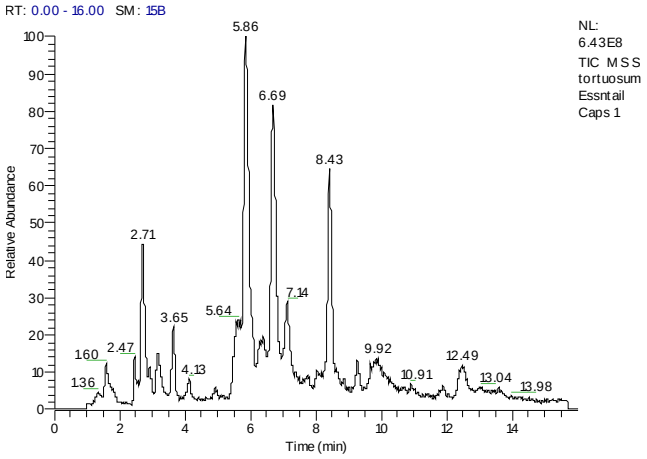
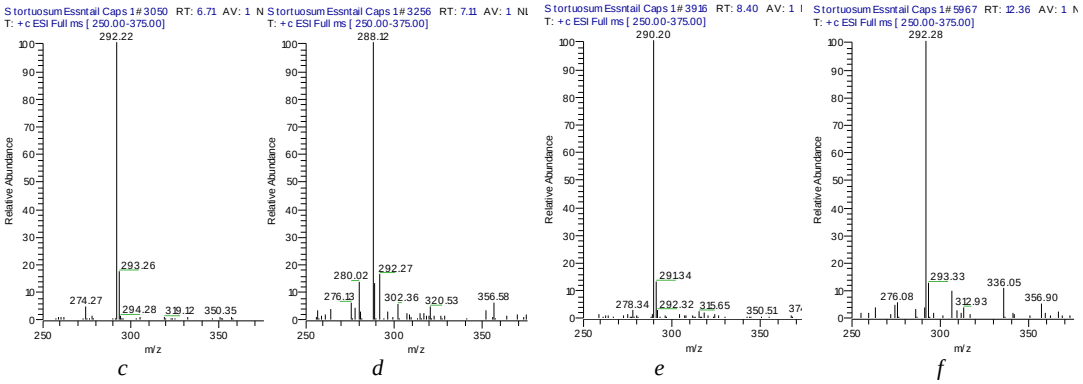


Figure 6.13b: TIC chromatogram of Sceletium capsules (Essential Source -Product C)



c *d* *e* *f*

Figure 6.13c-f: Ion spectra of the identified alkaloids in Sceletium tortuosum capsules (Essential Source - Product C)

6.4.13.4 Inference

The alkaloids mesembranol, mesembrenone and mesembrine were assayed by HPLC- UV and the product was found to contain 136.7, 25.0 and 18.4 µg/capsule, respectively. Δ^7Mesembrenone was not detected whereas the presence of epimesembranol (Figure 6.13f) was confirmed (RT 12.49 minutes) by MS.

6.4.14 *Sceletium tortuosum Capsules* (Manufactured by: Essential Source - Product D)

6.4.14.1 Description

Brown colored dried plant powder enclosed in a '0' size transparent hard gelatin capsule. The average weight was found to be 250 mg per capsule.

6.4.14.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 5.47, 6.03 and 7.10 minutes. The latter two peaks were identified as mesembranol and mesembrenone, respectively. Mesembrine and epimesembranol were not detected (Figure 6.14a).

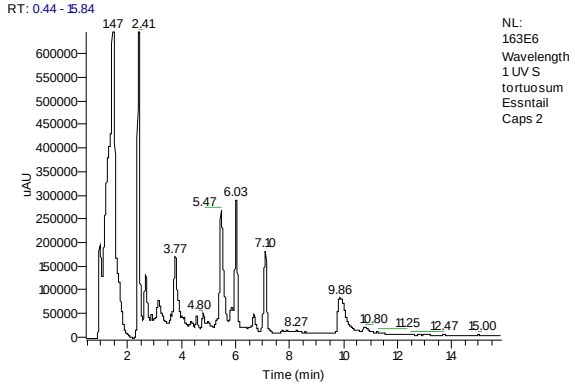


Figure 6.14a: HPLC-UV chromatogram of Sceletium tortuosum capsules (Essential Source - Product D)

6.4.14.3 ESI-MS profile

The MS profile showed ionized peaks at 5.55, 6.79, 7.16 and 8.43 minutes (Figure 6.14b) which corresponded to *m/z* 276, 292, 288 and 290 respectively (Figure 6.14c-f). The latter three *m/z* ratios correspond to alkaloids mesembranol, mesembrenone and mesembrine, respectively.

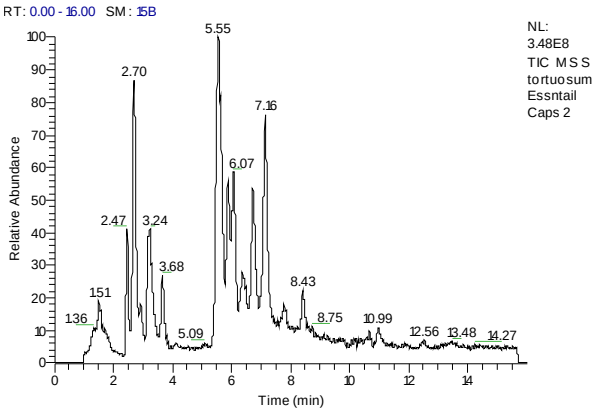
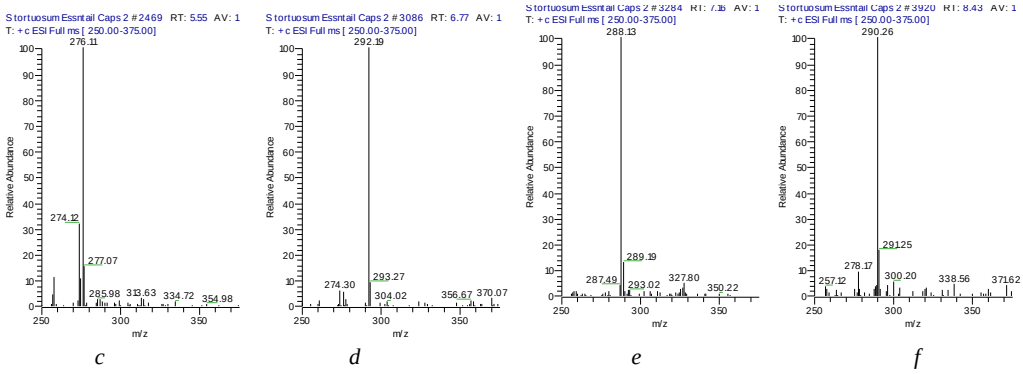


Figure 6.14b: TIC chromatogram of Sceletium capsules (Essential Source - Product D)



c *d* *e* *f*

Figure 6.14c-f: Ion spectra of the identified alkaloids in Sceletium tortuosum capsules (Essential Source – Product D)

6.4.14.4 Inference

The alkaloids were assayed by HPLC- UV and the product was found to contain 61.0 and 105.6 µg/capsule of mesembranol and mesembrenone, respectively. The compound Δ^7mesembrenone was not detected whereas the presence of mesembrine was confirmed by MS.

6.4.15 *Sceletium tortuosum Mother Tincture* (Manufactured by: Essential Source- Product E)

6.4.15.1 Description

Clear yellow liquid, with characteristic alcoholic odour.

6.4.15.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 5.97, 6.61and 7.07 minutes. The peaks at RT 6.61 and 7.07 were identified as mesembranol and mesembrenone (Figure 6.15a).

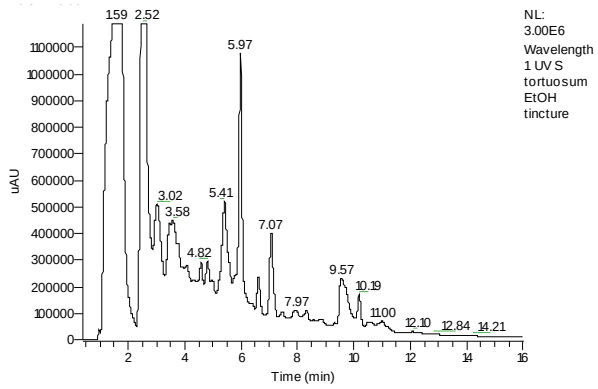


Figure 6.15a: HPLC-UV chromatogram of Sceletium tortuosum Mother tincture (Essential Source - Product E)

6.4.15.3 ESI-MS profile

The MS profile showed ionized peaks at 5.46, 6.68, 7.11 and 8.41 minutes (Figure 6.15b) which corresponded to *m/z* 276 (Figure 6.15c), 292, 288 and 290 respectively. The latter three *m/z* ratios correspond to the alkaloids, mesembranol, mesembrenone and mesembrine, respectively (Figure 6.15d–f).

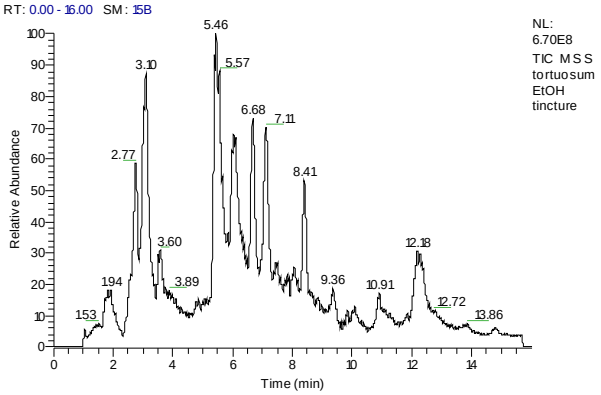
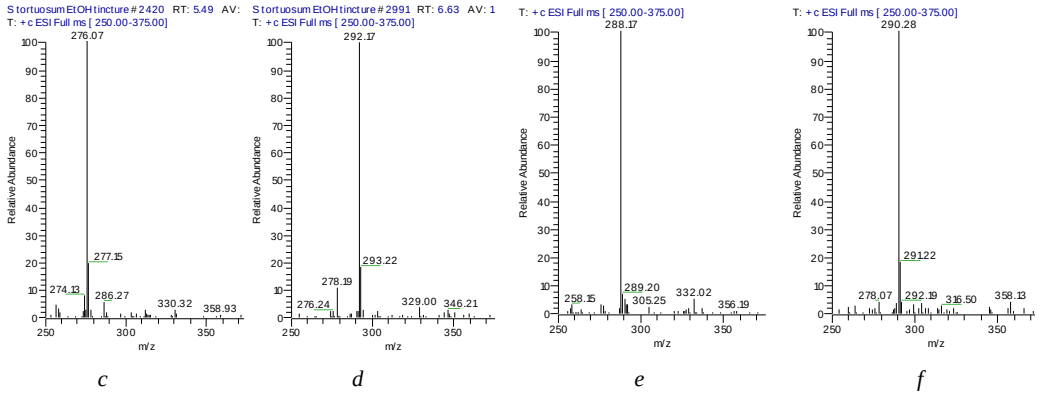


Figure 6.15b: TIC chromatogram of Sceletium tortuosum Mother tincture (Essential Source - Product E)



c *d* *e* *f*

Figure 6.15c–f: Ion spectra of the identified alkaloids in Sceletium tortuosum Mother tincture (Essential Source - Product E)

6.4.15.4 Inference:

The alkaloids were assayed by HPLC-UV and the product was found to contain 0.09% and 0.1% of mesembranol and mesembrenone respectively. The compound Δ^7mesembrenone was not detected and the presence of mesembrine and epimesembranol (Figure 6.15b) were confirmed by MS (RTs at 8.41 and 12.18 minutes, respectively).

6.4.16 *Sceletium tortuosum* Tablets (Manufactured by: Big Tree Health Products, Batch no. 7161)

6.4.16.1 Description

Pale yellow biconvex uncoated tablets with a few black and brown spots due to the plant material. Average weight: 501.3 mg/tablet.

6.4.16.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 6.00, 7.00, 7.43, 8.74 and 12.15 minutes, which correspond to Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol, respectively (Figure 6.16a).

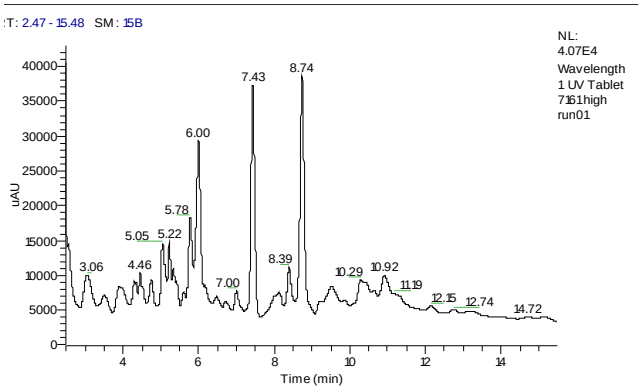


Figure 6.16a: HPLC-UV chromatogram of Sceletium tortuosum tablets (Big Tree Health Products, Batch no. 7161)

6.4.16.3 ESI-MS profile

The MS profile showed ionized peaks at 6.04, 7.11, 7.49, 8.80 and 12.77 minutes (Figure 6.16b) which corresponded to *m/z* 288, 288, 292, 290 and 292 respectively (Figure 6.16c-g). The *m/z* ratios corresponded to the alkaloids Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol respectively.

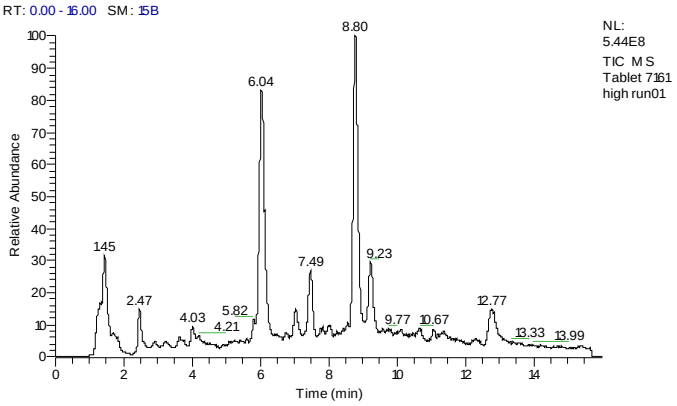
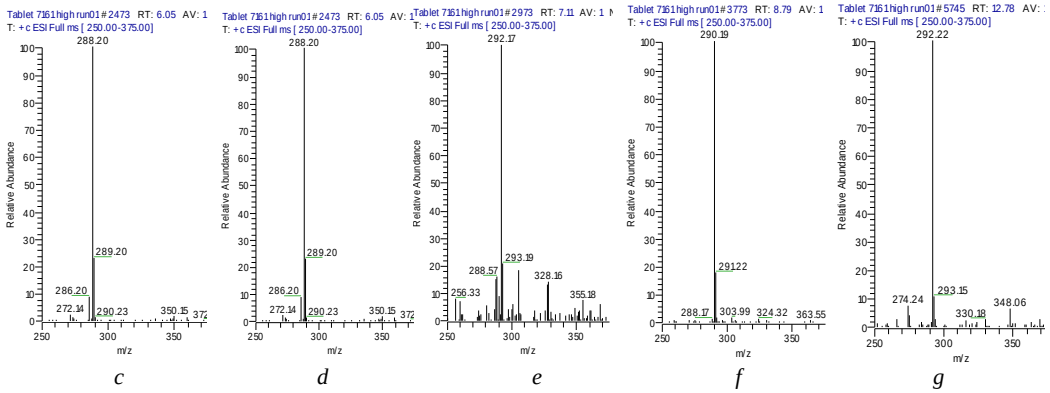


Figure 6.16b: TIC chromatogram of Sceletium tortuosum tablets (Big Tree Health Products, Batch no. 7161)



c *d* *e* *f* *g*

Figure 6.16c-g: Ion spectra of the identified alkaloids in Sceletium tortuosum Tablets – Big Tree Health Products (Batch no. 7161)

6.4.16.4 Inference

All the relevant alkaloids were assayed by HPLC and the product found to contain concentrations of 44.1, 5.30, 37.8, 116.2 and 27.2 µg/tablet for the alkaloids, Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol respectively. The MS detection positively identified the relevant alkaloids.

6.4.17 *Sceletium tortuosum* Tablets (Manufactured by: Big Tree Health Products Batch no. 9323)

6.4.17.1 Description

Pale yellow biconvex uncoated tablets with a few black and brown spots due to the plant material. Average weight: 499.4 mg/tablet.

6.4.17.2 HPLC-UV profile

The HPLC profile showed peaks at RTs 6.64, 7.05, 8.41 and 12.22 minutes, which correspond to mesembranol, mesembrenone, mesembrine and epimesembranol, respectively (Figure 6.17a).

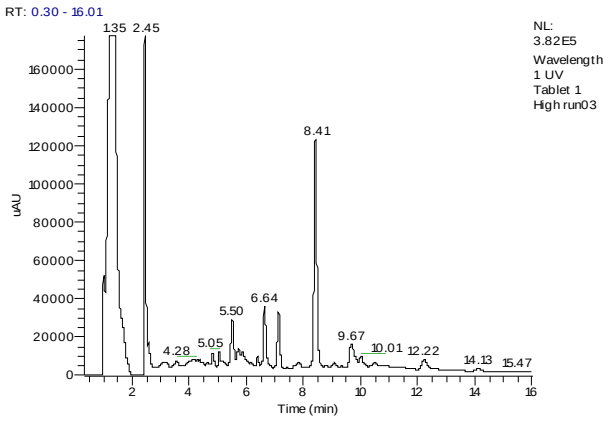


Figure 6.17a: HPLC-UV chromatogram of Sceletium tortuosum tablets (Big Tree Health Product, Batch no.9323)

6.4.17.3 ESI-MS profile

The MS profile showed ionized peaks at 5.79, 6.69, 7.18, 8.47 and 12.29 minutes (Figure 6.17b) which corresponded to *m/z* 288, 292, 288, 290 and 292 respectively (Figure 6.17c-g). The *m/z* ratios corresponded to the alkaloids Δ^7mesembrenone, mesembrenone and mesembrine respectively.

