CHEMICAL ANALYSIS OF MEDICINAL AND POISONOUS PLANTS OF FORENSIC IMPORTANCE IN SOUTH AFRICA

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TO GOD ALL THE GLORY

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ABBREVIATIONS

AAFS	:	American Association of Forensic Sciences
ADP	:	Adenosine diphosphate
amu	:	Atomic mass unit
APCI	:	Atmospheric pressure chemical ionisation
API	:	Atmospheric pressure ionisation
ARC	:	Agricultural Research Council
ATR	:	Atractyloside (referring to the ionic species in solution)
BPC	:	Bonded phase chromatography
BPI	:	Base peak intensity
CATR	:	Carboxyatractyloside
CHEX	:	Cyclohexane
CID or ISCID	:	Collision induced dissociation (also called In-source collision induced dissociation)
Da	:	Dalton
DEA	:	Dietylamine
EI	:	Electron impact ionisation
EMIT	:	Enzyme multiplied immunoassay technique
ESI	:	Electrospray ionisation
FCL JHB	:	Forensic Chemistry Laboratory Johannesburg
FIA	:	Flow injection analysis
FPIA	:	Fluorescent polarisation immunoassay
FTIR	:	Fourier transform infrared
GC	:	Gas chromatography (similar to GLC)
GC-EI-MS	:	Gas chromatography coupled to an electron impact mass selective detector
GC-MS	:	Gas chromatography coupled to mass spectrometry
GLC	:	Gas-liquid chromatography
GMP	:	Good manufacturing practice
GP	:	Gauteng Province
gpg	:	guinea pig
H or HETP	:	The height equivalent to a theoretical plate
HPLC	:	High performance liquid chromatography
HPLC-MS	:	High performance liquid chromatography coupled to mass spectrometry
I	:	Intensity
ID	:	Inner diameter
IP	:	Intraperitoneal
KATR	:	Atractyloside (referring to the salt containing two potassium ions)

LC	:	Liquid chromatography
LD_{50}	:	Lethal dose (where 50% of a population would die after administration of the dose)
LOD	:	Limit of detection
LOQ	:	Limit of quantification
muti	:	Traditional medicine derived from botanical/animal origin but may also contain pharmaceutical and/or industrial chemicals
N	:	Number of theoretical plates
NMR	:	Nuclear magnetic resonance
NWP	:	North-West Province
orl	:	oral
PBS	:	Phosphate-buffered saline solution
PDA	:	Photodiode array
PEEK	:	Polyetheretherketone
R	:	Resolution
RAU	:	Rand Afrikaans University
RIA	:	Radioimmunoassay
RSA	:	Republic of South Africa
RSD	:	Relative standard
R_t	:	Retention time (usually in minutes)
SAPS	:	South African Police Service
scu	:	subcutaneous
SIM	:	Single ion monitoring [similar to SIR (Single ion reaction)]
SOFT	:	Society of Forensic Toxicologists
SPE	:	Solid phase extraction
TIAFT	:	The International Association of Forensic Toxicologists
TIC	:	Total ion chromatogram
TLC	:	Thin-layer chromatography
TMD	:	Waters Thermabeam electron impact mass spectrometer
TOF	:	Time of flight
UJHB	:	University of Johannesburg
UV	:	Ultraviolet
V	:	Volt
WADA	:	World Anti-Doping Agency
ZMD	:	Z-Spray mass selective detector (1^{st} Generation)
ZQ	:	Z-Spray mass selective detector (3^{rd} Generation)

SUMMARY

The Forensic Chemistry Laboratory of Johannesburg (FCL JHB) is tasked with the chemical analysis of a variety of samples to assist in determining the cause of death where unnatural cause is suspected. Some of the samples submitted to the laboratory have a herbal or *muti* connotation, but a large portion of these cases turn out to have no herbal components present as only pharmaceutical or agricultural products are detected in these samples. This study combined, for the first time, forensic investigation, chemistry and botany to create a unique platform needed for the identification of poisonous plants and their components in forensic exhibits and viscera. The research was focussed on the poisonous plants previously detected at the laboratory, as well as the requests received for the analysis of *muti*/toxic plant components. The selection of plants included *Nicotiana glauca, Datura stramonium / Datura ferox*, *Callilepis laureola, Boophone disticha / Ammocharis coranica, Abrus precatorius, Ricinus communis, Nerium oleander / Thevetia peruviana* and *Bowiea volubilis.* All these species are known to have caused fatalities, hence their choice.

Nicotiana glauca has been implicated in the deaths of at least 15 people since 2001. It was previously detected by GC-MS (EI) in plant exhibits, but could not be detected in a viscera matrix. A selective extraction method for alkaloids was used to extract botanical and viscera samples. Anabasine was successfully detected on the HPLC-MS (EI) system but this detection technique was not considered sensitive enough. A very sensitive HPLC-MS method was developed on the ZMD detector by using electrospray technology. This method outperformed both electron impact detectors (GC and HPLC) and could detect 1ng/ml anabasine with relative ease in full scan mode.

Datura stramonium and *D. ferox* have not been previously positively linked to any human poisoning or death due to exposure to botanically derived products at the FCL JHB. Atropine and scopolamine were successfully ionised in ESI positive mode and could be detected at 10 pg/ml and 100 pg/ml level respectively. The identities of the compounds were confirmed by characteristic ISCID fragmentation patterns. The developed method was successfully applied to a suspected heart attack case. The results proved conclusively that the deceased was given *D. ferox* seeds as part of his meal and an overdose of atropine and scopolamine contributed to his death.

Callilepis laureola is reputed to be one of the more commonly used medicinal plants in South Africa, and although its use has been indicated by the specific mention of a possible nephrotoxin and/or hepatotoxin as causative agent, it has not been detected in any of the forensic chemistry laboratories in South Africa. This was mainly due to the absence of a reliable method for the

analysis of the main toxic component of *C. laureola*, atractyloside, by mass spectrometry. A sensitive and very selective HPLC-ESI-MS method was developed that could detect atractyloside, carboxyatractyloside and their monodesulfated analogues in botanical and viscera matrices. The method was successfully applied to a variety of forensic samples and proved that *C. laureola* may play an important role in herbal poisonings. In a selection of suspected herbal poisonings where the cause of poisoning was unknown, 30% of the samples tested positive for the presence of atractyloside, carboxyatractyloside or their monodesulfated analogues.

The bulbs of *Boophone disticha* are rich in isoquinoline alkaloids and some of the alkaloids were detected by GC-EI-MS and LC-EI-MS, but the detection of these alkaloids in viscera samples was not successful. A routine method used for the screening for drugs of abuse in forensic samples, were successfully used for the analysis of the bulb extracts of *B. disticha* and the bulb scales of *A. coranica*. The chromatographic profile of these two plants appeared very similar at a first glance, but a closer evaluation of the mass spectra highlighted significant differences between the two plants. Six alkaloids from *B. disticha* were isolated and characterised by LC-MS and NMR and these compounds were detected in suspected herbal poisoning cases. It has been shown that *B. disticha* is one of the commonly used plants to “clean the system” but frequently results in the death of the patient.

Abrus precatorius contains one of the most toxic compounds known to mankind, namely abrin that collectively refers to a group of glycoproteins. The seeds of *A. precatorius* also contain two indole alkaloids, abrine and hypaphorine. The two alkaloids were fractionated and characterised by LC-MS and NMR. Due to the fact that the instrumentation of the FCL JHB is not suited to the detection of proteins, an LC-ESI-MS method was developed for the detection of the two alkaloids in plant and viscera matrix as markers for *A. precatorius*. The presence of these two alkaloids was indicated on the TMD system (EI spectra) in a suspected herbal poisoning case. The LC-ESI-MS method was applied to the analysis of the samples and the absence of abrine and hypaphorine were proven in the samples.

Ricinus communis is similar to *A. precatorius* in that it also contains a group of extremely poisonous glycoproteins, collectively refered to as ricin. The analysis of *R. communis* seeds encountered the same problems as the analysis of *A. precatorius* seeds, and the analysis was again focused on the detection of the minor piperidine alkaloid ricinine. The LC-ESI-MS method developed for abrine was modified to detect ricinine and functioned well in botanical and viscera matrices. This method will enable the forensic analyst to detect ricinine in very low levels when the presence of ricinoleic acid in samples indicates the use of a *R. communis*-based product.