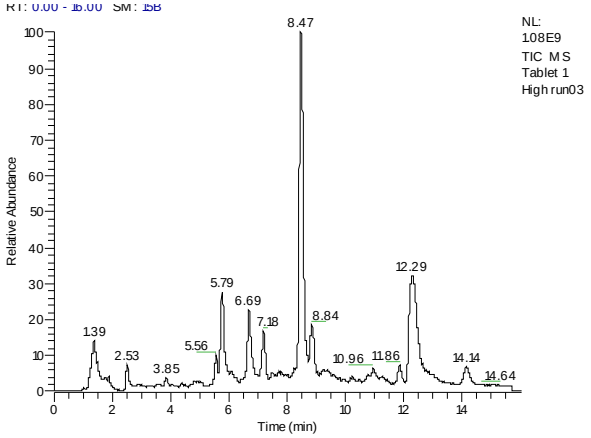


Figure 6.17b: TIC chromatogram of Sceletium tortuosum Tablets (Big Tree Health Products, Batch no. 9323)

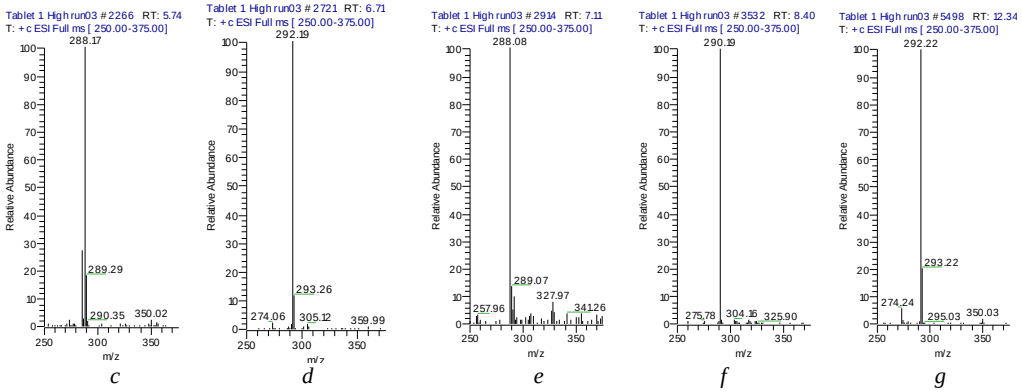


Figure 6.17c-g: Ion spectra of the identified alkaloids in Sceletium tortuosum tablets (Big Tree Health Products, Batch no.9323)

6.4.17.4 Inference

All the relevant alkaloids were assayed by HPLC and the product was found to contain concentrations of 42.1, 15.0, 184.8 and 27.2 µg/tablet for the alkaloids, mesembranol, mesembrenone, mesembrine and epimesembranol, respectively. The alkaloid, Δ^7mesembrenone, was found to be below the LoQ. The MS detection positively identified the relevant alkaloids.

6.5 CONCLUSIONS

6.5.1 *Sceletium* Plant Analysis

The *Sceletium* plant species which were grown under various conditions at different locations when analyzed showed major differences in their compositions of alkaloids. It is very important to note that the particular plant species clearly indicated that an enormous difference exists between the various species with respect to the presence and content of alkaloids. The *tortuosum* type of *Sceletium* species showed the presence of various alkaloids with variation in content and

composition. The *emarcidum* type of *Sceletium* species did not show presence of the relevant alkaloids. However e*marcidum* type plant species contained some unidentified compounds, which were not pursued further in this study. Furthermore, the presence of alkaloids in this particular species was not confirmed since treatment with Dragendorff's reagent yielded negative results. Mesembrine (~1%) was the major alkaloid found in *S. tortuosum* species along with other minor alkaloids Δ^7mesemberenone, mesembrenone and mesembranol and could only be quantified in some species of *S. tortuosum*.

6.5.2 *Sceletium* Plant Powder

The particular species viz. *S. rigidum,* S. *expansum, S. strictum* etc., of *Sceletium* in the *Sceletium* plant powder samples were mostly unknown since they were obtained/labelled only as *Sceletium tortuosum* and therefore assumed to be of the *tortuosum* type. This was due to the fact that most of the *Sceletium* cultivators knew the plant grown only as *Sceletium tortuosum* and had little knowledge regarding the taxonomy and other species of this genus. However, the analyses of these samples showed enormous variation of content and composition of the alkaloids. Of the analyzed samples only SRM02 contained 0.5% mesembrine and 0.4% mesembranol, with lower quantities of the minor alkaloids Δ^7mesembrenone and epimesembranol. One sample obtained as *Sceletium emarcidum* powder from Australia (www.herbalistics.com.au) showed complete absence of relevant alkaloids.

6.5.3 *Sceletium* Dosage Forms

Sceletium dosage forms in the form of tablets, capsules and a mother tincture also showed variations in content and composition of alkaloids. Since the available tablet samples were from one manufacturer (Big Tree Health Products) three samples of different batches were analyzed. The range of alkaloids were found to be similar, with mesembrine being the major alkaloid component. The analyzed batches 7161, 9232 and 9161 were found to contain 116.2, 185 and 121 µg per tablet of mesembrine, respectively. The minor alkaloids varied in content and Δ^7mesembrenone was detected in batch 9232 but found to be below the LoQ in this particular batch.

The chromatographic profiles of the capsule formulations manufactured by Herbal Care also showed variation in the composition of alkaloids. Product B contained none of the previously identified alkaloids but showed the presence of two unidentified compounds in the HPLC analysis.

The chromatographic profiles of the capsules manufactured by Essential Source showed a complex mixture of alkaloidal components. However, the major alkaloid present in Product C capsules was found to be mesembranol at a concentration of 136.7 μg/ capsule. The second sample Product D obtained from the same manufacturer had mesembrenone as the major alkaloid at concentration 105.6 μg/capsule.

In summary, the content of *Sceletium* alkaloids in plant material and formulated products are seen to show great variability with respect to both quality and quantity of particular alkaloids. The QC of *Sceletium* presents a formidable challenge in view of the fact that *Sceletium* plant and products containing *Sceletium,* contain a complex mixture of compounds. Hence, a prerequisite for appropriate QC of such materials necessitates the availability of relevant marker compounds as well as validated methods for the assay of these plants and formulated products. The above challenges were thus successfully achieved by the development and validation of appropriate qualitative and quantitative analytical methods which were successfully applied to fingerprint and assay *Sceletium* plant material and dosage forms. These methods therefore can be used for the QC of *Sceletium* plant material as well as for dosage forms containing *Sceletium*.

CHAPTER 7

CAPILLARY ELECTROPHORESIS OF *SCELETIUM* ALKALOIDS

7.1 INTRODUCTION

Capillary electrophoresis (CE) is a relatively new analytical technique which has gained popularity over the last 2 decades [78], mainly due to its wide application, high efficiency, short analysis times and importantly, low cost. This technique is well- suited for the analysis of natural products which generally contain multiple and complex chemical constituents that usually require lengthy analysis times and complex separation methods [79]. CE is a technique where charged components are separated in small capillaries based on their electrophoretic mobilities in an electrophoretic media under the influence of an applied electric field. This technique permits an efficient and rapid separation of compounds resulting in a relatively fast analysis [80].

The CE instrumentation is a simple set of components consisting of two buffer reservoirs, an anode and a cathode connected by a capillary which is inserted through a detector. A high voltage (HV) power supply to the buffer is used to complete the circuit [78] (Figure 7.1).

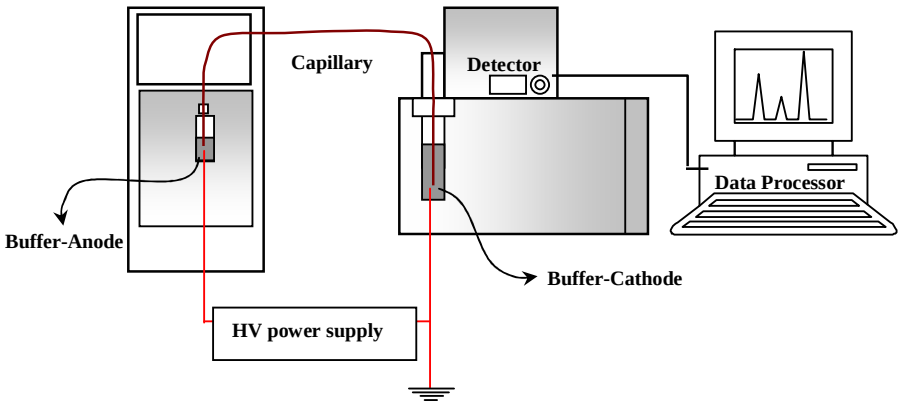


Figure 7.1: Schematic diagram of capillary electrophoresis instrument

7.2 ELECTROPHORESIS

Electrophoretic separation is based on differences in solute velocity in an electric field [80]. The electrophoretic separations are performed in an electrolyte solution known as the background

electrolyte (BGE). The pre-requisite of a BGE is to provide constant conditions of ionic strength, pH and maintain low current for these CE separations which are carried out in capillaries.
The principle of CE involves differential migration of charged species under the influence of an applied electrical field to separate components based on their electrophoretic mobilities and which relate to their respective mass to charge ratios [81]. The analytes are detected by employing various suitable detectors.

The velocity of the ion in an electrophoretic separation may be described as

$$v = \mu_{ep} E = \frac{\mu_{ep} V}{L} \qquad \textbf{(}\text{E- 1) [80]}$$

v = migration velocity, μ_{ep} = electrophoretic mobility, E = field strength, V = voltage applied across the capillary and L = capillary length

Thus the migration time't' taken by a charged molecule to travel from start to end of capillary is

$$t = \frac{L}{v} = \frac{l^2}{\mu_{ep} V} \qquad \text{(E-2) [80]}$$

Generally, small sample volumes are injected onto the anodic end through a narrow-bore capillary filled with buffer and ions are carried towards the cathode by a process known as electroosmotic flow (EOF) due to a phenomenon known as electroosmosis [80].

7.2.1 Electroosmosis

Electroosmosis (EO) is the flow of solvent due to an applied voltage. This electroosmotic flow is caused due to the negative charge on the interior wall of the capillary tube. The negative charge is caused by silanol groups of the capillary wall that form an electrical double layer. When the electric field is applied, the counter-ions (predominantly cations) accumulated on the negatively charged double layer, carry the bulk of the buffer solution towards the cathode. During the process, the neutral molecules also move towards the cathode with the EOF, which forms the basis of capillary zone electrophoresis (CZE). Typically the electrical double layer is hundreds of nanometers relative to the capillary radius which is usually about 50-100 μm, thus it is considered that the EOF originates from the capillary walls causing a "flat flow" profile (Figure 7.2) when compared to a pumping system like HPLC which has a parabolic flow through the column [80].

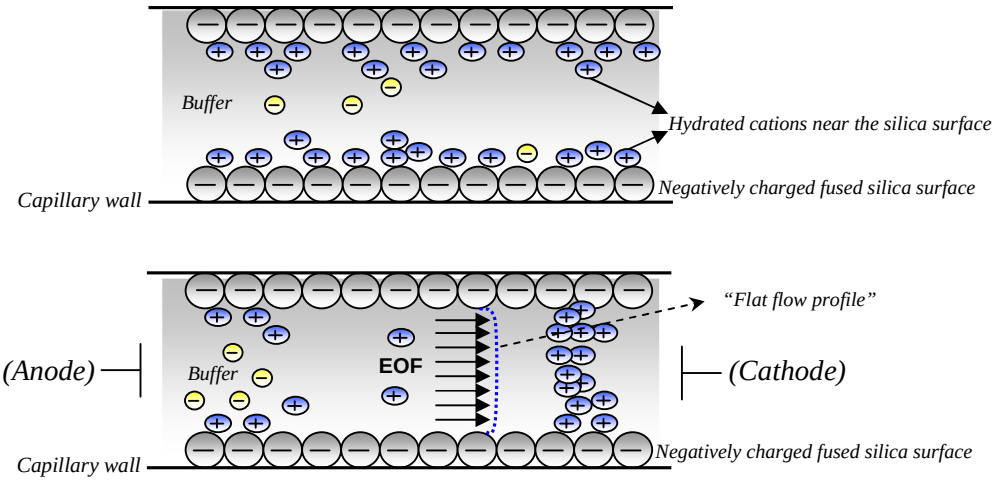


Figure 7.2: Schematic diagram showing "Flat flow profile" due to EOF in CE

When a mixture of substances in an ionic solution is introduced into a capillary separating system, these ionic substances are influenced by an applied potential. The effective mobilities of these ionic substances cause a difference in their migration velocities. These separate ionic zones are detected as individual substances over a period of time (Figure 7.3) [80].

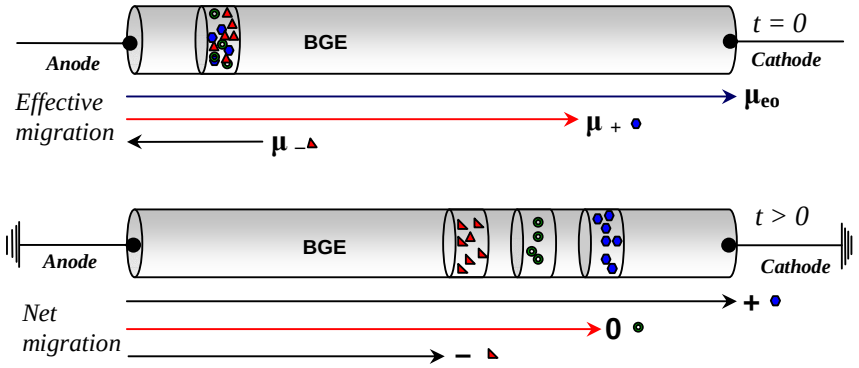


Figure 7.3: Schematic diagram of electrophoretic separation (+• = cation, –▲ = anion, 0 = neutral ion)

In the presence of an EOF, the migration velocity and time are given by:

$$v = \frac{(\mu_{eo} + \mu_{ep})V}{L} \quad \text{and} \quad t = \frac{L^2}{(\mu_{eo} + \mu_{ep})V} \qquad \text{(E-3 and 4) [80]}$$

v = migration velocity, μ_{ep} = electrophoretic mobility, E = field strength, V = voltage applied across the capillary and L = capillary length and μ_{eo} is coefficient for electroosmotic flow

Considering equation E-3, the migration of ions will be in the same direction when the EOF is higher in magnitude and travels opposite to all the anions in the buffer. In this situation, the non-ionic components will also be carried with the EOF causing their migration. In other words, the cations will have a strong tendency to move faster towards the cathode whilst the anions tend to migrate against the EOF and are the slowest to move towards the cathode, thereby achieving electrophoretic separation of charged components. The neutral ions move according to the EOF, thus the separation of components in a mixture is based on their respective electrophoretic mobilities. However, electroosmosis should effectively result in better separation of anions as they migrate against the EOF with consequent poor separation of cations which move faster towards the cathode. The magnitude of the EOF can be altered by selecting an appropriate ionic composition and an effective pH to achieve the required EOF, thus controlling the migration of solutes and thereby the separation.

The electrophoretic separation can be carried out in continuous or discontinuous electrolyte systems. When a continuous BGE solution is used, a continuum is formed along the migration path and provides an electrically conducting medium. The separation can be effected by a kinetic process or a steady state process by changing the properties of the BGE [80].

7.2.2 Modes of Capillary Electrophoresis

CE comprises of various modes of application techniques that have varied operative and separation characteristics. These techniques have evolved by a combination of chromatographic and electrophoretic techniques. The techniques are capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isotachophoresis (CITP), capillary isoelectric focusing (CIEF), micellar electrokinetic chromatography (MEKC), and capillary electro-chromatography (CEC) [80].

In CZE, CGE, MEKC and CEC, the BGE composition is maintained uniform throughout the migration path. As a result of this, the electric field strength and the effective mobilities of the charged species are stable, which results in migration of the analytes at constant, but different velocities [80].

In CIEF, the composition of the BGE is not maintained constant. The electric field and the effective mobilities may change along the migration path. At a point of time, certain components of the sample stop migrating and focus at a characteristic position based on their isoelectric points

e.g. ampholytes [80]. It is reported to be one of the most powerful techniques for the separation of protein mixtures [81].

CITP is a discontinuous electrolyte system, where the analytes migrate between two different electrolytes as distinct individual zones. The analytes condense between the leading and terminating electrolytes, producing a steady state migration comprising of consecutive sample zones [80] .

7.3 CAPILLARY ZONE ELECTROPHORESIS

CZE is fundamentally the simplest form of CE and the separation is effected due to the migration of solute in discrete zones at different velocities. In CZE, separation of both anionic and cationic components is possible due to the electroosmotic flow [80]. The differences required to resolve the ionic zones are dependent on the dispersive effects that act on the zones. The dispersive effects have to be controlled to achieve the desired separation as they may increase the zone length and cause a difference in mobilities which result in poor separation [82].

7.3.1 Dispersion Effects

Dispersion of a solute band is a common phenomenon in all separation techniques. This could be due to various factors that control the resolution of solutes. In CE, the separation is based on differences in solute mobilities and resolution of these solute zones depends on the band length of these zones. Thus, dispersion affects separation by increasing the zone length, resulting in changes in the mobility of solutes thereby leading to a decreased efficiency of CE system [82].

7.3.1.1 Joule Heat

The heat generated when an electric current is applied to an electrolyte system is known as joule heat. This causes an increase in temperature of the electrolyte system passing through a capillary. Fused silica being a high thermal conductor facilitates heat transfer across the capillary wall more effectively than the electrolyte. This effectively results in a temperature gradient to be set up with a higher temperature in the center and lower temperature on the capillary walls due to dissipation of heat through the walls (Figure 7.4). The temperature gradient is dependent on capillary diameter, buffer conductivity, and the applied potential. High efficiencies in CE systems can be attained by ensuring efficient heat dissipation, leading to a parabolic temperature gradient across the capillary, which can increase electrophoretic mobilities of the analytes [80].

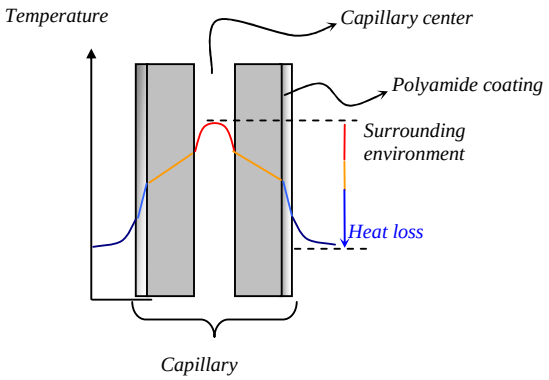


Figure 7.4: Schematic diagram o f heat dissipation in capillary electrophoresis

7.3.1.2 Adsorption effect

Peak distortion can be caused by adsorption of analytes to the capillary surface and this inhibits migration. This phenomenon is more pronounced for macromolecules like proteins [82].

7.3.1.3 Conductivity difference

This is caused when the analyte ions differ in mobility with respect to the buffer ions, generally due to differences in electrical conductivity leading to electro-dispersion. The differences in sample zone and running buffer conductivities can have three major effects:
1) Skewed peak shapes, 2) solute concentration or focusing (low conductivity sample), or solute defocusing (high conductivity sample) 3) temporary isotachophoretic states due to excess of a certain ion [82].