Nerium oleander is a common decorative garden plant that is used medicinally. The plant is rich in cardenolides with oleandrin the main compound. A reversed-phase chromatographic method with ESI mass spectral detection was developed to separate and detect 11 cardiac glycosides. The compounds were adequately separated to allow unambiguous identification, and displayed very stable cationisation with sodium. An extraction method was developed to extract the cardiac glycosides from the leaves of *N. oleander* and *Thevetia peruviana* and was also evaluated in a viscera matrix. The extraction method functioned well and extracted a variety of compounds that produced unique chromatographic fingerprints, allowing for the easy differentiation between the two plants. The method is ideally suited for the detection of oleandrin in high concentrations (full scan mode), low concentrations (selected masses) or trace levels (SIM analysis of ion clusters). The method is able to distinguish between extracts derived from *N. oleander* and *T. peruviana* and was able to detect and confirm neriifolin, odoroside and neritaloside in *N. oleander* leaf extracts. Analysis of forensic case exhibits were also successfully done with this method and performed well with liquid and solid matrices. With the new method oleandrin could be detected at trace levels in viscera samples that did not produce positive results in the past.

Bowiea volubilis is widely used as a medicinal plant, but is also an extremely toxic plant. It is freely available at traditional healer markets, and is one of the most highly traded plants on the Durban market. Despite the high usage of the plant, it has not been detected by any of the forensic laboratories in South Africa. Bovoside A, a bufadienolide, is reported to be the main cardiac glycoside in the bulb of *B. volubilis*. The cardiac glycoside method was successfully applied to the analysis of the bulb extract of *B. volubilis* and bovoside A was identified as the main bufadienolide present in the bulb. Bovoside A was fractionated and characterised by LC-MS. Four extracts of botanical origin could be successfully distinguished from each other by monitoring the main masses of bovoside A, oleandrin and thevetin A and thevetin B. These marker compounds were well separated from each other and made the identification of the botanical extracts quite easy, and the identity of each extract was confirmed by the mass spectrum of each peak.

OPSOMMING

Die Forensiese Chemiese Laboratorium in Johannesburg (FCL JHB) het die taak om deur middel van chemiese analise van ‘n verskeidenheid monsters die oorsaak van dood vanuit ‘n chemiese oogpunt te bepaal. Sommige van die monsters wat aan die laboratorium oorhandig word, is moontlik gekoppel aan kruie of muti, maar ‘n groot getal van hierdie sake bevat uiteindelik geen kruie komponente nie en daar word slegs farmaseutiese of landboukundige substanse in die monsters waargeneem. In hierdie studie word daar vir die eerste keer die dissiplines van forensiese ondersoek, chemie en plantkunde gekombineer om ‘n unieke platform te skep vir die identifisering van giftige plante en hulle komponenete in forensiese bewysstukke en menslike weefsel. Die navorsing was gefokus op die giftige plante wat voorheen in die laboratorium waargeneem is, sowel as die versoeke wat ontvang is vir die bepaling van muti/giftige plant komponente. Die plante wat geselekteer is sluit in *Nicotiana glauca, Datura stramonium/Datura ferox, Callilepis laureola, Boophone disticha/Ammocharis coranica, Abrus precatorius, Ricinus communis, Nerium oleander/Thevetia peruviana* en *Bowiea volubilis*. Al hierdie spesies is bekend daarvoor dat hulle reeds sterftes veroorsaak het en vandaar, die keuse om hulle in te sluit in die studie.

Nicotiana glauca is reeds gekoppel aan die dood van ten minste 15 mense sedert 2001. Dit is voorheen met behulp van GC-MS (EI) in plant bewysstukke waargeneem, maar kon nie in menslike weefsel opgespoor word nie. ‘n Selektiewe ekstraksie metode vir alkaloïede is gebruik om die plantaardige en weefsel monsters te ekstraheer. Anabasien is suksesvol op die HPLC-MS (EI) sisteem waargeneem, maar die metode was nie baie sensitief nie. ‘n Baie sensitiewe HPLC-MS metode is op die ZMD detektor ontwikkel deur gebruik te maak van elektrosproei tegnologie. Hierdie metode was superieur in vergelyking met elektronimpak detektore (GC en HPLC) en 1 ng/ml anabasien kon betreklik maklik waargeneem word in vol skanderingsanalise.

Datura stramonium and *D. ferox* is nog nooit voorheen gekoppel aan enige menslike vergiftiging of sterftes as gevolg van blootstelling aan produkte van botaniese oorsprong by die FCL JHB. Atropien en skopolamien is suksesvol geïoniseer in ESI positiewe fase en kon onderskeidelik tot op ‘n vlak van 10 pg/ml en 100 pg/ml waargeneem word. Die identiteit van die komponente is bevestig deur karakteristieke “ISCID” fragmentasie patrone. Die metode wat ontwikkel is, is suksesvol toegepas op ‘n saak waar ‘n hartaanval vermoed was. Die resultate het eenduidig aangetoon dat die ontslapene met *D. ferox* gevoer was, wat hy ingekry het as deel van sy maaltyd, en dat ‘n oordosis atropien en skopolamien bygedra het tot sy dood.

Callilepis laureola is bekend daarvoor dat dit een van die mees algemene medisinale plante in Suid-Afrika is en alhoewel ‘n moontlike nefrotoksien en/of hepatoksien die rede is vir sy toksisiteit,

is dit nog nooit voorheen waargneem by enige van die chemiese forensiese laboratoriums in Suid-Afrika nie. Die hoofrede daarvoor was die gebrek aan ‘n betroubare metode vir die analise van die toksiese komponent van *C. laureola*, atraktilosied, deur gebruik te maak van massa spektrometrie. ‘n Sensitiewe en baie selektiewe HPLC-ESI-MS metode is ontwikkel wat atraktilosied, karboksie-atraktilosied en hulle monosulfaat analoë in die plantaardige en weefsel matrikse kon waarneem. Die metode is suksesvol toegepas op ‘n verskeidenheid van forensiese monsters en het bewys dat *C. laureola* moontlik ‘n belangerike rol speel in vermeende kruie vergiftigingsake. In ‘n seleksie van vermeende kruie vergiftigings waar die oorsaak van vergiftiging onbekend is, het 30% van die monsters positief getoets vir die teenwoordigheid van atraktilosied, karboksie-atraktilosied of hulle monosulfaat analoë.

Die bolle van *Boophone disticha* is ryk aan isokwinolien alkaloïede en van die alkaloïede kon met GC-EI-MS asook LC-EI-MS waargeneem word, maar die waarneming van hierdie alkaloïede in weefsel monsters met hierdie metodes was onsuksesvol. ‘n Roetine metode, wat gebruik word vir die vinnige evaluering van monsters wat verband hou met dwelmmisbruik, is suksesvol aangewend vir die analise van die bolekstrakte van *B. disticha* en die eksterne skudblare van *A. coranica.* As ‘n eerste observasie het die chromatografiese profiele van die twee plante baie eenders vertoon, maar by nadere ondersoek van die massa spektra kon belangrike verskille tussen die twee komponente waargeneem word. Ses alkaloïede is geïsoleer en gekarakteriseer met LC-MS en KMR en hierdie komponente is daarna gereeld waargeneem in vermeende kruievergiftigingssake. Daar is aangetoon dat *B. disticha* een van die mees algemene plante is wat gebruik word om die “sisteem skoon te maak”, maar veroorsaak dikwels die dood van die pasiënt.

Abrus precatorius bevat een van die mees toksiese komponente bekend aan die mensdom, naamlik abrin, wat gesamentlik verwys na ‘n groep glikoproteïene. Die sade van *A. precatorius* bevat ook twee indool alkaloïede, abrien en hipaforien. Hierdie twee alkaloïede is gefraksioneer en gekarakteriseer met behulp van LC-MS en KMR. Aangesien die instrumente van die FCL JHB nie geskik is vir die waarneming van proteïene nie, is ‘n LC-ESI-MS metode ontwikkel vir die waarneming van hierdie twee alkaloïede in plant en weefsel monster matrikse. Die teenwoordigheid van die twee alkaloïede is aangedui deur die TMD sisteem (EI spektra) in ‘n vermeende kruie vergiftigingsaak. Die LC-ESI-MS metode is gebruik om die monsters te analiseer en die afwesigheid van abrien en hipaforien in die monsters, is bewys.

Ricinus communis is soortgelyk aan *A. precatorius* aangesien dit ook ‘n groep uiters giftige glikoproteïene bevat waarna gesamentlik verwys word as risien. Die analise van *R. communis* sade het soortgelyke probleme ondervind as die analise van *A. precatorius* sade, en daarom is die analise weereens gefokus op die waarneming van die kleiner piperidien alkaloïed, risinien. Die

LC-ESI-MS metode ontwikkel vir abrien is gemodifiseer om risinien waar te neem en was geskik vir die plantaardige sowel as weefsel matrikse. Hierdie metode sal ‘n forensiese analis in staat stel om baie lae vlakke risinien op te spoor waar die teenwoordigheid van risienolïensuur in monsters aandui dat ‘n produk, gebaseer op *R. communis, g*ebruik is.

Nerium oleander is ‘n algemene sierplant in tuine wat medisinaal gebruik word. Die plant is ryk aan kardenoliede waarvan oleandrien die hoofkomponent is. ‘n Omgekeerde fase chromatografiese metode met ESI massa spektrometrie as deteksie is ontwikkel om 11 hartglikosiede te skei en waar te neem. Die komponente is genoegsaam geskei om eenduidige identifikasie te verseker, en het baie stabiele kationisasie met natrium vertoon. ‘n Ekstraskie metode is ontwikkel om die hartglikosiede uit die blare van *N. oleander* en *Thevetia peruviana* te ekstraheer en is ook in weefsel matrikse getoets. Die ekstraksie metode was baie suksesvol en het ‘n verskeidenheid komponente geekstraheer wat gelei het tot unieke chromatografiese vingerafdrukke waarmee duidelike onderskeid getref kon word tussen die twee plante. Die metode is ideaal vir die waarneming van oleandrien in hoë konsentrasies (vol spektrum analise), lae konsentrasies (geselekteerde massas) of spoor hoeveelhede (“SIM” analise van ioon groepe). Die metode is in staat om te onderskei tussen die ekstrakte verkry uit *N. oleader* en *T. peruviana* en was ook in staat om neriifolien, odorosied en neritalosied in *N. oleander* blaarekstrakte waar te neem en te bevestig. Analise van forensiese bewysstukke is suksesvol toegepas en dit kan in vloeistof en soliede matrikse gebruik word. Met die nuwe metode kon oleandrien in spoor vlakke waargeneem word in weefsel monsters wat in die verlede geen positiewe resultate opgelewer het nie.

Bowiea volubilis word algemeen as ‘n medisinale plant gebruik, maar is ook baie toksies. Dit is vrylik by tradisionele genesermarkte beskikbaar en is een van die plante wat die meeste verkoop word op die Durbanse mark. Ten spyte van sy algemene gebruik is dit nog nooit voorheen deur enige van die forensiese laboratoriums in Suid-Afrika waargeneem nie. Bovosied A, ‘n bufadienolied, is die hoof hartglikosied in die bol van *B. volubilis*. Die hartglikosied metode is suksesvol toegepas op die analise van die bolekstrak van *B. volubilis* en bovosied A is geïdentifiseer as die hoof bufadienolied in die bol. Bovosied A is gefraksioneer en gekarakteriseer met behulp van LC-MS. Vier ekstrakte van plantaardige oorsprong kan met sukses van mekaar onderskei word deur die hoof massas van bovosied A, oleandrien en thevetien A en thevetien B te monitor. Hierdie merker komponente is baie goed van mekaar geskei en die identifikasie van die plantaardige ekstrakte was maklik, terwyl die identiteit van elke ekstrak bevestig is deur die massa spektrum van elke piek.

CHAPTER 1

AIM OF STUDY

This study is the first phase of an ongoing research project initiated to promote and enhance Forensic Chemistry in South Africa. It is a multidisciplinary investigation combining forensic investigation, chemistry and botany to create a unique platform needed by the forensic community of South Africa for the identification of poisonous plants especially those that are known to cause fatalities in humans from time to time. The main customer of the Forensic Chemistry Laboratory of Johannesburg (FCL JHB) is the South African Police Service (SAPS), and any procedures developed, or data produced by such methods, must comply with the stringent requirements of the SAPS (e.g. for use as evidence in court cases).

The project encompasses the identification of those medicinal and poisonous plants that were previously submitted to the FCL JHB, the development of extraction and analytical procedures for their poisonous components, and if possible, the application of the developed procedures to forensic investigations.

Various plant-derived compounds have been detected in forensic samples submitted to the FCL JHB. Most of the time these compounds were detected using a general extraction and screening procedure, but these methods were not developed or optimised to selectively extract and detect the compounds of interest. A few medicinal plants, for example *Callilepis laureola,* are implicated in fatalities [1] but have never been detected at the FCL JHB. Sometimes the forensic case files submitted to the Laboratory request the screening for a specific plant or plant-derived component, which might or might not be present. Most forensic case files that request screening for botanicals, request a general screening and do not supply detailed information nor a specific request, for example “Screen for herbal toxins” or “Screen for *muti*”.

The ideal forensic extraction procedure would be able to extract all the compounds present, or at least all compounds of interest. Similarly, the ideal forensic screening procedure would be able to detect all the compounds present with equal sensitivity. Although general extraction and screening procedures exist at the FCL JHB, these methods have not been evaluated as to their applicability to plant-derived compounds.

The real test of any developed method, whether an extraction procedure or a detection technique, is the application of these methods to real samples. This will prove the efficacy of the methods to deal with matrix effects and concentration differences.

The aim of the study can therefore be summarised as follows:

1. The unambiguous identification of those plant components previously detected at the Laboratory or that have been requested by the customers, focussing on lethal plant toxins.
2. Development of qualitative extraction procedures that would extract all the compounds of interest. This is typically not a single extraction procedure, but might be directed at classes of compounds (alkaloids, terpenoids or cardiac glycosides).
3. Development of chromatographic and detection techniques based on the compounds of interest and at the typical concentration in which these compounds would be present in the forensic samples.
4. The application (if possible) of the abovementioned extraction and detection procedures to forensic samples (crime scene exhibits and/or viscera samples). If this is not possible, the abovementioned procedures will be evaluated within a botanical matrix.
5. Quantification of forensically relevant compounds, as well as the determination of LOD and LOQ (depending on availability of reference standards).
6. All of the above must be done on commercially available equipment and columns, using off-the-shelf chemicals, utilising robust and reproducible methods to detect the compounds of interest in biological matrices.

This study will put forensic chemistry in South Africa on a new level and will greatly enhance the capability to screen forensic samples for botanically-derived compounds. Most of the compounds of interest have not been routinely detected in human viscera, and the developed methods will make a unique contribution to the analytical techniques used in forensic chemistry, toxicological studies and the screening of plant-derived products for toxic principles. It will allow the solving of a much larger percentage of fatal plant poisoning cases and suspected *muti* poisonings as was previously possible.

CHAPTER 2

INTRODUCTION

2.1 FORENSIC SCIENCE

The field of forensic science has come a very long way since its recorded beginnings in the 700s, when the Chinese used fingerprints to establish the identity of documents and clay sculptures. This field is one of the few areas of law enforcement where science, technology and crime solving meet. This combination supports the Theory of Transfer: “When two objects meet, some evidence of that meeting can later be found and verified”.

A few significant advances occurred in the years prior to 1800. In 1248, a book *Hsi Duan Yu (The Washing Away of Wrongs)* published by the Chinese, describing how to distinguish drowning from strangulation. It was the first recorded application of medical knowledge to the solving of crime. Paracelsus (1493 – 1541) introduced the use of drugs made from minerals [2]. On the subject of poisons, Paracelsus said “All substances are poisons: there is none which is not a poison. The right dose differentiates a poison from a remedy” [3]. In 1609, the first treatise on systematic document examination was published in France. In 1784, one of the first documented uses of physical matching saw an Englishman convicted of murder based on the torn edge of a wad of newspaper in a pistol that matched a piece remaining in his pocket [4].