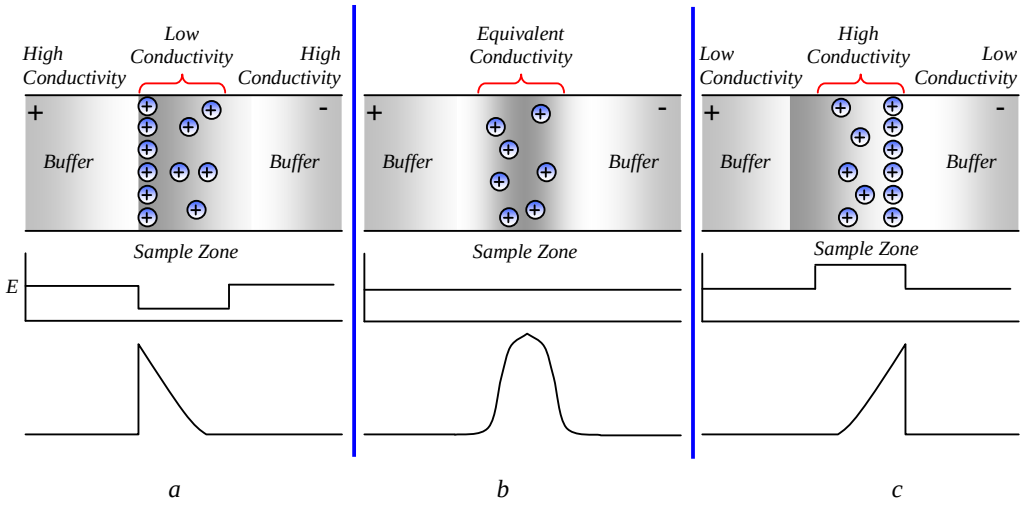


Figure 7.5 a, b and c: Schematic diagram for effect of conductivity on ionic analytes in CE [78, 82]

When the solute zone has lower mobility (low conductivity and higher resistance) than the running buffer, then the generated peaks show tailing (Figure 7.5a). Conversely, when the solute zone has a higher mobility than the running buffer, the generated peaks demonstrate fronting (Figure 7.5c). When the solute zone has the same ionic strength as the buffer, the peaks that are generated show symmetrical broadening. Since the conductivities are equivalent, there will be no peak distortions (Figure 7.5b) [78]. These peak shape distortions are caused by the differences in conductivity. Solute zones having higher conductivity and lower resistance cause higher mobility and diffuse towards the direction of migration encountering a higher voltage drop when entering the buffer zone. This causes the diffusing solute (anions) to accelerate away from the solute zone and results in zone fronting. As solutes at the trailing edge diffuse into the running buffer they also encounter an increased drop in voltage but in the same direction of migration and accelerate back into the solute zone, keeping the trailing edge sharp. Neutral species are unaffected by these conductivity differences [82].

Resolution of solute zones in CE is primarily driven by efficiency, not selectivity, which is in contrast to chromatography, which is usually the opposite. Due to sharp solute zones, even small differences in solute mobility permit adequate resolution [80].

7.3.2 Sample injection methods

To ensure high separation efficiency, the injection of the samples should not cause a significant broadening of the sample zone. To achieve this, the injection systems should be capable of reproducibly introducing small volumes of sample, such that there is no overloading of the capillary [78].

The sample is introduced directly into one end of the column, which effectively helps in keeping the sample zone to minimum. The most commonly used sample injection systems are electrokinetic and hydrodynamic injection [80] .

7.3.2.1 Electrokinetic injection

In this method, the capillary and high voltage electrode are immersed into sample buffer. The injection is started by applying voltage for a short period of time, which causes the sample buffer to enter the capillary due to electro-migration. This voltage causes electrophoretic migration of the sample ions and EOF of the sample solution. This effect can cause changes in electrophoretic mobilities of the ions in the sample and thus components of higher mobilities are injected in larger

quantities than the ions of lower mobility. Consequently, if the sample solution is different from the running buffer, there will be differences in conductivity, which, through alteration of the electrophoretic mobilities and EOF, can result in changes in the absolute quantity of sample injected. These effects have to be considered for optimization of CE methods [80].

7.3.2.2 Hydrodynamic injection

In this method, the sample is injected by gravity flow, vacuum or by the application of pressure. Gravity flow injection is carried out by placing the capillary in the sample solution and the sample container with the capillary is moved to a higher position than the opposite end for a short time period [78]. The quantity of injection is independent of electrophoretic mobility and the sample composition. In the case of pressurized injection, the sample container is subjected to a constant pressure for a short period of time, causing the sample to enter the capillary. Vacuum injection is done by application of vacuum at the opposite end of the capillary to suck the sample into the capillary. However, these techniques are associated with lower precision compared to the electrokinetic method, but have the advantage of not affecting the electrophoretic mobilities of the sample [80].

7.3.2.3 Other Injection Methods

Sample stacking is an injection procedure which concentrates dilute mixtures of ionic species before an electrophoretic separation, where the conductivity of the sample is lower than the running buffer. This causes the sample buffer to concentrate in a narrow zone or become “stacked” [78]. This phenomenon of stacking can be utilized by electrokinetic or hydrodynamic techniques to improve the efficiency of sample injection [80]. The technique of field amplified sample injection (FASI) is an enhanced system of sample stacking due to an applied voltage which results in a shorter plug of sample in the capillary. This injection system is selective to either negative or positive polarity and only one type of ion is introduced into the column [80].

7.4 CE ANALYSIS OF *SCELETIUM* ALKALOIDS

TLC, HPLC, GC and presently LCMS techniques have been successfully applied for use in phytochemical analyses. The use of CE for phytochemical research has been making good strides due to the fact that it is considered as a high efficiency, low cost method which is relatively simple [79]. CE analyses of natural products such as flavonoids, alkaloids, terpenoids, phenolic acids and

coumarins etc. have been reported in scientific literature. It has been mentioned that alkaloids are the second most analyzed substances by CE [79].

Alkaloids, being strong bases, [83] are good candidates for CE analysis as they are readily protonated and provide positive charge under the influence of low pH solutions [79]. Using this technique, many alkaloidal compounds have been studied and reported [79, 84].

General approaches for alkaloidal analysis using CE coupled with MS have been reported for indole alkaloids [84] as well as a report on the influence of alkaloidal structure on electrophoretic mobility [83]. CE methods, to my knowledge, have not yet been reported for *Sceletium* alkaloids in the scientific literature.

7.5 OBJECTIVES

The main objective of this study was to develop a simple, rapid, precise and reproducible analytical method for the separation of *Sceletium* alkaloids and also to apply the method for fingerprinting and assay of relevant alkaloids in dosages forms and plant material.

7.6 EXPERIMENTAL

7.6.1 CE Instrumentation

The CE instrumentation comprised a PrinCE (4tray) CE System Model 0500-002/OR and Diode array detector DAD-160, Model 0005-133. The CE system, detector operation and data processing were achieved by software DAx3D Data Acquisition and Analysis Version 8.0 (Prince Technologies B.V., Emmen, Netherlands). A Linear UV/Vis Model 200 ultraviolet detector (Linear Instruments Corp., Reno, NV, USA) was used and the data output from this detector was interfaced through a SATIN® box, to a Waters® Empower Chromatographic Manager (Waters Chromatography Division, Milford, MA, USA). The separation of alkaloids was carried out on a 50 cm effective length, fused silica capillary tubing 50µm I.D. x 360µm O.D. (Polymicro Technologies, L.L.C. Phoenix, Arizona, USA).

During the course of this study, the initial development was carried out using a DAD-160 detector and DAx3D software. However the method validation was carried out using a Linear UV/Vis detector and Waters® Empower.

7.6.1.1 Additional equipment

A Cole-Parmer ultrasonic bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used during sample extraction and a Mettler Toledo Electronic Balance, Type AG 135 and Mettler Toledo Electronic Micro Balance MX-5 (Mettler Toledo, Switzerland) were used for weighing reagents and standards respectively. A Crison GLP21 pH Meter (Crison, Barcelona, Spain) was used to measure and adjust the pH of the relevant solutions. HPLC grade water was purified by reverse osmosis process through a Milli-Q purification system and used to prepare the various solutions and buffers.

7.6.2 Materials and Reagents

Methanol 215 (HPLC grade) was purchased from Romil Ltd. (Cambdrige, Great Britain). Sodium dihydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_2O$) was obtained from Saarchem (Pty) Ltd. (Mudersdrift, South Africa). Sodium hydroxide (NaOH) procured from Associated Chemical Entreprises (Pty) Ltd. (Southdale, South Africa). Orthophosphoric acid (H_3PO_4) was purchased from Merck Chemicals (Pty) Ltd. (Wadeville, South Africa). Quinine hydrochloride was obtained from Sigma Chemical Company, St. Louis, MO, USA. Papaverine (base) was obtained from the Biopharmaceutics Research Institute, Rhodes University. *Sceletium* reference compounds and plant material and its products have been described and detailed in Chapters 2 and 3 respectively.

7.6.3 Capillary Conditioning

New capillaries of 65 cm were cut and an effective length to the window of 50 cm was made by using a gas flame. The capillaries were conditioned using a pressure of >2500 mbar using 1M NaOH solution for 30 minutes, 0.1 M NaOH solution for 30 minutes, followed by water for 40 minutes. Washing of capillaries between consecutive injections to ensure optimal charge density on the capillary wall during analytical work was done with water for 4 minutes, 1M NaOH for 2 minutes, 0.1M NaOH for 2 minutes and finally with water for 5 minutes. The buffers at the anode and cathode were replaced after each injection.

7.6.4 Preparation of Standard Solutions

All stock solutions were prepared from the reference standards which were individually weighed into volumetric flasks and dissolved in methanol. The required range of concentrations were

prepared by transferring aliquots of the stock solutions into volumetric flasks and mixed with running buffer and methanol to result in solutions containing 10% of the running buffer.

7.6.5 CZE Method Development for *Sceletium* Alkaloids

A method for the separation of alkaloids by CE reported by Unger *et al.* [84] was attempted to separate the *Sceletium* alkaloids. However, this method was specifically applied for the separation of indole alkaloids and could not be successfully adapted for the separation of the *Sceletium* alkaloids. Considering the fact that the *Sceletium* alkaloids are basic compounds (Table 7.1), orthophosphoric acid (H_3PO_4) was considered for use as a buffer.

Table 7.1
***pKa of *Sceletium* alkaloids**

Compound	pKa	Condition
Mesembrenone	8.58	Most basic
Epimesembranol	9.44	Most basic
Mesembrine	8.97	Most basic
Mesembranol	9.48	Most basic
Δ^7Mesembrenone	5.59	Most basic

**Data obtained from Scifinder Scholar " Calculated using advanced chemistry development (ACD/labs) software V8.14 for solaris"*

H_3PO_4 (25 mM) was used as the running buffer. The applied voltage was +15 kV with a voltage ramp of +6 kV/s and a sample containing 20 μg/ml of mesembrine base in methanol was injected electrokinetically at 2 kV for 0.2 minutes. The UV detector DAD-160 was set at 228 nm. The electropherogram showed one peak appearing at migration time (MT) 12.247 minutes (Figure 7.6). However, the peak showed tailing due to its lower mobility than that of the running buffer. The current generated was observed to be about 17.5 μA for an applied voltage of +15 kV.

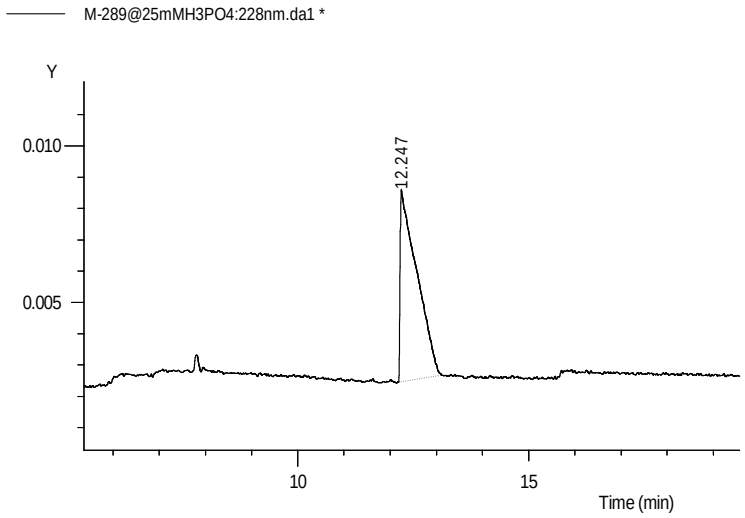


Figure 7.6: Electropherogram of mesembrine base in methanol with an applied voltage of 15kV and a running buffer of 25 mM H_3PO_4

The applied voltage was increased to +20 kV, which generated a current of about 25 μA. The resulting electropherogram showed an earlier peak occurring at 7.793 minutes with improved peak shape and reduced tailing (Figure 7.7).

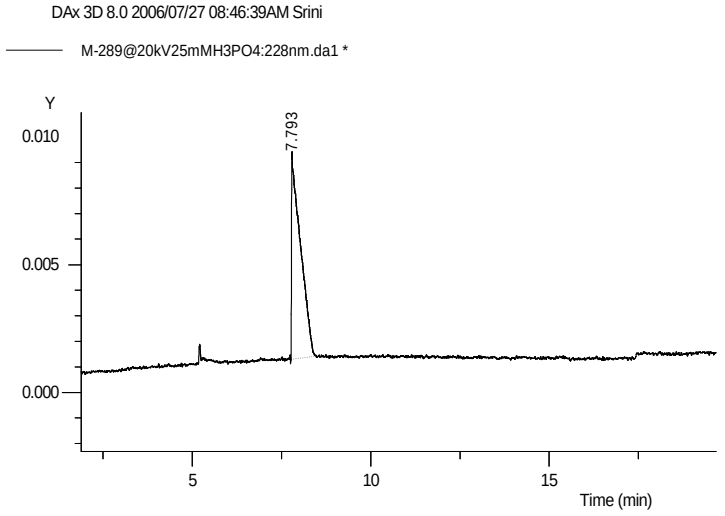


Figure 7.7: Electropherogram of mesembrine base in methanol with an applied voltage of +20kV and a running buffer of 25 mM H_3PO_4

Using the above-described conditions, a sample containing 20 μg/ml, of mesembrine and mesembrenone were injected, but the resulting electropherogram showed unresolved separation of the two compounds with MT 7.217 and 7.345 minutes for mesembrenone and mesembrine, respectively. Both co-eluting peaks were associated with poor peak shape (Figure 7.8).

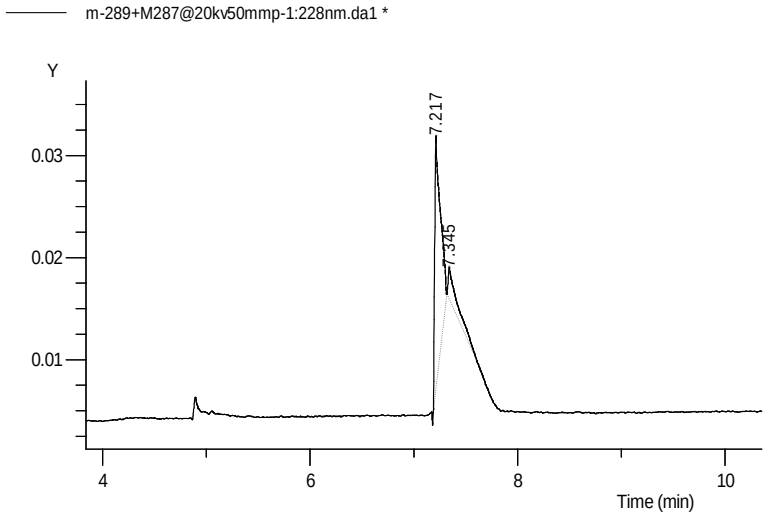


Figure 7.8: Electropherogram mesembrine base and mesembrenone in methanol with an applied voltage of +20kV and a running buffer of 25 mM H_3PO_4

The electrolyte concentration was increased to 50 mM H_3PO_4 and the system was run at an applied voltage of +15 kV which generated a current of 35 μA. The sample was prepared with the addition of 5% of the electrolyte to the methanolic solution. The separation and the peak shapes were found to be satisfactory with MTs of 7.107 and 7.297 minutes for mesembrenone and mesembrine (Figure 7.9). This could be explained by the fact that the conductivity of the analyte was close to the conductivity of the BGE.

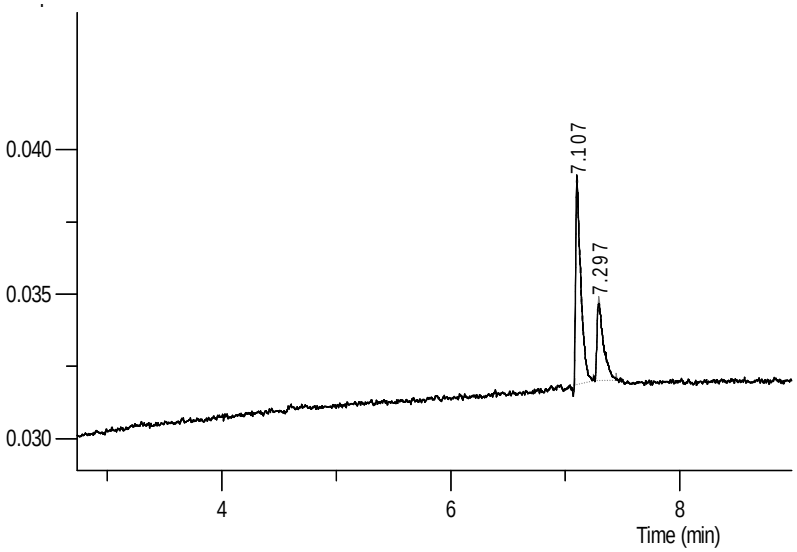


Figure 7.9: Electropherogram of mesembrine base and mesembrenone in methanol containing 5% BGE injected with an applied voltage of +20kV and a running buffer of 50 mM H_3PO_4

However, the electrolyte concentration had to be increased to 100 mM H_3PO_4 with an applied voltage of +20 kV to obtain separation of the compounds, mesembrine base, mesembrenone and Δ^7mesembrenone. The electrolyte generated a current of ~80 μA and the separation was found to

be satisfactory with the MT's of 7.240, 7.400 and 8.102 minutes for mesembrenone, mesembrine and Δ^7mesembrenone respectively (Figure 7.10).

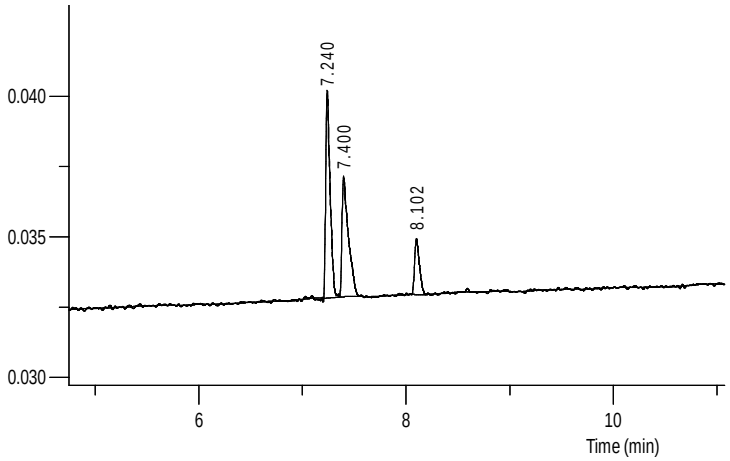


Figure 7.10: Electropherogram mesembrine base, mesembrenone and Δ^7mesembrenone in methanol containing 5% BGE injected with an applied voltage of +20kV and running buffer 100 mM H_3PO_4.