Forensic science is a scientific method of gathering and examining evidence. Crimes are solved by the systematic evaluation of the crime scene followed by the gathering of fingerprints, palm prints, footprints, and any physical pieces of evidence that could prove useful in the investigation. This is followed by a pathological examination during which tooth bite prints, physical scars or wounds are examined and recorded, followed by a detailed autopsy of the deceased leading to the collection of various biological samples like blood, urine, hair and other body tissues. Handwriting and typewriting samples as well as ballistics techniques are often used to identify criminals or criminal acts, but all of the forensic samples or techniques mentioned before have one thing in common – they all need sophisticated instrumentation and highly skilled staff.

2.2 FORENSIC TOXICOLOGY

Toxicology is the fundamental science of poisons. A poison is generally considered to be any substance that can cause severe injury or death as a result of a physicochemical interaction with living tissue. However, all substances are potential poisons since all of them can cause injury or death following excessive exposure [5].

Toxicity (the amount of poison or dose) is considered as a biological concept, and is therefore usually determined by some form of bioassay, but bioassays are hardly ever used nowadays in forensic toxicology. Chemical analyses are used to detect the presence of the toxin, measure its concentration and relate this to its known toxicity. Until the 19th century, physicians, lawyers and law enforcement officials harboured extremely faulty notions about the signs and symptoms of poisoning. It was traditionally believed that if a body was black, blue or spotted in places or "smelled bad", the deceased had died due to a poison. Other mistaken ideas were that the heart of a poisoned person could not be destroyed by fire, or that the body of a person dying from arsenic poisoning would not decay. Unless a poisoner was literally caught in the act, there was no way to establish that the victim died from a poison. In the early 18th century, a Dutch physician, Hermann Boerhave, theorised that various poisons in a hot, vaporous condition yielded typical odours. He placed substances suspected of containing poisons on hot coals and tested their smells. While Boerhave was not successful in applying his method, he was the first to suggest a chemical method for proving the presence of poison [6].

During the Middle Ages, professional poisoners sold their services to both royalty and the common populace. The most common poisons were of plant origin such as hemlock, aconite, and belladonna or toxic inorganic substances such as arsenic and mercury salts. During the French and Italian Renaissance, political assassination by poisoning was raised to a fine art by Pope Alexander VI and Cesare Borgia. The 1800's witnessed the development of forensic toxicology as a scientific discipline. In 1814, Mathieiv JB Orfila (1787 – 1853), the "father of toxicology", published *Traité des Poisons* – the first systemic approach to the study of the chemical and physiological nature of poisons [6].

The first successful isolation of an alkaloid poison was performed in 1850 by Jean Servials Stas, a Belgium chemist, using a solution of acetic acid and ethyl alcohol to extract nicotine from the tissues of the murdered Gustave Fougnie. Modified by the German chemist, Friedrich Otto, the Stas-Otto method was quickly applied to isolation of numerous alkaloid poisons, including colchicine, conine, morphine, narcotine and strychnine and this method is still used today.

In the second half of the 19th century, European toxicologists were in the forefront of the development and application of forensic sciences. Procedures were developed to isolate and detect alkaloids, heavy metals and volatile poisons. In America, Rudolph A Witthaus, Professor of Chemistry at Cornell University Medical School, made many contributions to toxicology and called attention to the new science by performing analyses for New York City in several famous poisoning cases: the murders of Helen Potts by Carlyle Harris and of Annie Sutherland by Dr Robert W Buchanan, both of whom used morphine. In 1911, Tracy C Becker and Professor Witthaus edited a four-volume work on medical jurisprudence, *Forensic Medicine and*

Toxicology, the first standard forensic textbook published in the US. In 1918, the City of New York established a medical examiner's system, and the appointment of Dr Alexander O Gettler as toxicologist marked the beginning of modern forensic toxicology in America [6].

In 1949, the American Academy of Forensic Sciences was established to support and further the practice of all phases of legal medicine in the US. Several other international, national and local forensic science organisations such as the Society of Forensic Toxicologists and the California Association of Toxicologists, offer forums for the exchange of scientific data pertaining to analytical techniques and case reports involving drugs and poisons. The International Association of Forensic Toxicologists (TIAFT) founded in 1963, with over 1400 members in at least 45 countries, permits worldwide cooperation in resolving technical problems confronting the toxicologist. In 1975, the American Board of Forensic Toxicology was organised to examine and certify forensic toxicologists [6]. Compared to the toxicological work done in industry or academic research, the task of the forensic toxicologist is compounded by limitations placed on the availability of analytical material, the time constraints usually placed on the process and the limited resources usually available at the government laboratories. Forensic toxicology demands an overall analytical system designed to exclude or indicate the presence of any poison in each of the chemical groups shown in Figure 2.1 [7]. Most of the numerous screening procedures reported in the literature are too limited to permit a confident negative report. All too often these screening procedures are class/group directed, for example drug oriented, even though many criminal poisonings result from compounds other than drugs [5].

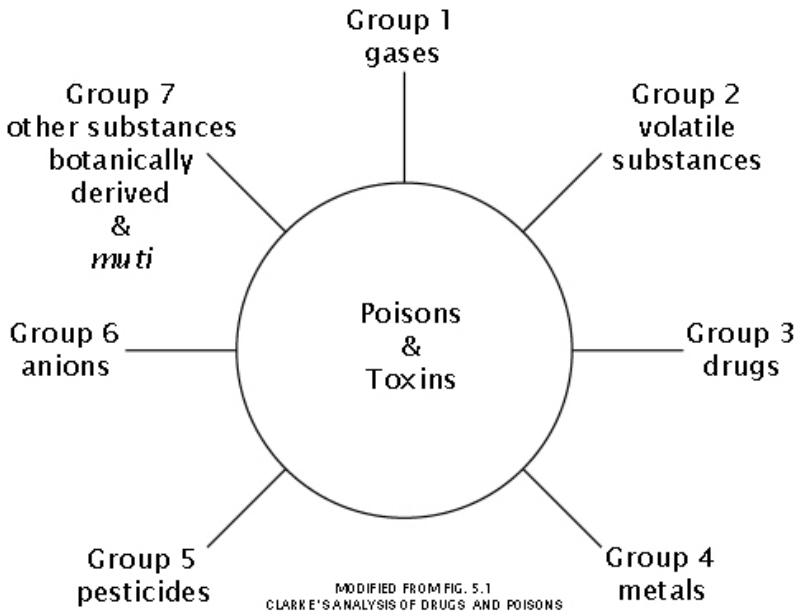


FIGURE 2.1

Chemical grouping of poisons

Apart from these analytical problems, the legal aspect of the work demands scrupulous attention to detail. Failure to make full descriptive notes on the items received, a simple error in the date the analysis was performed or neglecting to check reagent purity can become evidence of careless work in the hands of an astute lawyer.

The lawyer may, with justification, explore the extent of the toxicologist's experience and knowledge, demand a detailed account of the analytical methods and challenge the integrity of any opinion. The crucial evidence of identification and quantification of the poison may be faultless and the conclusions correct, but if the court's confidence in the forensic toxicologist as an unbiased scientific expert is destroyed, the case may be lost. A secure chain of custody of all the exhibits submitted also has to be proved [5].

Despite all this development and the formation of various forensic toxicological organizations, the basic principles of forensic toxicology still applies. Orfila [5] was well acquainted with the basic principles of forensic toxicology, and the guiding principles he established nearly 200 years ago are still applicable. These may be summarised as follows:

- All chemists who undertake this work must have toxicological experience.
- The analyst must be given a complete case history that contains all the information available.
- All the evidential material, suitably labelled and sealed in clean containers, must be submitted and examined.
- All the known identification tests should be applied and adequate notes made at the time.
- All the necessary reagents used for these tests should be pure, and blank tests should be performed to establish this fact.

All tests should be repeated, and compared with control samples to which the indicated poison has been added. Strict adherence to these principles makes forensic toxicology one of the slowest and most expensive forms of analysis. However, this must be accepted not only to ensure justice for the poisoned victim and for the accused, but also to protect the integrity and reputation of the analyst and the laboratory he or she represents [5]. It is good advice to any forensic toxicologist to remember Orfila's motto "The presence of a poison must be proved in the blood and organs before it can be considered as a cause of death". The four stages of any toxicological examination are:

1. Detection – to detect any drugs or poisons in the samples submitted by means of screening procedures.
2. Identification – to identify conclusively any drugs, metabolites or poisons present by means of specific relevant physicochemical tests.

3. Quantification – to quantify accurately those drugs, metabolites or poisons present. The quantification may be hampered by the availability of suitable standards.
4. Interpretation – to interpret the analytical findings (identification and if applicable – quantification) in the context of the case, the information given and the questions asked by the investigating officer.

The detection of a drug or poison is the most difficult part of the analytical toxicology process, as the nature of the poison is seldom known. Screening tests for any possible drugs or poisons should be used where there is no information available pertaining to the identity of the drug or poison. General screening methods are usually more flexible than special methods and can therefore be applied to a wide variety of materials. They are essential for the investigation of unknown poisonings, and have some advantages even when the toxic agent is known or suspected. The deaths investigated by toxicologists include deaths from drug abuse, accidental poisoning, suicidal poisoning and homicidal poisoning.

2.2.1 Deaths from drug abuse

Drug abuse, the non-medical use of drugs or other chemicals for the purpose of changing mood or inducing euphoria, is the source of many poisonings. Drug abuse may involve the use of illicit drugs such as heroin or phencyclidine, the use of restricted or controlled drugs such as cocaine, barbiturates and amphetamine, or use of chemicals in a manner contrary to the intended purpose – such as inhaling solvents and aerosol products [6]. In a broader sense, drug abuse may also include the excessive use of legal substances such as alcohol and prescription drugs. Alcohol is a self-limiting poison as people usually lose consciousness before a lethal dose is ingested and therefore overdose deaths due to the ingestion of excessive quantities of alcohol are uncommon. However, numerous accidental deaths occur from the concurrent ingestion of potent prescription drugs and alcohol [6]. It must be noted that many drugs were initially derived from plant material (crude morphine from *Papaver somniferum* L., cocaine obtained from coca, the dried leaves of *Erythroxylum coca* and other species of *Erythroxylum*) or are plant products chemically modified to produce more potent drugs (morphine acetylated with acetic anhydride to produce heroin) [5].

2.2.2 Accidental poisoning

Most of the accidental poisonings occur in the home. The main cause of accidental poisoning in children occur due to the carelessness of adults who do not store poisonous compounds such as prescription drugs, detergents, pesticides and household cleaners in such a way that they can not be reached by children who are generally curious and adventurous in nature. Adult poisoning occur usually as a result of mislabelling of products, which are not stored in their original containers. In industry, accidental poisoning is usually the result of carelessness or mishaps that

expose the workers to toxic substances [6]. Another agent that commonly leads to accidental poisoning is carbon monoxide, which is quite frequently generated by fossil fuel devices that is used in enclosed spaces without adequate ventilation. A few examples of such devices would be primus (paraffin) stoves and coal fires that are commonly made within informal dwellings during cold winter spells.

2.2.3 Suicidal poisoning

In suicide, poisoning is a common manner of death. The most common suicidal agent is carbon monoxide, which is usually generated from automobile exhausts. While cyanide, arsenic and other well-known poisons may occasionally be used as suicidal agents, most deaths result from prescription drugs. Nowadays, most suicidal poisonings involve multiple drug ingestion. By analysing the gastric and intestinal contents, blood, urine and the major organs of the body, the toxicologist can establish beyond doubt that the deceased could not have taken such a dose accidentally [6]. In South Africa a new trend has developed among the black population to commit suicide by the ingestion of a nematicide called Aldicarb (Temik):

2-Methyl–2-(methylthio)propanal-*O*-[(methylamino)carbonyl]oxime.

The use of plants or plant-based material to commit suicide has not been reported, largely due to the uncoordinated collection of forensic and toxicology data in South Africa. The reluctance of the South African population to report the abuse or misuse of traditional medicine or *muti* also contributes to the low number of cases that are actually reported and documented.

2.2.4 Homicidal poisoning

Accidental and suicidal poisonings are common today, but murder by poison is rare in the first world countries. However, in third world countries such as in Africa and the Eastern Pacific rim, murder by poison is not uncommon. To determine that a person died as a result of homicidal poisoning is one of the most difficult types of investigation. The general evidence of poisoning is obtained from knowledge of the symptoms displayed by the decedent before death, the post-mortem examination of the body by the pathologist and the isolation and identification of the poison by the toxicologist. Murder by poison most commonly occur within the home and the physician will seldom suspect a bereaved family member. Also, there are rarely any symptoms of poisoning that cannot equally well be caused by disease. The pathologist can recognise the effects of certain poisons at autopsy, but most poisons do not produce observable changes in body tissue and in most cases, toxicological analysis produces the evidence for murder by poison [6].

The true incidence of poisoning in South Africa is not well documented. This is mainly caused by fragmented reporting, some reporting only covers certain regions of the country or refer to a specific hospital or poison centre [8]. Another cause is the poor evaluation of the crime scene, the lack of knowledge as to which samples should be taken and submitted for analysis, and a general unwillingness of people to get involved by supplying crucial information. Poisoning due to plant material (herbal poisoning) is also poorly reported, partly because of the unwillingness of people to admit using traditional medicine derived from plant material, but also because of the fear that the cultural heritage of the people will be “stolen” and commercialised.

The modern forensic toxicologist utilises a variety of analytical instrumentation and techniques to carry out his or her mission. In South Africa, the forensic analyst is often confronted with unique analysis due to the use of traditional medicines (*muti*), which have medicinal value but can cause death or injury when used incorrectly. In most of the cases, the methods required for the analysis of these compounds do not exist and have to be developed.

Generally, the most difficult part of any toxicology analysis is the process of isolating the toxic substance from the biological matrix. This must be accomplished in a manner that provides a concentrated sample that contains the compound(s) of interest, but is relatively free of interference by naturally occurring substances. Once the compound is isolated, purified and concentrated, identification and quantification become possible.

In order to provide the pathologist or investigating officer with sound information as to the state of intoxication or poisoning, degree of impairment or contribution to a fatality, the toxicologist must be informed as to the nature and circumstances of the intoxication as well as the quantity or concentration of the substance involved [6]. Once again the toxicologist in South Africa is often faced with a situation where the possible cause of death is unknown, especially where *muti* was involved as some *muti* does not produce immediate effects or show immediate symptoms, and some pathologists are not familiar with the pharmacological action nor the organ / cellular damage caused by some plants used as traditional medicine.

The procedure utilised in a typical toxicology section of a laboratory may include preliminary examination of materials following its extraction and isolation by using chemical spot tests, which by colour production may indicate the presence of a type of drug or poison. More sophisticated techniques include radioimmunoassay (RIA), thin-layer chromatography (TLC), ultraviolet spectroscopy (UV); infrared spectroscopy (IR); high performance liquid chromatography (HPLC) and gas chromatography (GC). The application of mass spectrometry (MS) is now commonly utilised for positive identification of drugs and toxic compounds [9].

2.3 MEDICINAL PLANTS

It is estimated by the World Health Organisation (WHO) that approximately 75 – 80% of the world's population uses plant medicines either in part or entirely. For many this is out of necessity because they cannot afford the high cost of pharmaceutical drugs or do not have access to university-trained medical practitioners. Growing numbers of American health care consumers are turning to plant medicines for many reasons – low cost and seeking natural alternatives with fewer side effects are commonly cited [10]. In Africa, traditional healers and remedies made from plants play an important role in the health of millions of people. The relative ratios of traditional practitioners and university-trained doctors in relation to the whole population in African countries are revealing. In Ghana for example, in Kwahu district, for every traditional practitioner there are 224 people, compared to one university trained doctor for nearly 21 000 people. The same applies to Swaziland, where there are 110 people for every traditional healer whereas for every university-trained doctor there are 10 000 people [11, 12, 13].