The conditions described above were also applied to a mixture of mesembranol and epimesembranol. The separation was found to be satisfactory with MTs of 7.823 and 8.028 minutes for epimesembranol and mesembranol respectively (Figure 7.11).

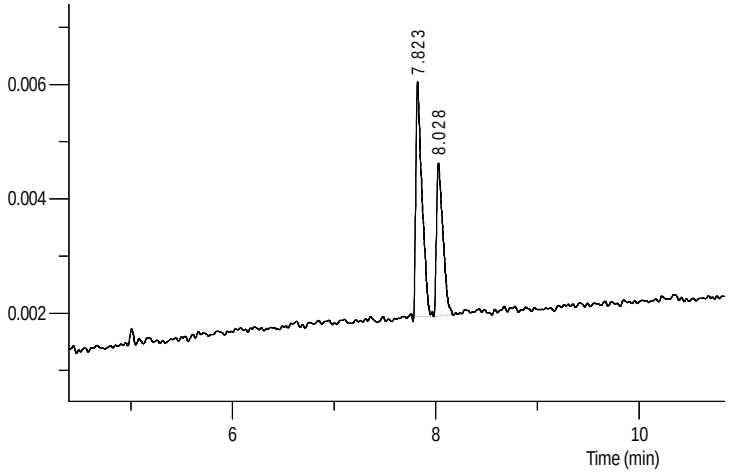


Figure 7.11: Electropherogram of epimesembranol and mesembranol in methanol containing 5% BGE injected with an applied voltage of +20kV and a running buffer of 100 mM H_3PO_4

However, the electrolyte system was not suitable to effect the separation of the compounds, epimesembranol and mesembrine by this method. The peaks were observed at MTs 7.122 and 7.195 minutes for epimesembranol and mesembrine respectively (Figure 7.12).

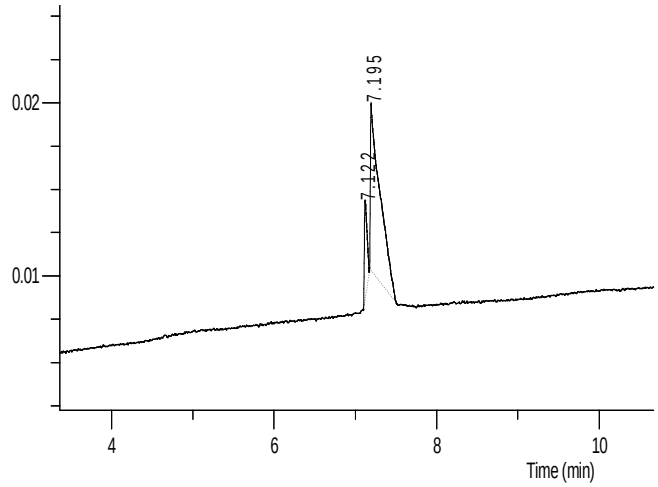


Figure 7.12: Electropherogram of mesembrine base and epimesembranol in methanol containing 5% BGE, injected with an applied voltage of +20kV and running buffer 100 mM H_3PO_4

A mixture of 5 alkaloids was injected to confirm the resolution between mesembrine and epimesembranol which showed poor resolution with MT 7.618 minutes for epimesembranol + mesembrine. The other alkaloids were well-resolved with MTs 7.480, 7.818, 8.365 minutes for mesembrenone, mesembranol and Δ^7mesembrenone respectively (Figure 7.13).

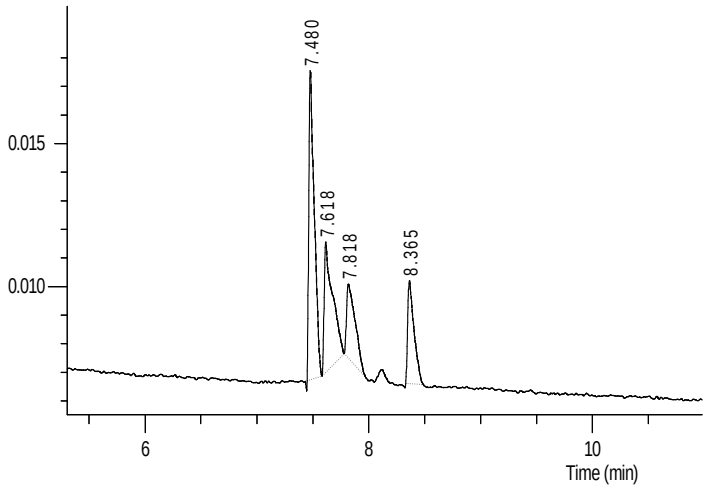


Figure 7.13: Electropherogram of 100 µg/ml concentrations each of Δ^7mesembrenone, mesembrenone, mesembranol, mesembrine and epimesembranol in methanol with 10% BGE injected at 20kV with running buffer of 100 mM H_3PO_4

The above alkaloids were scanned between 400 nm and 200 nm using the PDA detector and showed UV absorption maxima at 228 and 298 nm for Δ^7mesembrenone whereas other alkaloids showed maxima at 228 and 278 nm (Figure 7.14), which were comparable with the maxima obtained during the HPLC-PDA analysis.

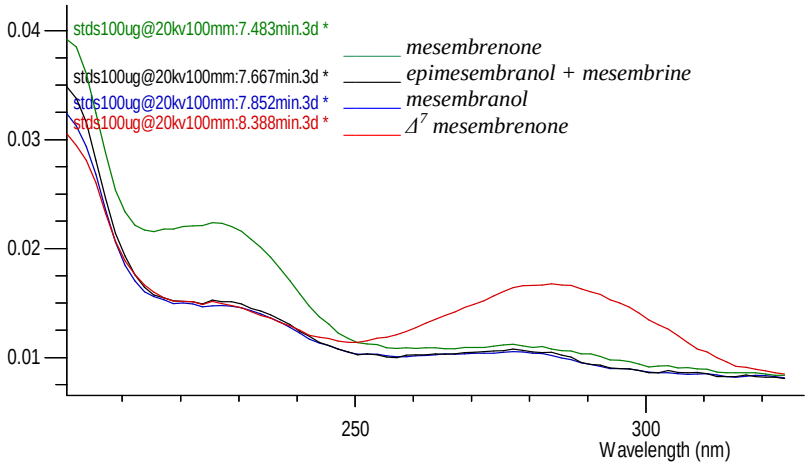


Figure 7.14: UV absorption maxima of Sceletium alkaloids using the DAD-160 PDA detector

A minimal separation between epimesembranol and mesembrine was achieved by increasing the electrolyte concentration to 200 mM H_3PO_4 injected at +20 kV which generated a current of ~95 µA. The sample was prepared by increasing the electrolyte concentration to 10% in methanol. The observed MTs of 9.855, 10.037, 10.107, 10.295 and 10.842 minutes corresponded to mesembrenone, epimesembranol, mesembrine, mesembranol and Δ^7mesembrenone respectively (Figure 7.15).

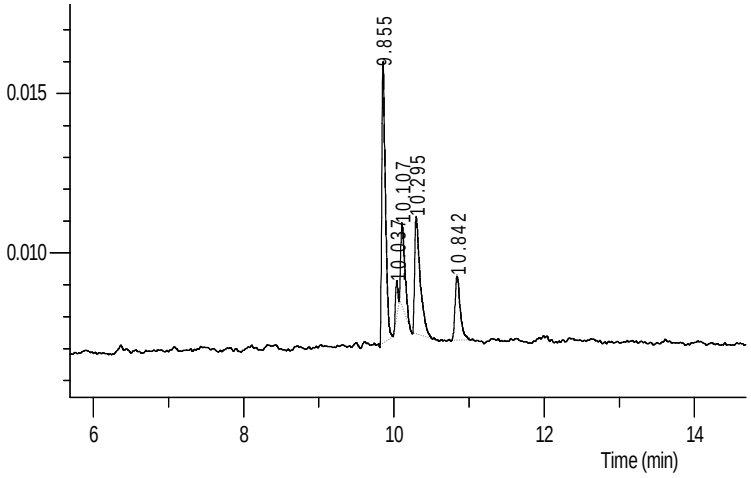


Figure 7.15: Electropherogram of 100 µg/ml concentrations each of Δ^7mesembrenone, mesembrenone, mesembranol, mesembrine and epimesembranol in methanol, with 10% BGE concentration, injected at +20kVwith running buffer of 200 mM H_3PO_4

7.6.6 Selection of Internal standard

The sample injection systems in commercial CE instruments are generally nanolitre volumes, which are prone to high injection volume variability. The use of an internal standard is highly recommended in such conditions to improve the precision of the injected samples [85]. Since the MTs of the alkaloids are close, identification of individual alkaloids could be difficult without a reference internal standard such that the MT's may be calculated as relative migration time (RMT).

The pre-requisite for an internal standard is that it should not interfere with the primary compounds present in the sample and should have good sensitivity in the analyzed conditions. In this regard, the alkaloids papaverine and quinine hydrochloride were considered for further studies. A 100 µg/ml concentration of papaverine (Figure 7.16) in methanol with 10% electrolyte concentration was analyzed using the above described conditions and it had a MT of 11.83 minutes (Figure 7.17).

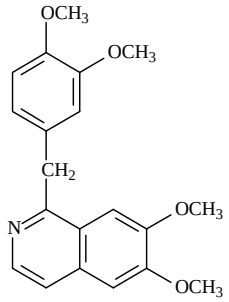


Figure 7.16 Structure of papaverine base

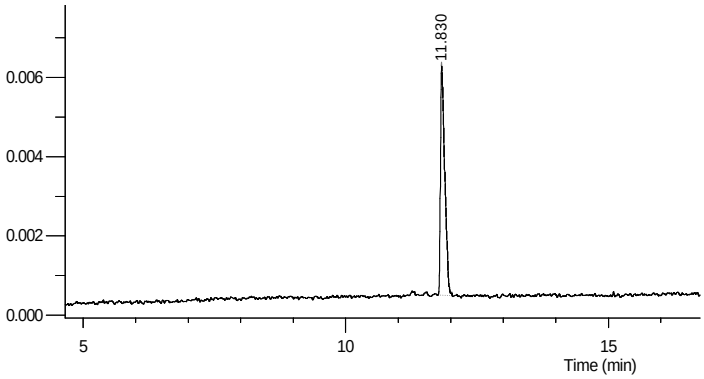


Figure 7.17: Electropherogram of 100 µg/ml concentration papaverine in methanol with 10% BGE concentration, injected at +20kV with running buffer of 200 mM H_3PO_4

However, papaverine co-eluted with mesembrenone at MT 11.650 minutes (Figure 7.18) hence, papaverine was not suitable for use as an internal standard.

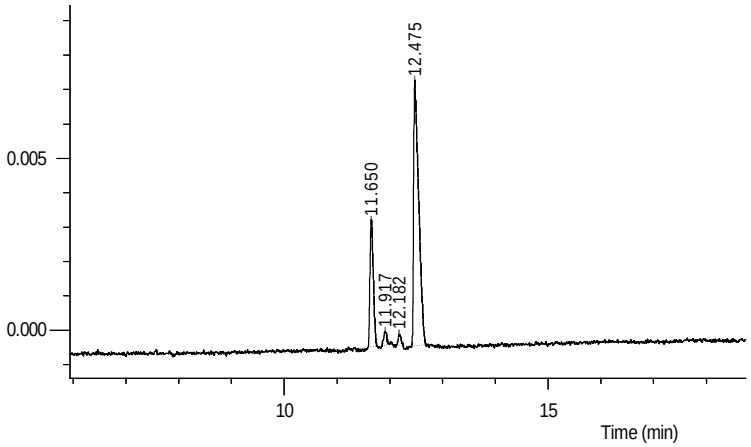


Figure 7.18: Electropherogram of 100μg/mL concentrations of papaverine and Sceletium standard alkaloids in methanol with 10% BGE concentration, injected at +20kV with a running buffer of 200 mM H_3PO_4

Quinine hydrochloride (QHCl) (Figure 7.19) analyzed under similar conditions showed a MT of 6.860 minutes (Figure 7.20) which was an ideal elution time as it did not interfere with mesembrenone eluting at MT 11.477 (Figure 7.21).

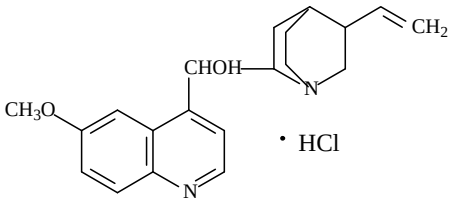


Figure 7.19 Structure of quinine hydrochloride

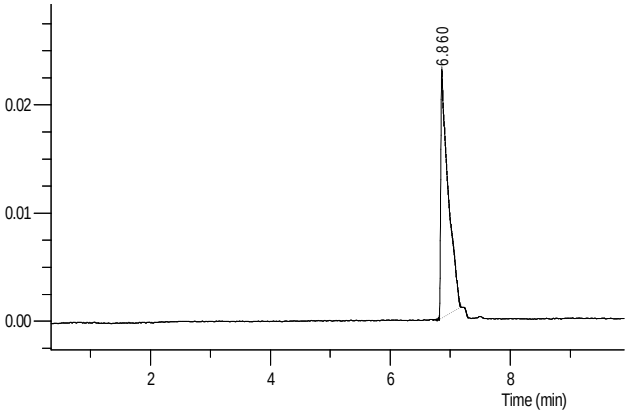


Figure 7.20: 100 μg/mL concentrations of quinine hydrochloride in methanol with 10% BGE concentration, injected at +20kV running buffer 200 mM H_3PO_4

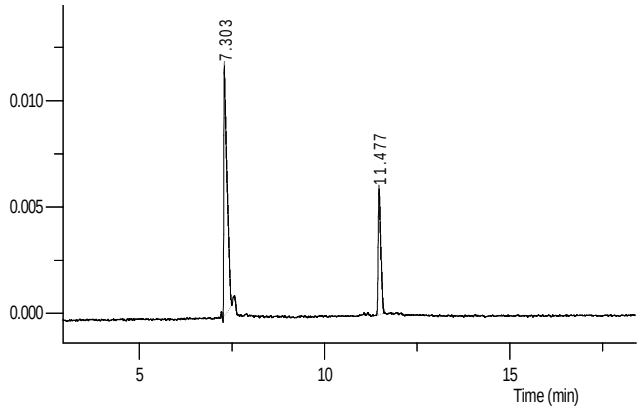


Figure 7.21: Electropherogram of 100 µg/mL concentrations of quinine hydrochloride and Sceletium standard alkaloids in methanol with 10% BGE concentration, injected at +20kV with a running buffer of 200 mM H_3PO_4

Although the method using H_3PO_4 buffer resulted in partial resolution between the various alkaloids and internal standard, the current that was generated during the analysis was very high. This can lead to undesirable joule heating and have deleterious consequences. In view of the need to keep joule heat at a minimum for effective resolution, a 50 mM $NaH_2PO_4.2H_2O$ (pH 4.5) running buffer was used to reduce the current. The *Sceletium* alkaloids were analyzed with an applied voltage of +20 kV which resulted in a current of ~26 µA and MTs of 10.375, 10.557, 10.667, 10.738 and 21.150 minutes for mesembrenone, epimesembranol, mesembrine, mesembranol and Δ^7mesembrenone respectively. However, it can be seen that resolution was poor with Δ^7 mesembrenone eluting very late at 21.150 minutes (Figure 7.22).

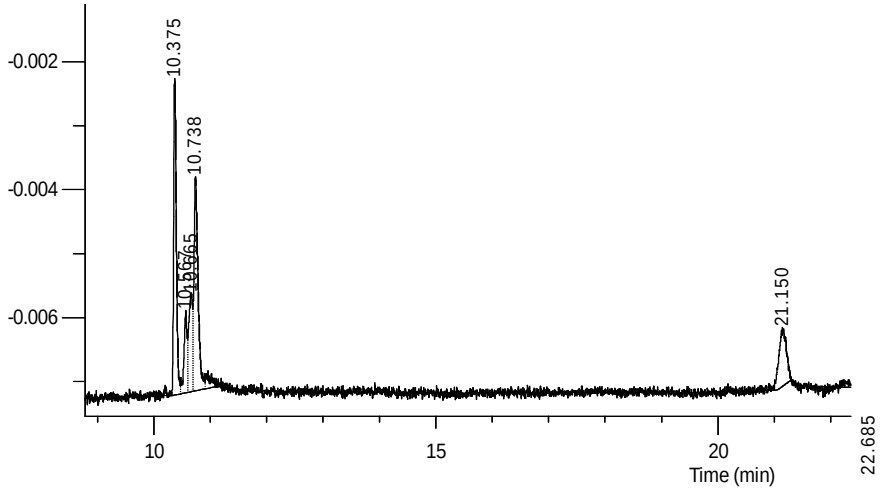


Figure 7.22: Electropherogram of 100 µg/ml concentration each of Δ^7mesembrenone, mesembrenone, mesembranol, mesembrine and epimesembranol in methanol with 10% BGE concentration injected at +20kV with a running buffer of 50 mM NaH_2PO_4 (pH 4.5).

The buffer pH was adjusted to 2.5 using H_3PO_4 and CE carried out with an applied voltage of +20 kV which generated a current of ~30 µA. Under these conditions, resolution of the individual peaks was slightly improved with MTs of 10.058, 10.302, 10.430, 10.543 and 18.343 minutes

which correspond to mesembrenone, epimesembranol, mesembrine, mesembranol and Δ^7mesembrenone respectively (Figure 7.23).

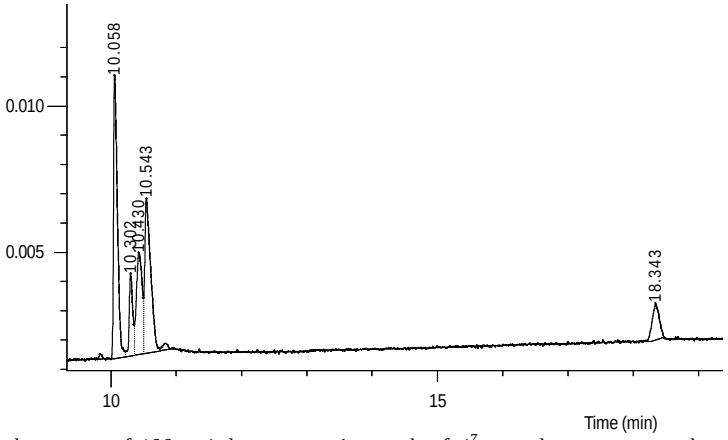


Figure 7.23: Electropherogram of 100 µg/ml concentration each of Δ^7mesembrenone, mesembrenone, mesembranol, mesembrine and epimesembranol in methanol with 10% BGE concentration injected at +20kV with a running buffer of 50 mM NaH_2PO_4 (pH 2.5)

The buffer system was further modified by adjusting the pH to 1.5 using H_3PO_4. The applied voltage of +16 kV with a voltage ramp of +6 kV/s generated a current of ~70 µA and samples were injected electrokinetically at 2 kV for 0.2 minutes. The separation was substantially improved and the MTs observed at 9.993, 10.177, 10.305, 10.473 and 11.345 minutes corresponded to mesembrenone, epimesembranol, mesembrine, mesembranol and Δ^7mesembrenone, respectively (Figure 7.24). These conditions were subsequently used for further analytical work.

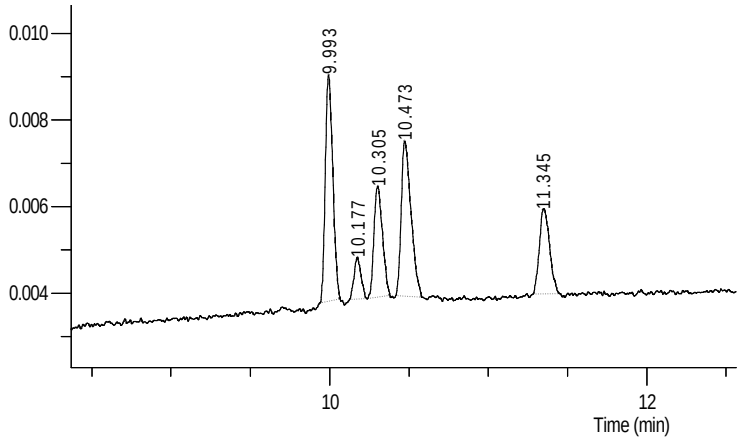


Figure 7.24: Electropherogram of 100 µg/ml concentrations each of Δ^7mesembrenone, mesembrenone, mesembranol, mesembrine and epimesembranol in methanol with 10% BGE concentration, injected at +16kV with a running buffer of 50 mM NaH_2PO_4 (pH 1.5)

Method validation was carried out using a Linear UV/Vis detector and the data output from the detector was interfaced through a SATIN® box, to a Waters Empower® Chromatographic Manager. A typical electropherogram obtained for a 60 µg/ml *Sceletium* standard spiked with 40 µg/ml of QHCl internal standard is shown in Figure 7.25.

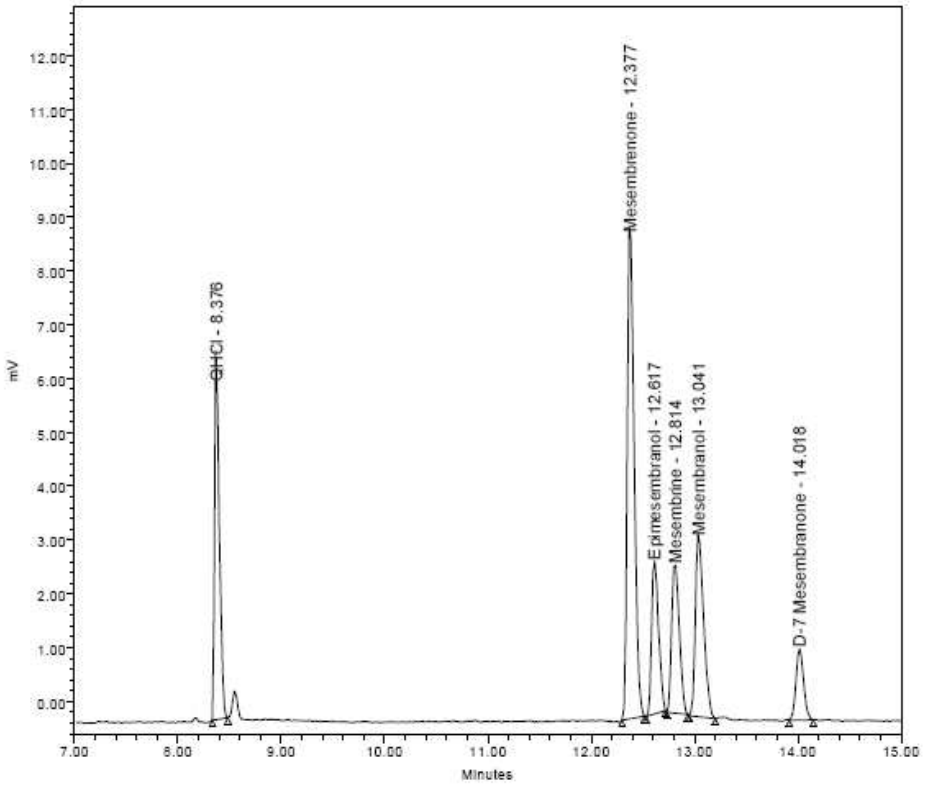


Figure 7.25: Typical electropherogram obtained from the Linear UV/Vis detector for a 60 µg/ml concentrations each of, mesembrenone, epimesembranol, mesembrine, mesembranol, Δ^7mesembrenone and 40 µg/ml QHCl internal standard in methanol with 10% BGE concentration, injected at +16kV with a running buffer of 50 mM NaH_2PO_4 (pH 1.5)

The tablet sample (Big Tree Health Products, Batch no. 9161) was extracted in methanol and mixed with running buffer to result in a solution of methanol containing 10% buffer. The sample was spiked with internal standard (QHCl) to obtain a final concentration of 75 mg/ml and 40 μg/ml respectively. A typical electropherogram from the analysis of the tablet sample is shown in Figure 7.26.

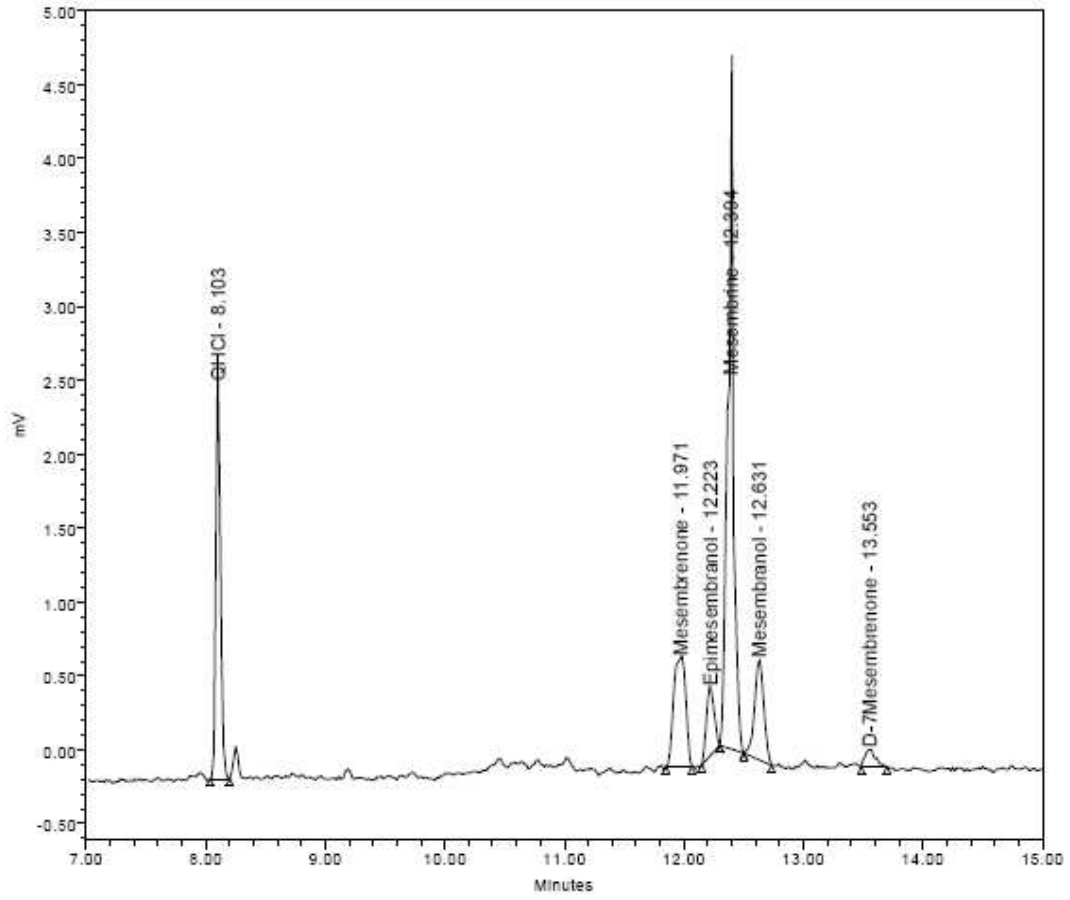


Figure 7.26: Typical electropherogram obtained from the Linear UV/Vis detector for tablet sample in methanol spiked with 40 μg/ml QHCl, with 10% BGE concentration, injected at +16kV with a running buffer of 50 mM NaH_2PO_4 (pH 1.5)

Sceletium plant material (SRM02) was extracted as previously described. The sample was spiked with internal standard (QHCl) to obtain a final concentration of 50 mg/ml and 40 µg/ml, respectively. A typical electropherogram of the plant material is shown in Figure 7.27.

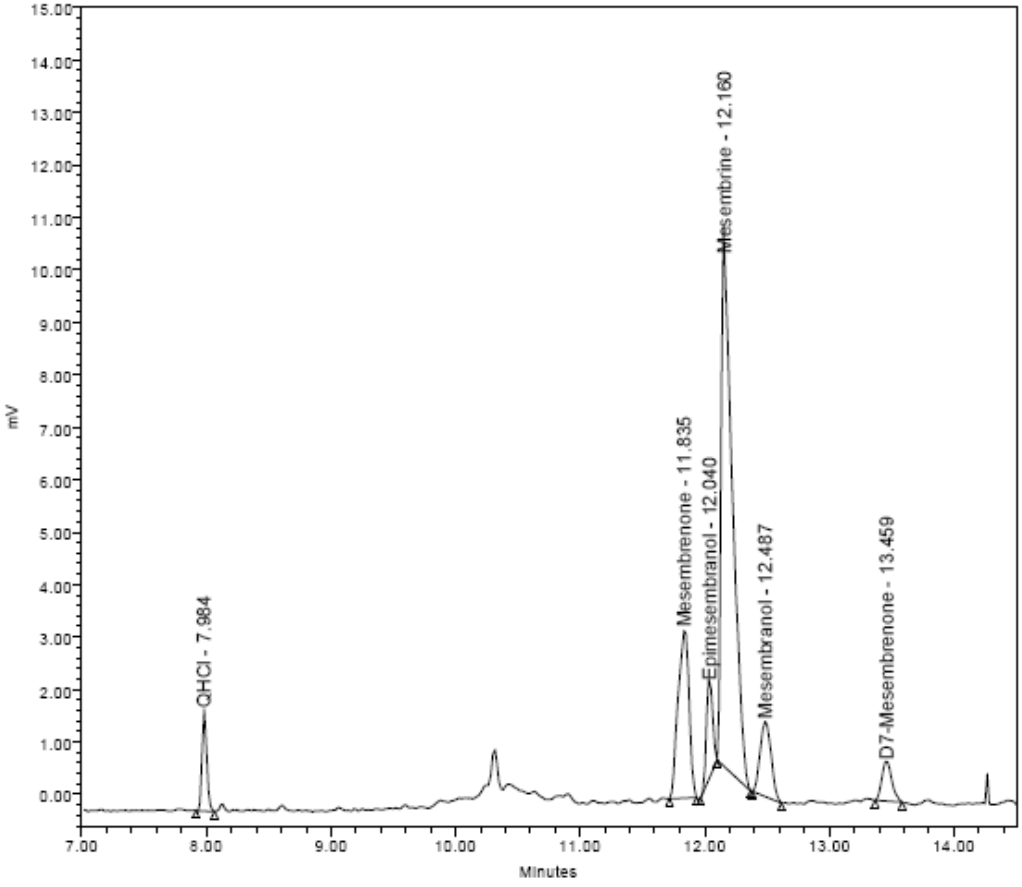


Figure 7.27: Typical electropherogram obtained from the Linear UV/Vis detector for Sceletium plant material in methanol spiked with 40 µg/ml QHCl with 10% BGE concentration injected at +16kV with a running buffer of 50 mM NaH_2PO_4 (pH 1.5)

7.6.7 Method Validation

Validation of the method was carried out using *Sceletium* tablets (Big Tree Health Products, Batch no.9961).

7.6.7.1 Preparation of Standard and Sample Solutions

Standard methanolic stock solutions (1mg/ml) of Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine hydrochloride, epimesembranol and quinine hydrochloride (QHCl) were prepared. Calibration standards were prepared to obtain 6 calibrators in the concentration range of 2.5 – 80 μg/ml of each alkaloid, a concentration of 40 μg/ml of QHCl in methanol and the final concentration of 10% BGE in each of the standard solutions.

Sceletium tablets were crushed and extracted with methanol. Tablet samples were weighed and concentrations corresponding to 25, 50 and 75 mg/ml provided the low, medium and high samples for precision studies respectively. The sample preparations were also prepared to obtain 10% BGE in each sample. The solutions were passed through 0.45 μm PVDF membrane filters before injecting into the CE system.

7.6.7.2 Accuracy and Precision Studies

Accuracy and precision studies were performed by separately preparing solutions which were appropriately diluted to obtain final concentrations of 20 and 60 μg/ml of each of the 5 alkaloids at each concentration for use as QC standards. The samples for the precision studies were prepared from commercially purchased *S. tortuosum* tablets (manufactured by Big Tree Health Products, Cape Town, South Africa, Batch no. 9961) and made up in methanol as previously described, with the aid of sonication for 20 minutes. Thereafter, the preparations included BGE at a final concentration of 10% in each sample. The samples were filtered through 0.45 μm PVDF membrane filters prior to analysis.

7.6.7.3 Recovery Studies- Tablets and Placebo Matrix

Recovery studies were carried out by preparing in triplicate, tablet samples containing 12 mg/ml of crushed tablets. Standard solutions of mesembrine hydrochloride were used to spike the tablet preparations to obtain final concentrations of 6, 12 and 24 μg/ml of the mesembrine standard.

The tablet placebo matrix was prepared by mixing the tablet excipents as described in Chapter 5. Three samples each of tablet placebo matrix (12 mg/ml) were prepared individually by spiking with the 5 alkaloid standard mixture to obtain final spiked concentrations of 4 μg, 8 μg and 12 μg/ml of each of the alkaloids in each of the placebo sample preparations.

7.6.7.4 Limit of Quantitation and Limit of Detection

A solution of a standard stock mixture containing the 5 alkaloids was diluted appropriately to obtain concentrations for the estimation of LoD and LoQ according to a signal to noise (S/N) ratio of 3:1 and 10:1 respectively.

7.6.7.5 Results and Discussion

7.6.7.5.1 Linearity

Calibration curves were constructed by plotting the peak area ratio of each alkaloid/QHCl versus the concentration corresponding to that alkaloid on each of three days. The curves obtained were found to be linear with determination coefficients greater than 0.99 for all alkaloids except Δ^7mesembrenone which was found to be greater than 0.97. The relevant data are provided in Table 7.2.

Table 7.2
Linear ranges and coefficients of determination (CZE)

Name of the compound	Day	y=mx + c linear model	Determination coefficient (R^2)
Mesembrenone	Day 1	y = 0.0947x + 0.3096	0.9988
	Day 2	y = 0.1006x + 0.3204	0.9984
	Day 3	y = 0.0950x + 0.3106	0.9988
Epimesembranol	Day 1	y = 0.0318x + 0.0997	0.9885
	Day 2	y = 0.0362x + .06180	0.9980
	Day 3	y = 0.0327x + 0.0971	0.9989
Mesembrine HCl	Day 1	y = 0.0353x + 0.0626	0.9970
	Day 2	y = 0.3620x + 0.0618	0.9950
	Day 3	y = 0.0352x + 0.0621	0.9964
Mesembranol	Day 1	y = 0.0426x + 0.1271	0.9978
	Day 2	y = 0.0414x + 0.1244	0.9996
	Day 3	y = 0.0430x + 0.1237	0.9962
Δ^7Mesembrenone	Day 1	y = 0.0152x + 0.0111	0.9907
	Day 2	y = 0.0129x + 0.0121	0.9738
	Day 3	y = 0.0134x + 0.0127	0.9724

7.6.7.5.2 Precision and Accuracy

The studies were performed using QC samples that were prepared separately on each day of the analysis. The inter-day RSD values obtained were less than 7% for the *Sceletium* alkaloid QC standards. The accuracy of the method was found to be between 90.6-107% for all five compounds. The results tabulated are shown in Tables 7.3, 7.4, 7.5, 7.6 and 7.7.

Table 7.3
Accuracy: Mesembrenone (CZE)

Mesembrenone	Day	Actual Weight (µg/ml)	Calculated Weight (µg/ml)	% Accuracy	Inter-day % RSD
QC1	1	17.60	17.85	101.40	1.40
	2	20.40	20.67	101.50	
	3	20.40	19.90	99.00	
QC 2	1	52.80	54.80	103.80	6.80
	2	61.20	55.46	90.60	
	3	61.20	57.80	94.40	

Table 7.4
Accuracy: Epimesembranol (CZE)

Epimesembranol	Day	Actual Weight (µg/ml)	Calculated Weight (µg/ml)	% Accuracy	Inter-day % RSD
QC1	1	20.40	22.00	108.0	5.90
	2	20.80	19.80	95.20	
	3	20.40	21.80	101.90	
QC2	1	61.20	56.60	92.50	6.30
	2	62.40	57.40	92.00	
	3	61.20	63.10	103.10	

Table 7.5
Accuracy: Mesembrine HCl (CZE)

Mesembrine HCl	Day	Actual Weight (µg/ml)	Calculated Weight (µg/ml)	% Accuracy	Inter-day % RSD
QC 1	1	20.80	20.90	100.50	2.10
	2	20.00	20.90	104.50	
	3	19.20	19.50	101.60	
QC 2	1	62.40	57.70	92.50	3.50
	2	60.00	59.10	98.50	
	3	57.60	53.30	92.50	

Table 7.6
Accuracy: Mesembranol (CZE)

Mesembranol	Day	Actual Weight (µg/ml)	Calculated Weight (µg/ml)	% Accuracy	Inter-day % RSD
QC 1	1	20.40	19.30	94.60	2.30
	2	19.60	18.70	90.30	
	3	21.20	19.90	93.90	
QC 2	1	61.20	60.40	98.70	3.20
	2	58.80	55.00	92.30	
	3	63.30	60.10	94.90	

Table 7.7
Accuracy: Δ^7Mesembrenone (CZE)

Δ^7Mesembrenone	Day	Actual Weight (µg/ml)	Calculated Weight (µg/ml)	% Accuracy	Inter-day % RSD
QC 1	1	19.20	19.70	102.60	3.00
	2	18.40	17.80	96.70	
	3	17.60	17.70	99.80	
QC 2	1	57.60	55.20	95.80	6.60
	2	55.20	60.10	108.90	
	3	52.80	57.60	104.20	

7.6.7.5.3 Precision Studies on Tablet Formulation

The RSD for inter-day precision are shown in Table 7.8. The concentrations of the alkaloids, epimesembranol, mesembrine and mesembranol were determined in all three samples. However, whilst the presence of mesembrenone and Δ^7mesembrenone were evident in all three concentrations of the tablet samples, only the high sample preparation showed quantifiable amounts of mesembrenone whereas the content of Δ^7mesembrenone was below the LoQ in all three samples. The content of alkaloids presented as microgram per tablet, are shown in Table 7.9.