In the past, modern science has considered methods of traditional knowledge as primitive and during the colonial era traditional medical practices were often declared as illegal by the colonial authorities. Consequently, doctors and health personnel have in most cases continued to shun traditional practitioners despite their contribution to meeting the basic health needs of the population, especially the rural people in developing countries. However, recent progress in the fields of environmental science, immunology, medical botany and pharmacognosy, have led researchers to appreciate in a new way the precise descriptive capacity and rationality of various traditional taxonomies as well as the effectiveness of the treatments employed [11].

The importance of medicinal plants in South African traditional healing systems cannot be overemphasized. These medicinal plants known in South Africa as *muti*, play an indispensable role in the healing practice of traditional healers in the country. There are approximately 200 000 traditional healers in South Africa, with 70 – 80% of the black population consulting these health practitioners. Many of these customers or patients obtain plant medicines from the traditional healers. The *muti* industry is a multi million Rand industry in South Africa and is continually growing [14]. Southern Africa, and in particular South Africa, is rich in plant diversity with some 30 000 species of flowering plants. This represents nearly 10% of the world's higher plants. Great cultural diversity also exist within this region and many people are dependent on the plant diversity and use plants in their day-to-day activities that include food, water, fuel and medicine to mention a few. Taking into consideration this broad and diverse plant base of South Africa, it is not surprising to find that approximately 3000 species of plants are used as various medicines and *muti*. Of these plants, some 350 species are regularly used and traded as medicinal plants or herbal preparations. [15].

Extreme pressures on wild reserves of plants have come about mostly in the last 10 years. Factors, among others, that have contributed towards the rise in commercialization of *muti* are the rapid growth and urbanization of the black population as well as the affordability of these plant medicines. In 1992 the number of gatherers selling *muti* plants in Johannesburg was fewer than 10 gatherers on Soms Traditional Healer's market. Today, Soms is a sprawling market overflowing with roots, barks and bulbs with well over 100 gatherers selling their products there. *Muti* traders, which include the traditional healers, used to obtain their plants from gatherers who would supply door to door. At present, however, the gatherers prefer to sell their plants directly to any interested party on the open-air markets such as Soms. Although the harvesting of medicinal plants used to be the domain of trained and ecologically responsible healers, many of the current gatherers in South Africa have a poor knowledge of what the plants they gather are used for and they practice non-sustainable and destructive harvesting techniques [14].

Currently traditional African medicine supplied by sangomas, nyangas and other informal vendors do not fall under the National Drug policy. On the other hand, marketed labelled medicines, internationally established herbal formulations and nutritionals are currently regulated or will soon be regulated.

Medicines regulation, in the public interest, comprises of three integral aspects: quality, safety and efficacy. In terms of quality the hygienic and contamination situation relating to the traditional medicines is a concern as they are sold on pavements and markets which are often tainted with sputum, urine and faeces, contrasting with the GMP pharmaceutical manufacturing standards which are necessary for production and packaging of other medicines. Regarding efficacy and safety, it is imperative to evaluate the relative risk from traditional African herbs and substances, since some 75% of the African population is estimated to use traditional substances, usually in combinations. According to the electronic database established by Noristan Laboratories, now with the Medical Research Council (MRC), of the 350 plant extracts assayed, some 79% showed definite pharmacological activity, and 12% definite toxic effects [16].

According to J. Kaufman *et. al* [17], "Information on cause of death among adults in sub-Saharan Africa is essentially non-existent". The author of this report estimates that annually several thousand deaths from traditional African medicines occur. Dr. M. Stewart, recently retired from the Department of Chemical Pathology, S.A. Institute for Medical Research, concurs with this assessment, having previously confirmed "70 traditional African medicine deaths in 8 months at the Coronation Hospital in Johannesburg". It is significant that the major poisoning symptoms and causes of death from traditional African medicines closely mirror the major symptoms and causes of death (besides infections) among the black population: diarrhoea, vomiting, hypoglycaemia, renal and/or hepatic failure, respiratory distress and cardiac failure [16].

The crude death rate in South Africa is 8.9 per 1000 [18], meaning that approximately 400 000 of 40 million South Africans die each year. In the RSA 20% of all deaths are of unknown causes. Deaths from traditional African medicines could constitute a portion of this approximately 80 000 deaths.

Prof. Pieter Joubert ex Medunsa, whose work is widely respected, published and cited nationally and internationally, has determined that “in developing countries, besides infectious conditions, acute poisoning with pesticides, paraffin (kerosene) and traditional medicines are the main causes of morbidity, whilst acute poisonings with traditional medicines is the main cause of mortality ” [19]. In a project covering 1981 – 1982 at Ga-Rankuwa Hospital, Pretoria, Joubert determined that whilst only 18% of all acute poisonings were due to traditional medicines, most (86%) of all deaths from acute poisoning were as a result of poisonings with traditional medicines [20]. In a continuing project covering 1981 – 1985, also at Ga-Rankuwa Hospital, Joubert once again determined that “the main poisoning causes were paraffin (59%), but with low mortality (2%), whilst poisoning with traditional medicines resulted in high mortality (15%) and accounted for 51% of all deaths as a result of acute poisoning, always accidental. Vomiting, diarrhoea and abdominal pains were the most frequent encountered symptoms, whilst lungs, liver and central nervous system were commonly affected. The traditional healer was the main source (83%), while 11% was bought from African medicine shops [21]. The plants most frequently implicated in the poisonings at Garankuwa Hospital were *Jatropha curcas, Ricinus communis* and *Datura stramonium* [22].

The Zulu population uses a wide variety of toxic plants for medicinal purposes [23]. A medicinal plant associated with many fatalities is *Callilepis laureola*, also known as *Impila* which ironically means “health” in Zulu [24]. With approximately 50% of the population using *Impila* in KwaZulu-Natal, it is the second most widely used medicinal plant and has been extensively reported [25]. *Impila* induces severe toxicity and cause the following: acute renal failure and fatal liver necrosis. Thus, it is nephrotoxic, hepatotoxic and also causes hypoglycaemia. It causes alteration in consciousness, vomiting, diarrhoea, convulsions and the rootstock is toxic and can be fatal if ingested in small quantities [16].

Many black South African women use traditional African herbal remedies as antenatal medications or to induce or augment labour. Very little is known about the pharmacology and potential toxicity of plants used in herbal remedies, but several of these plants are known to be poisonous. Some of the herbs are toxic in large doses, and there are about 36 plants in South Africa used to induce labour, of which 15 are toxic [26, 27, 28].

Traditional medicines can be beneficial, dangerous or useless in a pharmacological or psychological capacity, their dangers being mainly as direct irritants or as hepatic, renal or cardiac toxins. Mild to moderate toxicity from short or long term use is difficult to separate from the symptoms of the original illness. Greater absorption from enemas, coupled with irritant proctitis and perforation of the rectal / colon membranes during administration, indicate that enemas result in a high mortality [25]. Traditional medicines (or *muti*) are usually administered orally or as an enema by a traditional healer. Gastro-intestinal irritation was the most common syndrome (54%) experienced after traditional medicine administration. Cardiac glycosides are often suspected (44%) in autopsies (FCL JHB data base) where death was presumed to be caused by herbal medicine. It is concluded that in patients with gastro-intestinal symptoms, traditional medicine containing cardiac glycosides should be expected [29]. It must be taken into consideration that this high percentage of positives for cardiac glycosides were linked to HPLC-PDA results, which based the identification of cardiac glycosides on the non-specific PDA spectra of these compounds.

Toxicity related to traditional African medicines is becoming more widely recognized. Accidental herbal toxicity occurs not only as a result of the lack of pharmaceutical quality control in harvesting and preparation, but also because these remedies are believed to be harmless. Treatment in most cases of plant poisoning remains symptomatic, with few antidotes available [30].

To fully understand the active ingredients in the different plant species which may have beneficial effects or which may be poisonous, reliable analytical methods have to be developed to study the remedies of traditional healers and to help them in developing safe medicine for their patients. Very few analytical methods currently exist to identify the active ingredients in the different plants of interest.

2.4 ANALYTICAL METHODS USED IN FORENSIC TOXICOLOGY

When tablets or capsules are found in the immediate vicinity of /or in the possession of an unconscious patient, it is reasonable to assume that a drug overdose has been taken. If the patient responds to supportive treatment and no active measures are contemplated, toxicological analyses are of historical interest only. However, when circumstantial evidence is lacking, a diagnosis of poisoning may be difficult to sustain simply on the basis of clinical examination, since coma or death induced by drugs is not readily differentiated from that caused by disease processes. The role of the laboratory is important in the case of poisoning cases. While numerous chemical methods are available to the toxicologist, only a few of the more common

procedures are discussed. All of these methods can be applied to qualitative (identification) and quantitative (concentration) analysis.

2.4.1 Colour test

A colour test is a chemical procedure in which the substance tested is acted on by a reagent, which causes a change in the reagent, thereby producing a characteristic colour or colour change. The greatest utility of colour tests in toxicology is the rapid screening of urine specimens, as the urine may be analysed directly without time-consuming extraction procedures. A false positive result, that is the development of a colour when the correct compound is not present, is one of the drawbacks of this method. The forensic analyst must be aware of the limitations of this method and particularly the sources of false-positive reactions [6].

2.4.2 Microdiffusion test

Microdiffusion analysis is used for the rapid isolation and detection of volatile poisons. Numerous volatile poisons and gases may be detected by microdiffusion techniques; they include acetaldehyde, carbon monoxide, hydrogen cyanide, ethanol, fluoride, halogenated hydrocarbons and methanol [6].

2.4.3 Immunoassays

Immunoassay is a technique which requires antibodies that bind tightly to the drug of interest and only weakly or not at all to other substances. At present, there are three commercially available systems, widely used in forensic toxicology: enzyme multiplied immunoassay technique (EMIT), fluorescent polarisation immunoassay (FPIA) and radioimmunoassay (RIA). An immunoassay consists of a mixture of the drug-specific antibody and a “labelled drug” for which the antibody was prepared. The “label” may be a radioactive atom (RIA), or chemically attached fluorescent compound (FPIA), or an enzyme (EMIT). When a sample containing the drug of interest is added to the mixture, it competes with the “labelled drug” for binding to the antibody. The presence of the drug sought is indicated by a change in radioactivity (RIA), fluorescence polarization (FPIA) or enzyme reaction rate (EMIT). These techniques may be used for both quantitative and qualitative analysis. The techniques are rapid and often simple to apply [6] but suffer from false-positive reactions.

2.4.4 Chromatography

Chromatography is a separation technique and is a term that literally means “colour writing” and the origin of the word is attributed to the Russian botanist Tswett [31] who coined the term to identify the method by which he successfully separated plant extracts into different coloured zones in the early 1900s [32]. The term has lost its original meaning in the wealth of separations

performed on colourless substances. The components of a sample mixture are distributed between two phases, one of which is stationary while the second one, the mobile phase, percolates through a matrix or over the surface of a fixed phase. Although there are many varieties of chromatographic analysis the three most commonly applied by toxicologists and forensic analysts are thin-layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC) [6].

2.4.4.1 Thin-layer chromatography (TLC)

In TLC, the stationary phase is a "thin layer" of an absorbent, usually silica gel, which is spread on a solid support. Concentrated sample extracts and drug standards are applied as a series of spots along the bottom of the plate and placed in a closed tank in which the absorbent layer makes contact with the "developing solvent" (mobile phase) below the applied spots. The solvent moves up the plate by capillary action, dissolving and separating the components of the extracts. The presence of drug is visualised by spraying or dipping into the various reagents, which produce coloured reactions with particular compounds. There are numerous TLC spray reagents to choose from, but the toxicologist must be guided by the chemical nature of the compounds of interest. If a compound from the extract migrates the same distance and reacts to the applied sprays in the same manner as the reference drug / compound, the toxicologist then has a tentative identification of the compound, which must be confirmed by another chemical or analytical test.

2.4.4.2 Gas chromatography (GC)

In GLC, or more commonly called GC, the mobile phase is an inert carrier gas for example helium or nitrogen which flows through a column packed with a solid support coated with a liquid stationary phase (packed column) or over a stationary (liquid) phase coating the walls of a narrow column (capillary column). Numerous types of liquid materials are available, and the forensic analyst varies the stationary phase depending upon the nature of the compounds or groups of compounds he wishes to separate and identify. An extract of a specimen chromatographed under the same conditions as reference drugs and producing a peak at the same time would be tentatively positive for the reference drug in the spectrum. Gas chromatography is particularly suitable for the analysis of volatile substances such as alcohols [6].

The analysis of natural products, herbal formulations and *muti* samples by GC is usually hampered by various problems. The analysis of the volatile fraction of these type of samples usually do not pose too great a challenge – as long as the compounds of interest can be efficiently volatilised from the matrix without contaminating the inlet, analytical column or detector. The less volatile fraction, however, requires derivatisation to enhance the volatility of

the compound(s) of interest. Another limitation of GC and ultimately GC-EI-MS systems is the molecular size of the compound(s) of interest. These systems work well when analysing compounds less than 600 amu (maximum 1000 amu) but fail to effectively separate and detect larger molecules. Although newer mass detectors are capable of detecting macro molecules, the inlet systems (GC) still suffer from the initial limitations. Another limitation of using GC or GC-MS for the analysis of herbal products and *muti* is the thermal instability of many of the active compounds found in these samples.

Although HPLC and HPLC-MS (especially HPLC-EI-MS) are not suited for the analysis of volatile compounds, it is ideally suited for the analysis of the less volatile to non-volatile fraction of these samples, and typically do not suffer the abovementioned problems.

2.4.4.3 High Performance Liquid Chromatography

The modern form of column chromatography has been called high performance chromatography, high-pressure, high-resolution and high-speed liquid chromatography. However, the abbreviation HPLC is now universally understood to describe the technique that separates mixtures on columns filled with small particles (typically 10 µm or less in diameter) by elution with a liquid under pressure. The essential equipment consists of an eluent reservoir, a high pressure pump, an injector for introducing the sample, a stainless steel column containing the packing material, a detector and a data recording device. Thus HPLC is similar to other types of chromatography in having a stationary phase (packing material) and a mobile phase (eluent) [5].

HPLC offers many advantages over traditional LC:

(a) Speed of analysis
(b) Drastic improvement in resolution
(c) Sensitivity - various highly specific and sensitive detectors are HPLC compatible
(d) Reusable columns
(e) Ideal for large molecules and ionic species as well as non-volatile compounds
(f) Easy sample recovery
(g) Easily automated for unattended operation

HPLC Columns

The column is the heart of the chromatographic system and the success or failure of an analysis depends on the choice of column and proper operating conditions which include the proper selection of mobile phase and the pH of the mobile phase (Figure 2.3). Columns are almost invariably made from high quality stainless steel but can also be constructed from PEEK material. Columns are generally operated at ambient temperatures, but elevated or lowered

temperatures may be used to afford a difficult separation. To ensure stable chromatography and reproducible retention times at any time of the day, the column is usually electrically thermostated at a temperature 5 ^{o}C higher than the highest ambient temperature.

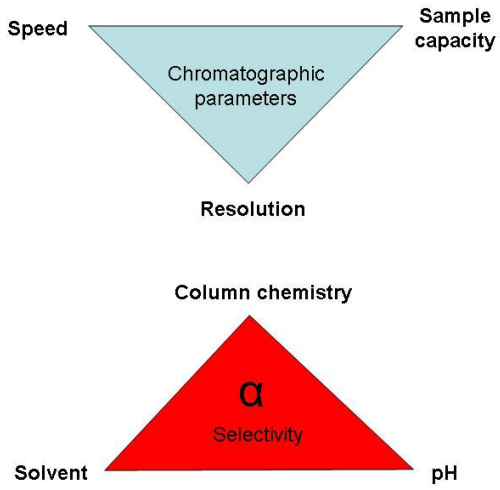


FIGURE 2.2

Factors affecting chromatography and selectivity

Gradient elution

Gradient elution is defined as increasing the strength of the mobile phase during a chromatographic analysis. The net effect of gradient elution is to shorten the retention time of compounds strongly retained on columns. Gradient elution offers several advantages:

(a) Total analysis time can be reduced significantly
(b) Overall resolution per unit time of a mixture is increased
(c) Peak shape is usually improved (less tailing)
(d) Effective sensitivity is increased since there is little variation in peak shape
(e) Removal of well-retained compounds that might bleed off in the next analysis
(This is extremely important when working in a biological matrix).