Table 7.8

Precision studies of *Sceletium* tablets (CZE)

Compound	Content in µg/ tablet (± SD)			n=3
Mesembrenone	**Day 1**	**Day 2**	**Day 3**	**Inter-day % RSD**
Low	—	—	—	—
Medium	—	—	—	—
High	11.93 (±1.85)	10.31 (±1.00)	11.84 (±1.84)	7.90
Epimesembranol				
Low	10.00 (±1.24)	9.30 (±1.10)	11.10 (±0.93)	8.50
Medium	9.34 (±1.26)	10.27 (±0.31)	10.12 (±1.50)	4.80
High	11.17 (±1.22)	12.12 (±1.56)	11.00 (±0.92)	5.10
Mesembrine				
Low	162.52 (±6.51)	162.13 (±5.65)	164.91 (±5.60)	0.90
Medium	169.80 (±0.13)	167.47 (±1.22)	162.15 (±3.21)	2.20
High	167.84 (±10.86)	164.00 (±12.20)	159.00 (±6.50)	2.70
Mesembranol				
Low	32.52 (±7.14)	37.50 (±1.55)	38.27 (±3.47)	8.70
Medium	38.46 (±1.37)	38.38 (±1.82)	36.20 (±1.81)	3.80
High	36.80 (±1.40)	37.50 (±1.10)	36.10 (±3.57)	1.90
Δ^7Mesembrenone	—	—	—	—
Low	—	—	—	—
Medium	—	—	—	—
High	—	—	—	—

Average weight of tablet = 497.3 mg, SD= Standard deviation, — = Below LoQ
Low = 25 mg/ml (n=3), Medium = 50 mg/ml (n=3), High = 75 mg/ml (n=3); Total samples n=9 each day

Table 7.9

Content of identified *Sceletium* alkaloids per tablet

Compound	Content in µg/tablet
Mesembrenone	11.3
Epimesembranol	10.5
Mesembrine	164.3
Mesembranol	36.9
Δ^7Mesembrenone	—

— = Below LoQ

7.6.7.5.4 Recovery Studies – Tablet Formulation

Since the mesembrine content was higher than the other alkaloids in these tablets, only the content of mesembrine was assessed. This was necessary since the lowest calibrator for all alkaloids was 2.5 μg/ml, the concentrations of the other 4 alkaloids in the sample preparation would be below the LoQ.

The values obtained resulted in recoveries between 91%-106% calculated as mesembrine base over the range of spiked concentrations used with RSDs of less than 9%. The results are shown in Table 7.10.

The recoveries from the spiked mesembrine HCl from the placebo tablet matrix samples yielded values ranging between 99%-113% with inter-day RSD of less than 6%. The results are tabulated in Table 7.11.

Table 7.10
Recovery studies of Mesembrine from tablet dosage form (CZE)

Sample	Day-1				Day-2				Day-3				Inter-day Recovery % RSD (n=3)
	Mesembrine (µg/ml added)/12 mg dosage/ml	Observed content (µg/12 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrine** (µg/ml added)/12 mg dosage/ml	Observed content (µg/12 mg dosage/ml)	% Recovery	% RSD (n=3)	**Mesembrine** (µg/ml added)/12 mg dosage/ml	Observed content (µg/12 mg dosage/ml)	% Recovery	% RSD (n=3)	
*Content	4.02				3.97				4.13				
Low	(+5.55)[1] 9.57[2]	9.25 (± 0.51)	96.66	4.90	(+5.44) 9.41	9.12 (± 0.37)	96.92	4.10	(+5.12) 9.25	9.12 (±0.40)	98.60	4.40	1.10
Medium	(+11.09) 15.11	14.46 (±1.04)	95.70	7.20	(+10.88) 14.85	15.43 (±1.30)	103.91	8.42	(+10.24) 14.37	15.20 (±1.01)	105.80	6.64	5.27
High	(+22.19) 26.21	25.02 (±1.36)	95.46	5.44	(+21.76) 25.73	25.30 (±0.90)	98.33	3.60	(+20.48) 24.61	22.60 (±0.81)	91.83	3.60	3.42

* Actual content[1]; spiked amount[2]; represents the total content, i.e. spiked plus original content (follows through the table), (±SD), Average weight = 497.30 mg/ tablet

Table 7.11
Recovery studies of Mesembrine from tablet matrix (CZE)

Tablet Matrix Sample	Day 1			Day 2			Day 3			
	Mesembrine content in matrix (µg/ml)	Observed content (µg/ml)	% Recovery	**Mesembrine** content in matrix (µg/ml)	Observed content (µg/ml)	% Recovery	**Mesembrine** content in matrix (µg/ml)	Observed content (µg/ml)	% Recovery	**Inter-day Recovery % RSD (n=3)**
Low	4.16	4.18	100.30	3.68	3.82	103.74	3.84	4.20	109.42	4.40
Medium	8.32	7.86	94.45	7.36	8.51	108.17	7.68	7.61	99.10	7.00
High	12.48	12.67	101.56	11.04	12.38	112.15	11.52	12.44	108.00	5.00

7.6.7.5.5 Limit of Detection and Limit of Quantitation

The LoD and LoQ for mesembrenone were found to be 1 μg/ml and 2 μg/ml respectively. The LoDs and LoQs for mesembrine, mesembranol and epimesembranol were found to be 1.5 μg/ml and 2.5 μg/ml, respectively whereas the values for Δ^7Mesembrenone were estimated at 2.5 and 3.5 μg/ml for the LoD and LoQ respectively.

7.7 CONCLUSIONS

CE methods have been conspicuously absent in the scientific literature. Hence, in view of the advantages and potential offered by CE, a method was developed and validated for application of CE to fingerprint the presence of alkaloids as well as for use as an assay method for the quantitative analysis of alkaloids in *Sceletium* products.

Due to the fact that the CE system involves the use of aqueous-based electrolytes and relatively cheaper uncoated fused silica capillaries for the separation compared to the more expensive HPLC columns, it is considered as a far more economical procedure, which is also easier to transfer between laboratories [85].

Since the identified markers have closely related structures, of which two alkaloids, mesembranol and epimesembranol were epimers, the higher resolution capability of CZE is an attractive incentive to use this method in efficiently separating the multi-component alkaloidal mix in *Sceletium* as well as in accomplishing this in relatively short analysis times. The sample preparations were relatively simple and involved a one step methanol extraction which provided reproducible results. Furthermore, the PDA detector was extremely valuable for peak identification and homogeneity testing during the initial method development.

In summary, a relatively simple CZE method was developed to identify 5 alkaloids and quantify mesembrine, an important *Sceletium* alkaloid. The method has been shown to have the necessary accuracy, precision and reproducibility for the rapid fingerprinting and quantitative analysis of mesembrine in *Sceletium* products.

These findings indicate that CE should be considered as an alternative and viable option for the fingerprinting and assay of phyto-pharmaceuticals, and as such, can be used as an important QC tool in the quest to determine the quality of complementary medicines.

CHAPTER 8

FERMENTATION STUDIES OF *SCELETIUM* PLANTS

8.1 INTRODUCTION

Phytochemical constituents in plants are reported to vary due to differences in growth conditions, mode of harvesting, time of harvesting, drying and storing the harvested material [86]. There are studies that have shown variations between plants grown in a field and greenhouse [87]. The geographical influence on chemical components in cultivated plants has also been reported [51]. For example, *Sceletium* species cultivated in Germany did not form alkaloids whereas those cultivated in the USA contained these components [88]. Such variations have important implications for the content and consistency of phyto-pharmaceutical products. In some cases, where plants are reported to be processed in a specific manner, for example, fermentation before production of products, further variations may result.

Sceletium plants and their products have been purported to improve mood and decrease anxiety, especially when the plant material has been fermented and either chewed or smoked. The fermentation process in which whole plant material is crushed and then placed in sealed containers for several days and dried under the sun is purported to improve the potency and efficacy of the preparation. Fermentation has been highlighted as one of the important treatments of *Sceletium* [51] and this process has been used for advertising increased potency of such products which are marketed as dried and fermented plant powder and used in preparations of commercial phyto-pharmaceutical dosage forms containing *Sceletium*.

Smith *et al.* [51] in their review published in 1996 detailed the fermentation technique based on information obtained from a traditional user of *Sceletium*. The process involved crushing the plant material using stones and allowing the crushed plant material to remain in a bag for about 2-3 days after which the bag is opened, mixed and closed again. Fermentation is then continued for 8 days and then the contents are removed from the bag and spread out to dry in sun. It was emphasized that if the described process is not followed, the product would not be effective for its indicated mood elevating effects.

Smith *et al.* in 1998 [55], reported that the constituents were of higher concentration only after fermentation. It has been suggested that the crushing process, prior to anaerobic fermentation,

introduces oxalate-degrading microbes into the skin or plastic bag thereby eliminating the potential toxic effects of oxalic acid, which is removed by this traditional 'fermentation' process while retaining alkaloids. In their work, they described that *kougoed*, prepared from fermenting *Sceletium tortuosum*, was screened for the presence of the mesembrine alkaloids using gas chromatography (GC) with a NPD and based on their mass spectra, three alkaloids, 4'-O-demethylmesembrenol, mesembrine and mesembrenone were identified [55]. The levels, as well as the ratios of the three alkaloids were reported to have changed remarkably. Substantial increases in total alkaloid levels were observed when the *Sceletium* material was crushed, bruised and extracted prior to drying whereas no such changes occurred when intact plants were oven dried at 80°C and then extracted. *Kougoed*, when prepared by the contemporary plastic bag method has been reported to contain the mesembrine alkaloids at levels and ratios substantially different from those of unfermented material. In addition, a marked decline was seen in the content of 4'-*O*-demethylmesembrenol and mesembrine levels, whilst the content of mesembrenone increased significantly. When fresh plant material of *Sceletium* was crushed and immediately dried at 80°C, the chromatographic profile was observed to be similar to that of a fermented sample [55].

It has been suggested that an enzymatic reaction may occur during the process of bruising the plants and that these reactions may explain the changes in alkaloid ratio and content. Also, the temperature (80°C) probably influences the changes in alkaloidal content. The report further mentioned a second experiment which was carried out by suspending the crushed plants in liquid nitrogen in order to assess the enzymatic influence. When the frozen plant material was re-suspended in water, the enzymatic activity resumed but was eliminated by boiling in ethanol. The authors thus suggested that the essential step in the production of *kougoed* may therefore not entirely be due to "fermentation" but that crushing the plant material and consequent mixing of cellular material may also be equally necessary. Based on these results, it was suggested that instead of performing a "traditional" fermentation, simply crushing and drying at 80°C may be a quick alternative method to modify the alkaloid content. A further traditional method of preparation of *Sceletium* involves heating crushed material under a fire which is also a rapid method of preparation purported to provide the same or similar changes in alkaloidal content. However, the alkaloidal content following this particular process has not been reported. Hence, it appears that such treatments have a rational pharmacological basis and have evolved over many generations through trial-and-error experimentation by indigenous people of southern Africa [55].

There are many websites on the internet which discuss the process of fermentation and make claims that products subjected to a fermentation process result in enhanced effects. The website described below suggests a 'Do it yourself' fermentation procedure (Figure 8.1a).

Figure 8.1a: Website on preparation of 'Kanna' from Sceletium
http://www.herbalistics.com.au/shop/product_info.php?products_id=139 , date assessed 18-8-2006

The website further describes the step-wise process, the containers used in fermentation process and the yields that will be obtained by this process (Figure 8.1b).

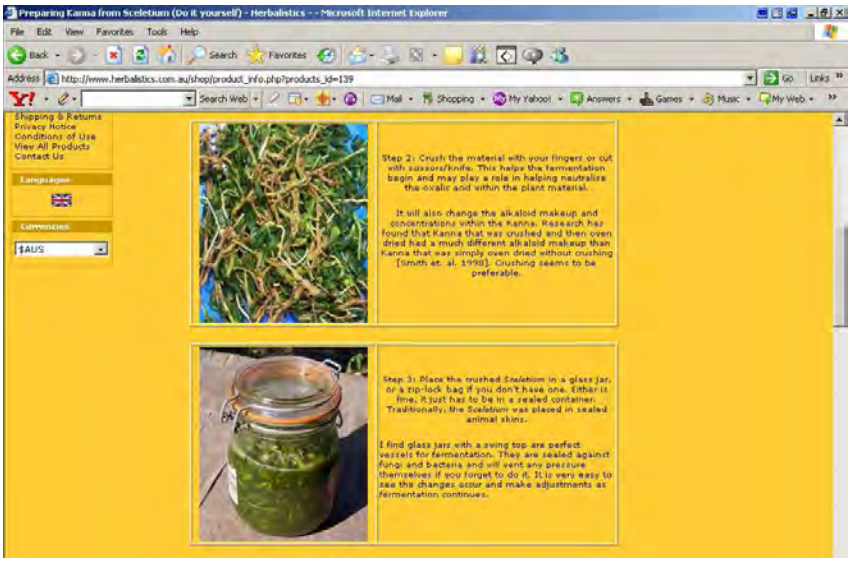


Figure 8.1b: Website on preparation of 'Kanna' from Sceletium showing fermentation of a sample.
http://www.herbalistics.com.au/shop/product_info.php?products_id=139, date assessed 18-8-2006

8.2 STUDY OBJECTIVE

The main objective of this study was to carry out a fermentation process under controlled conditions and to monitor the change in alkaloidal content using a quantitative HPLC/PDA method and also the LCMS method for their identification.

8.3 EXPERIMENTAL

8.3.1 Reagents and Materials

All reagents and materials used have previously been described and have been detailed in Chapters 4 and 5. *Sceletium* reference compounds and plant material and its products have been described and detailed in Chapters 2 and 3, respectively.

8.3.2 Instrumentation

The HPLC and LCMS systems and equipment used have previously been described in Chapters 4 and 5. A Hot air oven Model FSIE and a low temperature incubator, Model L.T.I.E (Labcon (Pty) Limited, Krugersdorp, South Africa) were used to dry the samples and for the temperature controlled studies respectively.

8.3.3 Preparation of Standard Solutions and Samples

Standard methanolic stock solutions (1mg/ml) of Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine hydrochloride and epimesembranol were prepared. A working stock solution was prepared and diluted to obtain a concentration of 100 µg/ml of each alkaloid. Standard solutions comprising a set of nine calibrators in the concentration range of 400-30,000 ng/ml were prepared for HPLC-UV analysis.

The *Sceletium tortuosum* plant (SP04, GRA) was used for this study. Five grams of crushed plant material was extracted twice using 2 portions of 20 ml methanol which were combined and made up to 50 ml with methanol. The solution was filtered and 1.0 ml was diluted to 10 ml and an aliquot of 10 µl was injected into the HPLC.

8.3.4 Methods

Two separate fermentation studies were carried out. The first study was performed during December 2005 and January 2006. The samples for fermentation were made from the aerial plant parts of *Sceletium tortuosum* (75 g) which was transferred into a polythene bag (Figure 8.2) and carefully hand crushed using fingers, which yielded a watery plant mass (Figure 8.3). The second study was carried out in a similar manner using ~ 130 g of the same plant's arial parts during November 2006. The study periods were chosen during November-January to coincide with the summer season and hot days, since the natural habitat of *Sceletium* is in the hot and arid Karoo regions of South Africa.

Figure 8.2: Arial parts of S. tortuosum collected into polythene bag

Figure 8.3: Crushed plant material for fermentation

The initial analysis for alkaloid content was performed on the wet mass, sampled on Day 1 immediately after crushing and rest of the material was left to ferment under sunlight during the day and remained in place through the night. Subsequent samples were collected on each day at the 24^{th} hour for a period of 10 days and analyzed by HPLC.

In order to reproduce the results obtained by Smith *et al.* [55], one plant sample was separately crushed and dried at 80°C for 5 hours to investigate whether drying the crushed plant at 80°C would provide similar results to the fermented sample which showed an increase in mesembrenone content.

The second study was carried out on similar lines of the first study for 14 days. However, this analysis included analysis by LCMS as well as HPLC.

A parallel study was designed to study the transformation of the alkaloids mesembrine and Δ^7mesembrenone. Standard mesembrine hydrochloride was prepared in water to obtain a solution of 0.5 mg/ml. The sample was exposed to the same fermentation conditions used for the plant samples. The solution was sampled on each day at the same times of the plant sample preparation and analyzed under the same conditions.

A further study was designed to study the effect of light and temperature on different solutions of mesembrine hydrochloride, methanol and water respectively. The samples were divided into two sets which were protected from light. One set was maintained at 40°C in a low temperature incubator and the other at ambient temperature (~ 22°C).

8.3.5 Observations and Results

8.3.5.1 Sceletium Plant Fermentation Studies

The first study showed interesting transformation of two main alkaloids. It was found that for mesembrine and Δ^7mesembrenone, the content of the former decreased whilst the latter increased. A previous study by Smith *et al* reported the transformation of mesembrine and a non-specified mesembrenone and showed similar trends [55].

The first sample on Day 1, analyzed immediately after crushing, showed a concentration of 1.33% mesembrine and the presence of Δ^7mesembrenone which was confirmed by PDA analysis, albeit at very low detection levels (< LoQ) (Figure 8.4). However, the crushed sample which had been dried at 80°C showed no significant change in mesembrine content (1.12%) to the initial content found on Day 1 and the Δ^7 mesembrenone content was still below the LoQ (Figure 8.5) in contrast to the results reported by *Smith et al.* [55] who found high concentrations of mesembrenone under similar conditions.

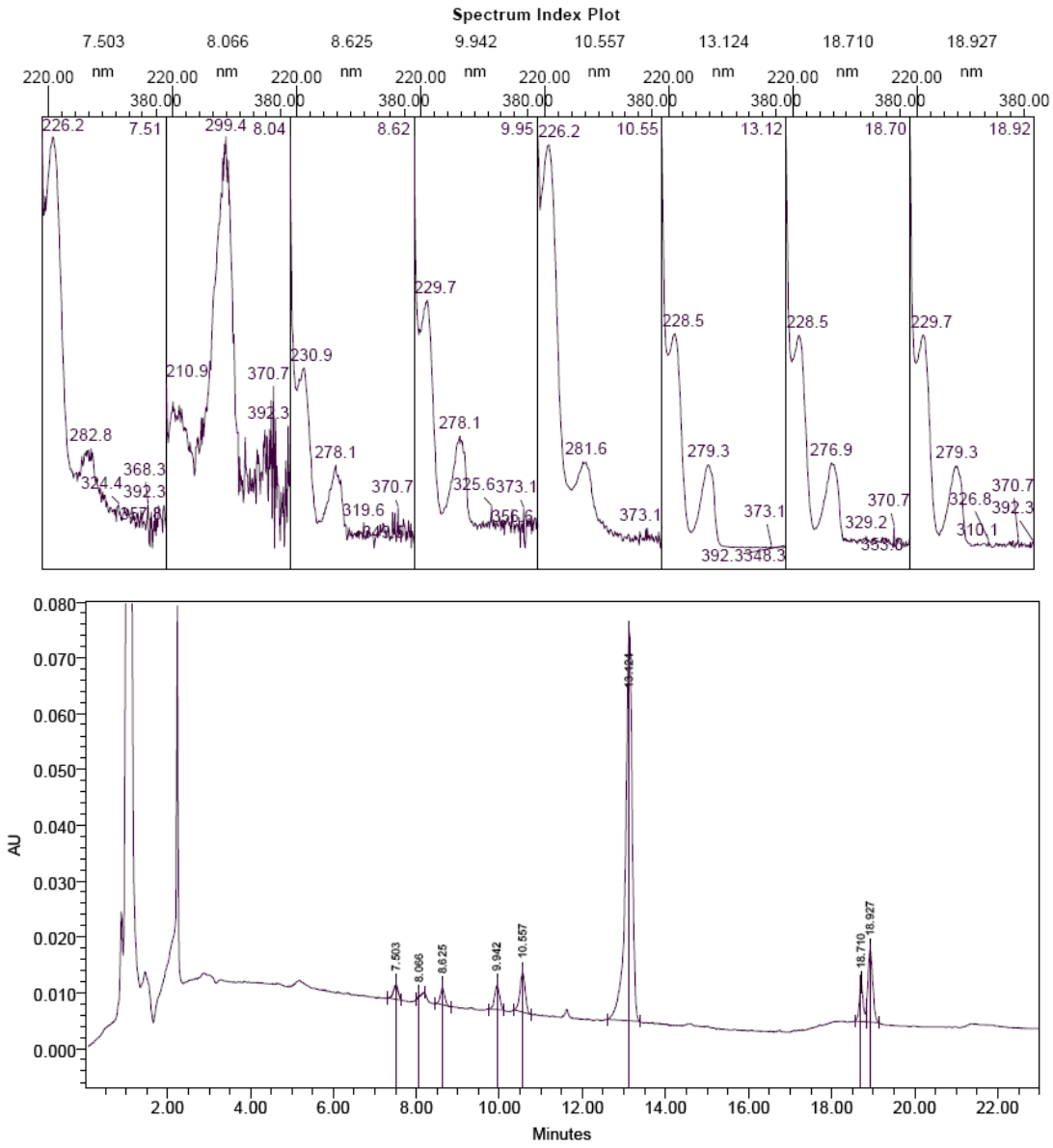


Figure 8.4: Initial crushed plant material on Day 1 (Study 1). Top segment - PDA-UV scan of the relevant alkaloids. Bottom segment - HPLC chromatogram - Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol at 8.066, 9.942, 10.557, 13.12 and 18.97 minutes respectively

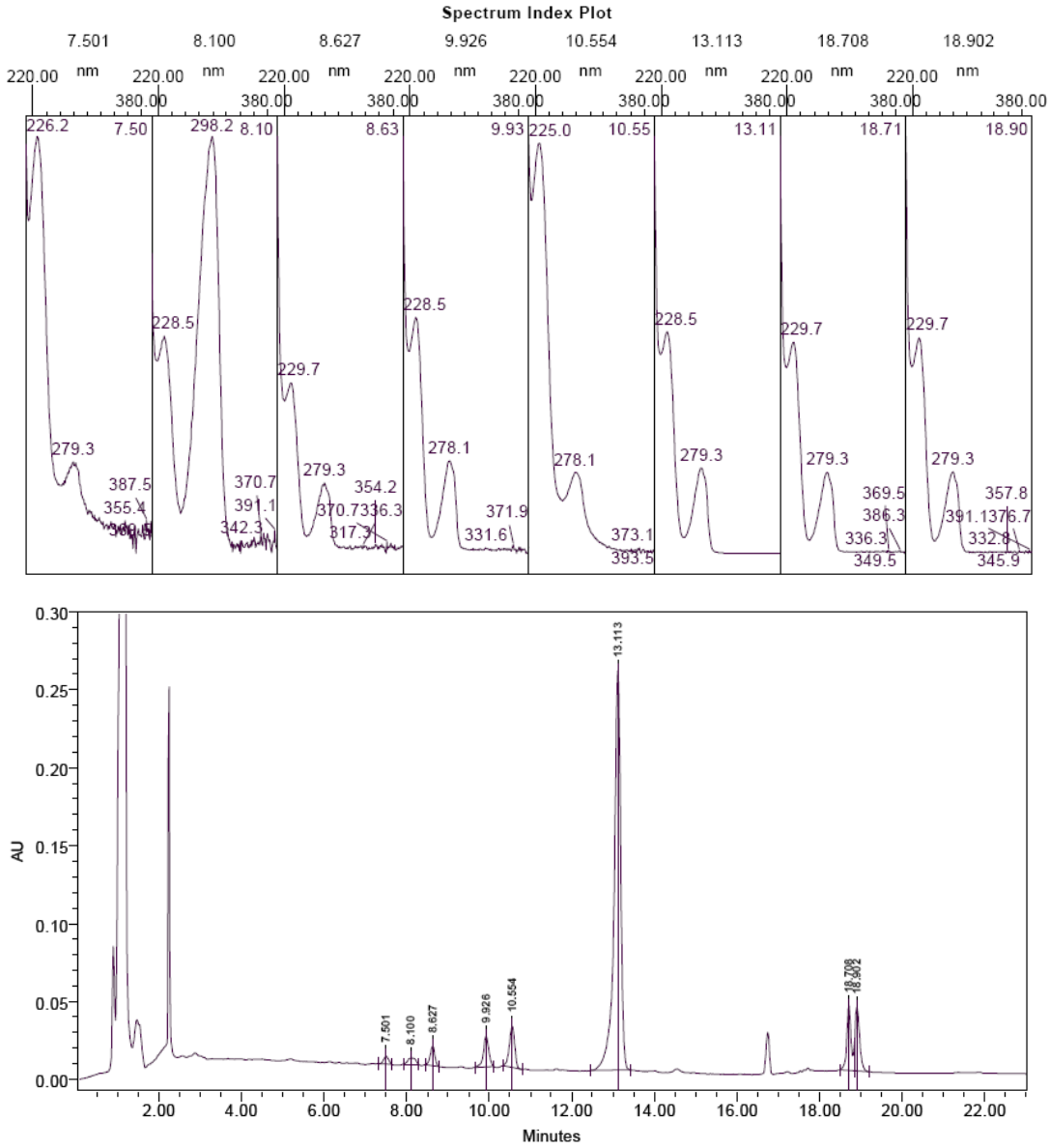


Figure 8.5: Crushed plant material on Day 1 - sample dried at 80°C (Study 1). Top segment PDA-UV scan. Bottom segment HPLC chromatogram - Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol at 8.100, 9.926, 10.554, 13.113 and 18.902 minutes respectively

The sample on Day 5 showed concentrations of Δ^7mesembrenone, now >LoQ, of 0.07% with the mesembrine content decreased to 0.68% (Figure 8.6).

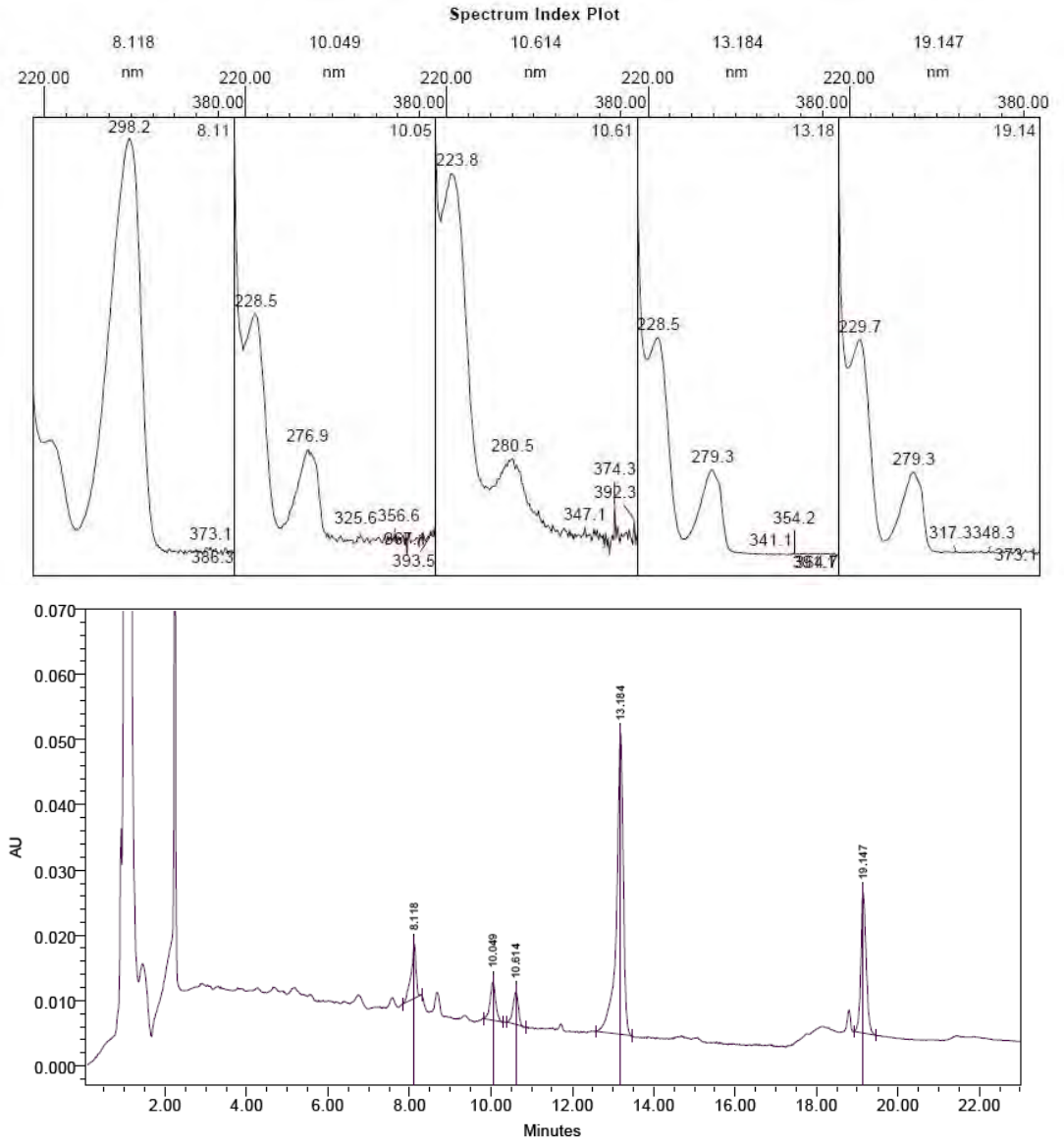


Figure 8.6: Crushed plant material on Day 5 sample (Study 1). Top segment - PDA-UV scan. Bottom segment - HPLC chromatogram - Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol at 8.118, 10.049, 10.614, 13.184 and 19.147 minutes respectively

During the course of the study, the mesembrine content showed a steady decline from an initial 1.33% to 0.05% on the 10th day. On the other hand, the content of Δ^7mesembrenone was found to increase from below the LoQ on days 1 to 4 and 0.11% on the 10th day (Figure 8.7).

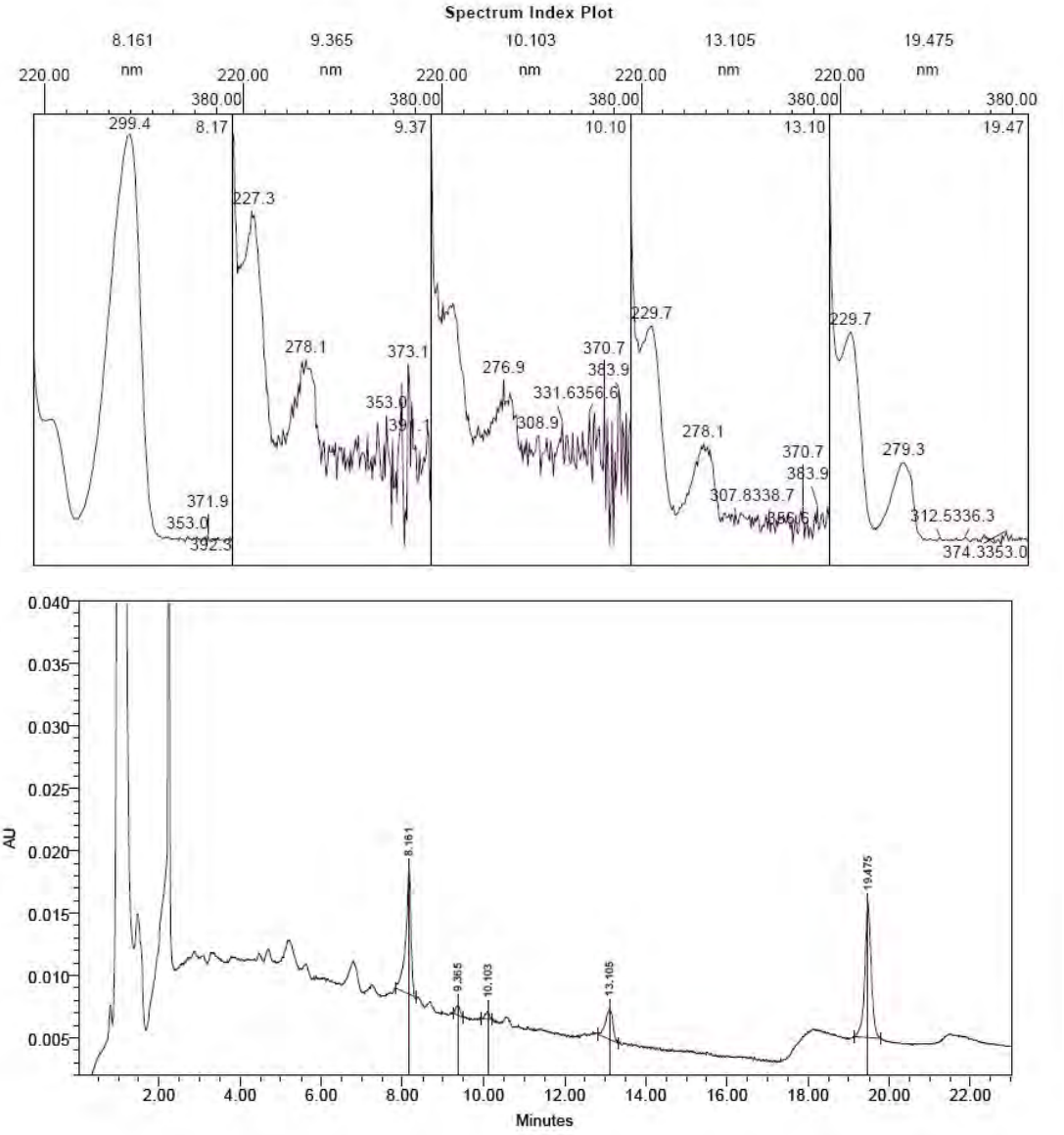


Figure 8.7: Crushed plant material on Day 10 sample (Study 1). Top segment - PDA-UV scan. Bottom segment - HPLC chromatogram - Δ^7mesembrenone, mesembranol, mesembrenone, mesembrine and epimesembranol at 8.161, 9.365, 10.103, 13.105 and 19.475 minutes

The graphical representation of the mesembrine and Δ^7mesembrenone content is shown in (Figure 8.8). It was also observed that no significant change in content of mesembranol, mesembrenone and epimesembranol occurred during the entire fermentation process (content of mesembranol, mesembrenone and epimesembranol were found to be reasonably constant at ~0.14%, ~0.15% and ~0.4%, respectively).

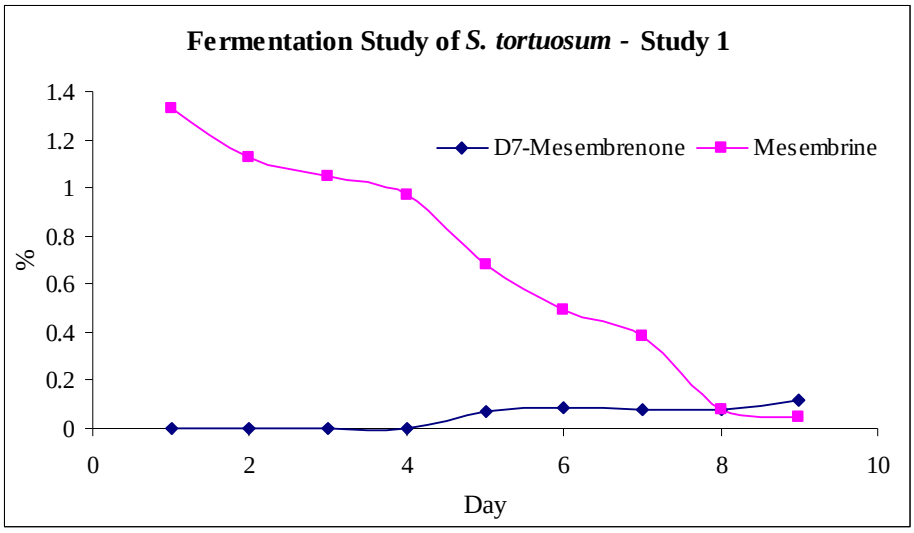


Figure 8.8: Fermentation of Sceletium tortuosum - Study 1

The second study was carried out for 14 days and also showed a decrease in mesembrine content with a concurrent increase in Δ^7mesembrenone. However, the tranformations were slower compared to the first study. The initial mesembrine content for the Day 1 sample was found to be 2.2% with mesembrine content decreasing to 0.8% by Day 14. Whilst the Δ^7Mesembrenone content was found to be below the LoQ from days 1-5, a value above the LoQ of 0.06% was subsequently determined and which increased to 0.18% on Day 14. The graphical representation of the percentage content is shown in (Figure 8.9).

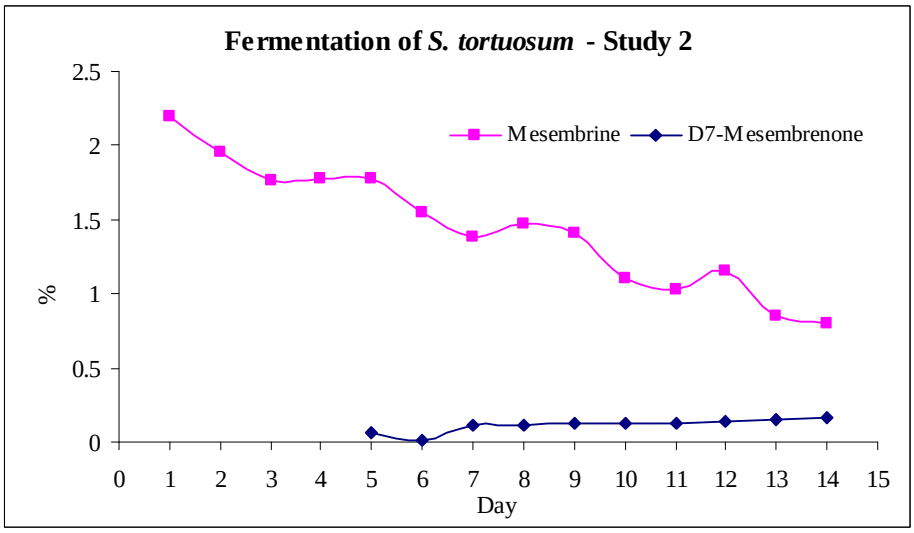
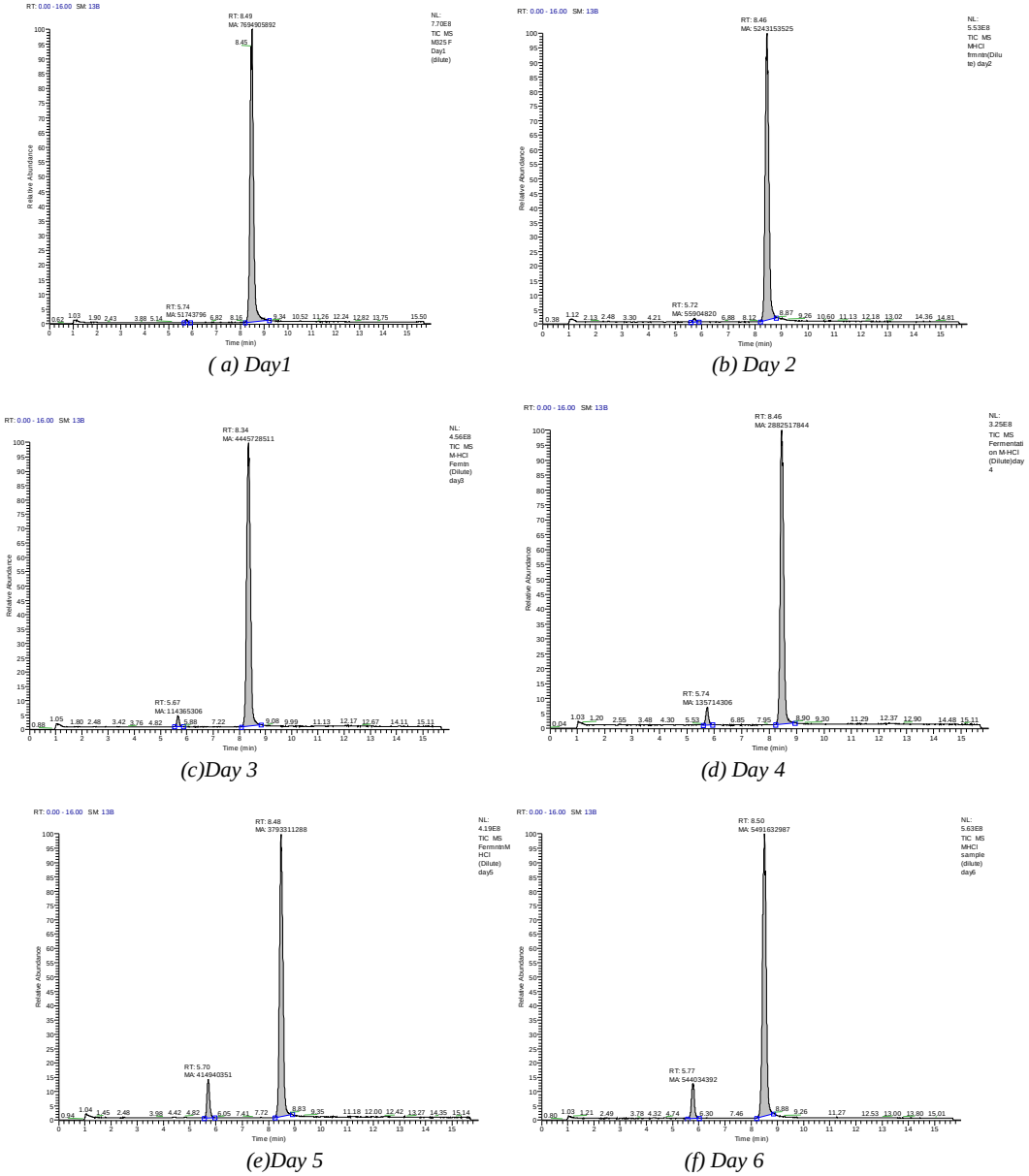


Figure 8.9: Fermentation of Sceletium tortuosum – Study 2

8.3.5.2 Mesembrine Hydrochloride Studies

Since the results obtained from the plant studies showed transformation of mesembrine and Δ^7mesembrine, it was important to study pure compound, mesembrine hydrochloride in water under similar conditions of exposure to that used for plant fermentation. The compound showed a gradual transformation to Δ^7mesembrenone over a period of 14 days (Figure 8.10a–n). On day 14, only 35% mesembrine remained whereas 65% Δ^7mesembrenone was now found in the solution (Figure 8.10n). Interestingly, no alkaloids were found in the same solution when tested after 20 days (Figure 8.10o).

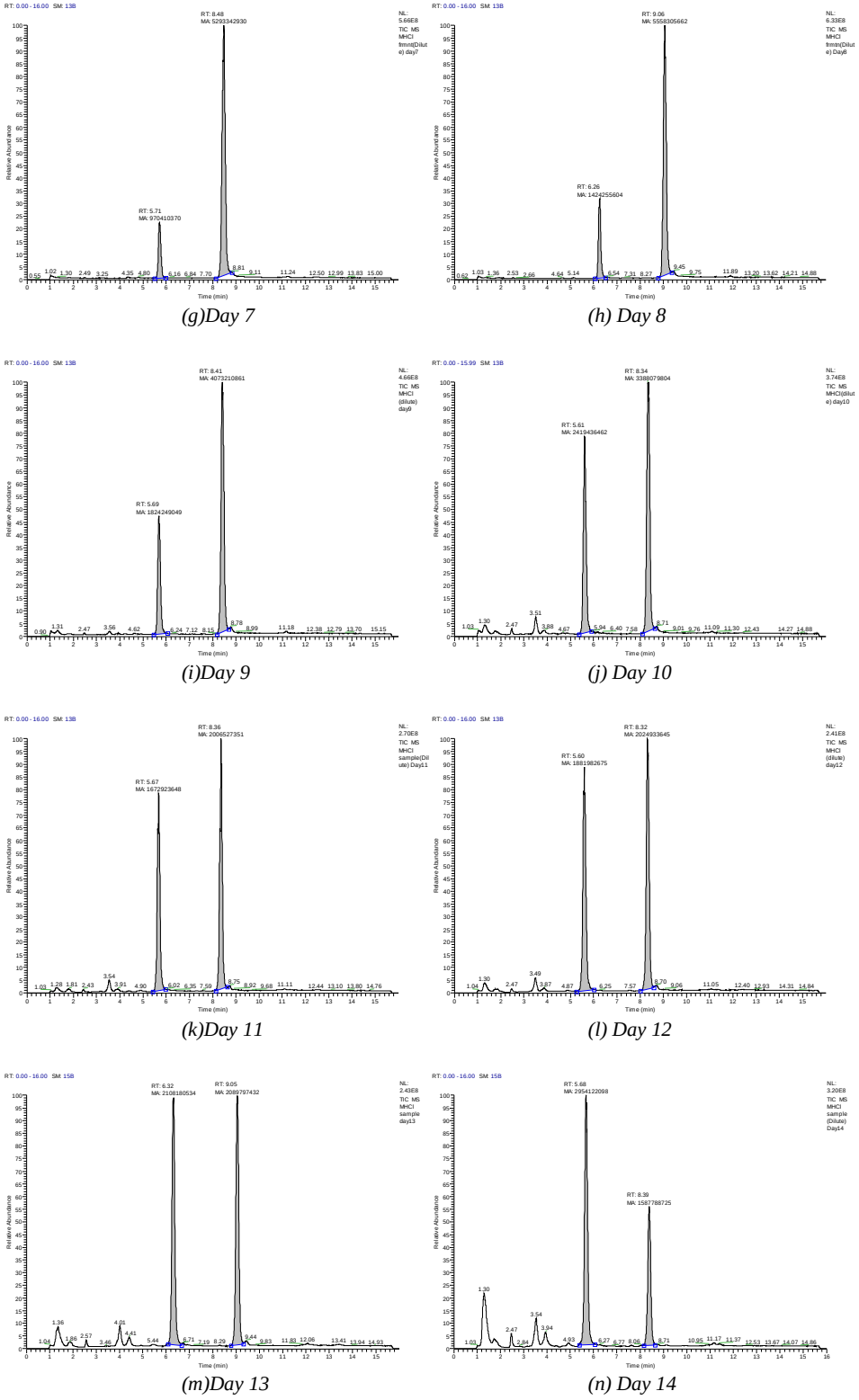


(a) Day1 *(b) Day 2*

(c)Day 3 *(d) Day 4*

(e)Day 5 *(f) Day 6*

Figure 8.10a–f: LCMS-TIC chromatograms of mesembrine HCl transformation to Δ^7mesembrenone- Day 1 to 6



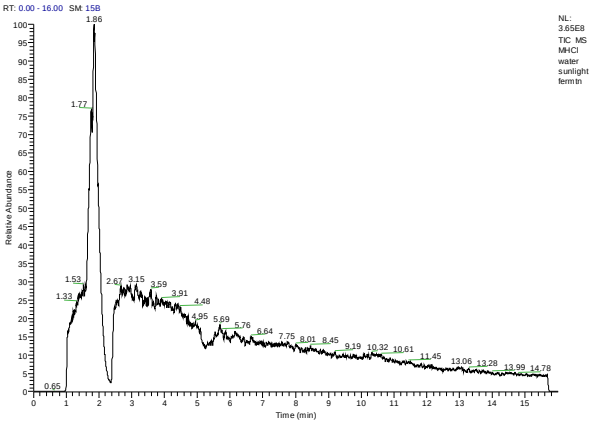
(g)Day 7 *(h) Day 8*

(i)Day 9 *(j) Day 10*

(k)Day 11 *(l) Day 12*

(m)Day 13 *(n) Day 14*

Figure 8.10g–n: LCMS-TIC chromatograms of mesembrine HCl transformation to Δ^7mesembrenone- Day 7 to 14



(o) Day 20

Figure 8.10o: LCMS-TIC chromatograms of mesembrine HCl transformation to Δ^7mesembrenone- Day 20

In contrast, the sample in methanol showed no such transformation. It thus seems that the alkaloids appear to be stabilized in the presence of methanol (Figure 8.11) whereas the same alkaloids in aqueous solutions appear to be influenced by temperature and light which resulted in their respective transformations.

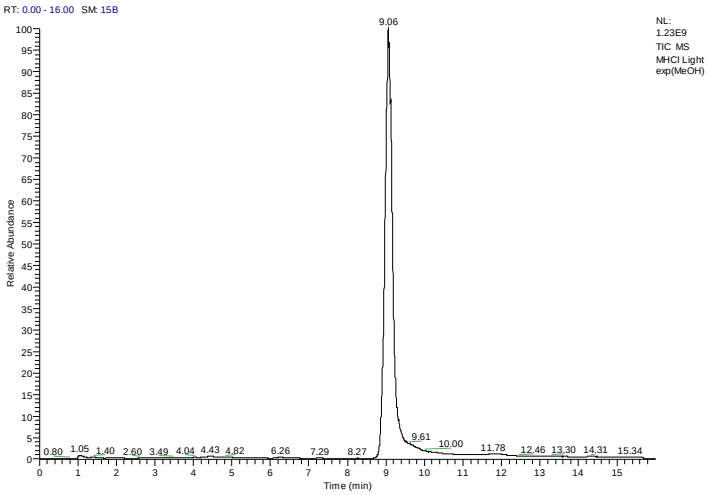


Figure 8.11: LCMS-TIC chromatogram of mesembrine HCl in methanol exposed to sunlight

In order to confirm the influence of light and temperature, a study was conducted on samples in water under light protected conditions carried out at room temperature (22 ± 2°C) (Figure 8.12) and at 40°C (Figure 8.13) where no change in mesembrine occurred. This study thus confirmed the influence of light and temperature on the stability of mesembrine in aqueous solution.

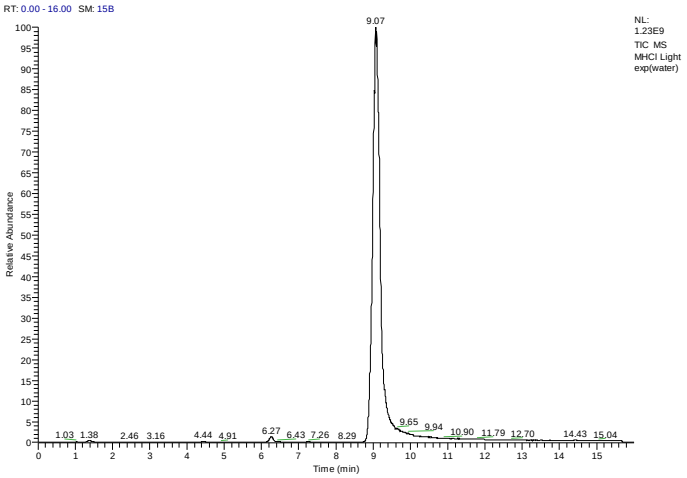


Figure 8.12: LCMS-TIC chromatogram of mesembrine HCl in water protected from sunlight at room temperature

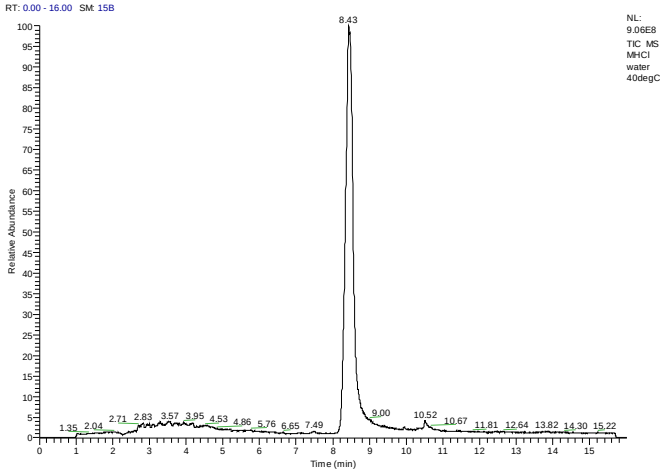


Figure 8.13: LCMS-TIC chromatogram of mesembrine HCl in water protected from sunlight maintained at 40°C

8.4 CONCLUSIONS

In summary, the studies show that the fermentation process transforms mesembrine to Δ^7mesembrenone and requires an aqueous environment together with the presence of light to facilitate such a change. The HPLC and LCMS methods were employed to monitor the respective alkaloids and both fermentation studies showed reproducible results. These studies indicate that if mesembrine is the alkaloid that is purported to cause the claimed biological activity/pharmacological effect, then the claims of more effective material due to fermentation are questionable. Furthermore, the suggested enzymatic activity during fermentation of *Sceletium* as reported by Smith *et al* [55] needs to be further investigated. Such a study could involve the addition of a specific enzyme inhibitor during the fermentation process and subsequent monitoring of the content of mesembrine and Δ^7mesembrenone.

CHAPTER 9

CONCLUDING REMARKS

The identified alkaloids in *Sceletium,* mesembrine, mesembrenone, Δ^7mesembrenone, mesembranol and epimesembranol *were found to be* present in varying amounts in both raw material and its dosage forms. This underlined a number of unique issues with respect to the quality and related purported therapeutic efficacy of *Sceletium*. Since these particular alkaloids are not commercially available, procedures are required for their extraction and purification from plant material for use as markers in order to develop analytical methods for assay and QC of the plant material and dosage forms.

The need for qualified reference substances is of paramount importance in the development and validation of analytical methods. The alkaloids, mesembrine, mesembrenone and Δ^7mesembrenone, were isolated and purified whereas mesembranol and epimesembranol were synthesized. All these compounds were characterized by various spectral methods and were subsequently used as markers for the development and validation of analytical methods for the assay and QC of *Sceletium* plant material and pharmaceutical dosage forms.

A relatively simple HPLC method was developed and validated and which conformed to all parameters of analytical method validation. The method and the markers were used to identify and quantify several *Sceletium* alkaloids. Furthermore, this method was found to efficiently separate the alkaloidal markers from complex components present in plant material as well as from excipients in the tablet dosage forms using a simple one step methanol extraction procedure. The method was successfully applied for the assay and QC of *Sceletium* plant material and its dosage forms.

The application of LCMS for the analysis of *Sceletium* plant material and dosage forms for several of the alkaloidal components was successfully developed and validated. The efficiency of the LCMS method was enhanced by the HPLC separation which resolved the closely related alkaloidal compounds present in *Sceletium*. The soft ionization method was found to be selective and sensitive for *Sceletium* alkaloids. The specificity provided by the LCMS method resulted in unequivocal identification of the alkaloids based on their *m/z* values and can readily be applied for

the QC of those compounds and products. This method was successfully used for the chemo-taxonomy of some species and available dosage forms of *Sceletium*.

Application of CE is an exciting prospect for high efficiency separation of multi-component systems. The use of aqueous-based electrolytes and relatively cheaper uncoated fused silica capillaries makes it more economical compared to systems that require the use of organic solvents. The CZE method was validated and applied for fingerprinting the relevant alkaloids and also as an assay method for the quantitative analysis of mesembrine in *Sceletium* products. These findings indicate that CZE should be considered as an alternative and viable option for fingerprinting and QC of phyto-pharmaceuticals.

The application of the HPLC and LCMS methods provided valuable insight into the fermentation process. The transformation of mesembrine to Δ^7mesembrenone was monitored under controlled conditions with reproducible results. The study confirmed that mesembrine in aqueous solution under the influence of sunlight is unstable. It was found that an aqueous environment together with the presence of light may facilitate this transformation during the fermentation process.

The quality of herbal medicines is presently a major concern worldwide. Herbal preparations are generally consumed as non-prescription and over the counter (OTC) products. Selective analytical methods and monographs have to be developed for the standardization of herbal products due to inherent variations of the constituents in their source plants. During the course of this project, it was observed that there is an increased interest in *Sceletium* cultivation for the herbal product market. The cultivators of these herbal plants have contributed much to this study and have shown a keen interest in improving the quality of their products.

This study has provided the relevant analytical markers, validated analytical methods, and equally important, the necessary technical support for cultivators to identify the correct species and an insight into the chemical constituents of *Sceletium*. Thus, the objective of applying pharmaceutical analysis for the assay and QC of *Sceletium* and its products has been successfully achieved.

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