Gradients may be introduced in a stepwise manner or continuously by gradually increasing the solvent strength (organic content) according to a pre-programmed gradient profile.

The mobile phase (eluent)

In liquid chromatography the composition of the solvent or mobile phase is one of the variables influencing the separation. The mobile phase should:

(a) Be pure – it should not contain any contaminants – especially metals

(b) Not degrade or destroy the packing (high pH eluents degrade silica-based stationary phases)

(c) Be compatible with the detector (especially in coupling HPLC systems to mass spectrometers)

(d) Dissolve the sample

(e) Have a low viscosity

(f) Permit easy sample recovery, if desired

(g) Be commercially available at a reasonable price

(h) Should not chemically react with the stationary phase, thereby altering the stationary phase and creating an unique stationary phase.

2.4.5 COLUMN THEORY

The successful chromatographic separation involves making a compromise between chromatographic resolution, sample capacity, and analysis time.

Resolution

The goal of chromatography is to separate components of a sample within a reasonable period of time into separate bands or peaks as they migrate through the column. The resolution, R, between two peaks can be quantitatively measured using the following equation:

$$R = (t_{R2} - t_{R1}) / ((w_2 + w_1)/2) = 2\Delta t/(w_2 + w_1)$$

Where

- t_{R2} and t_{R1} are retention times of the retained components measured at the peakmaximum
- Δt is the difference between t_{R2} and t_{R1}
- w_2 and w_1 are the peak widths in units of time measurement at the base of the peaks.

Generally, a resolution value of 1.0 or greater is required for good quantitative or qualitative work. Improving column efficiency by decreasing the width of the band is the most desirable way of improving resolution since it saves time. Column efficiency is a function of column parameters, such as solvent flow rate and viscosity, the particle size of the column packing, the type of column packing as well as the method of packing the column, and the chemical processes used to limit unwanted chromatographic processes due to unwanted active sites (silanol activity). It is also possible to improve the resolution by changing the column selectivity, but if the efficiency is constant, increasing the resolution usually involves longer elution times for a particular system or the use of an alternative column chemistry (packing) or mobile phase system.

Column efficiency

Column efficiency can be measured quantitatively by the following equation:

$$N = 16\ (t_R/w) = 5.5(t_R/w_{1/2})$$

Where

- w is the peak width at the baseline, $w_{1/2}$ the width at ½ peak height
- t_R is the retention time of retained compound

This equation compares the width of the peak to the length of time the component has been on the column. Thus, an efficient column keeps the bands from spreading and/or gives very narrow peaks. The value for N is called the number of theoretical plates. It is a useful measure of the performance of the chromatographic column because, to a first approximation, the value of N is independent of the retention time. That is, the number of theoretical plates calculated for a peak at 3 minutes will be the same as the value calculated for peaks emerging at 10 minutes from the same column if extra-column effects are absent. Generally, the measurement involving the peak width at ½ height ($w_{1/2}$) has been found to be most useful since it can be carried out on peaks that are not completely resolved or display a slight tailing.

The number of theoretical plates is proportional to column length. Generally longer columns have more plates, but the pressure drop is greater. The height equivalent to theoretical plate, H or HETP, is the preferred measure of column efficiency because it allows a comparison between columns of different lengths:

$$H = HETP = L / N$$

Where

- L is the length of the column (usually in millimetres)
- N is the number of theoretical plates.

Columns for high speed liquid chromatography generally have plate heights in the range of 0.01 to 1.0 mm. The value of H can also be used as an evaluation criterion for columns as the better columns have smaller values for H than less efficient columns.

Band broadening can also occur in the injector, detector and interconnecting tubing. It is extremely important to keep the system volume and length of interconnecting tubing to a minimum and to ensure proper connections with negligible dead volume at each connection

point. Another way to improve the resolution in an HPLC separation is to change the selectivity of the column. This may be done by changing the stationary phase, the column chemistry used or the mobile phase composition. The newest trend is to decrease the particle to 1.7 µm and to increase the flow rate to afford ultra-fast chromatography with unparalleled resolution and sensitivity. This trend unfortunately requires special hardware that can produce and withstand pressures up to 20 000 psi and specially designed detectors with state-of-the-art electronics to detect transient chromatographic peaks.

Distribution coefficient

The distribution coefficient equals the concentration of solute in the stationary phase divided by the concentration in the mobile phase. The distribution coefficient is often called:

Partition coefficient in liquid chromatography

Permeation coefficient in exclusion chromatography

Adsorption coefficient in liquid adsorption chromatography and

Distribution coefficient in ion-exchange chromatography

Column selectivity is improved by changing the distribution coefficient K and/or the stationary phase volume V_s. The distribution coefficient is changed as follows:

(1) Changing mobile phase – this can be done by increasing polarity, pH and/or ionic strength. This is used in gradient elution
(2) Changing stationary phase – this may be achieved by changing pore size of the gels, modifying surfaces of adsorbents, changing liquid used as stationary phase
(3) Changing temperature – in certain instances such as ion-exchange and exclusion chromatography, the selectivity of the column can be changed by changing the temperature
(4) Changing nature of solute – an example here is to eliminate the charge on an amino acid by changing pH of the mobile phase to reduce affinity for ion-exchange resins or by forming an ion pair (also called ion-pairing chromatography).

Choosing column parameters

Three critical qualities of chromatographic separations are resolution, speed and sample capacity, and usually results in a compromise between these factors (Figure 2.2). Generally, resolution is improved by sacrificing speed and sample size. In complex analysis good resolution between peaks are critical and is usually improved by changing the selectivity of the analytical column (Figure 2.2).

The length of the column is a very important parameter in separation, and doubling of the length results in doubling of the retention and separation of the zones. High speed is achieved by making the column as short as possible consistent with the resolution required, as well as operating at high solvent flow rates.
A second important parameter is the column diameter and typical column diameters for analytical separations are between 2 and 4 mm, while for preparative work, diameters of 8 to 50 mm are more appropriate. The sample capacity of a column increases as the square of the diameter, but flow inequalities and packing difficulties may prohibit indefinite increases.

The particle size depends on the type of support used. Small porous particles offer relatively high surface area and if uniformly packed, give columns with small inter-particle void spaces and excellent efficiency. Sample size capacity of these columns is relatively high. The disadvantages of small particles are high resistance to liquid flow and manufacturing as well as packing difficulties. Resolution and capacity are improved by using the smallest particles possible.

The choice of the correct stationary phase is very important for the best possible separation and analysis. The stationary phase must be insoluble in the mobile phase. For example, silica is very soluble in alkaline solutions with a pH greater than 7.5, and acids with a pH lower than 2. This led to the development of hybrid (silicon-carbon) particle technology that combined the best characteristics of silica and polymer packing (Xterra column technology by the Waters Corporation). This stationary phase is a great improvement of the traditional silica stationary phase or even the more resent polymeric phases due to the following characteristics:

- pH stability from pH 1 to 12 (Xterra columns last 7 times longer at extreme pH conditions)
- stable to rapid mobile phase composition changes
- stable to rapid flow rate changes
- display unique “dual mode” retention mechanism for polar-imbedded stationary phases.

Other parameters which are also important in choosing the best conditions for separation include:

Gradient elution - the gradient profile should be kept as simple as possible;
Flow rate - this will influence resolution and speed of separation and column efficiency;
Column pressure - this is generated by the resistance of the column to liquid flow and many column parameters can influence the pressure;
Column temperature - changing the temperature can have an influence on the resolution, but may also increase the solubility of the sample [33].

2.4.6 HPLC-MS SYSTEMS (LC-MS)

The major technical challenge in liquid chromatography-mass spectrometry is interfacing the chromatographic and mass spectrometric components. The mass spectrometers used with HPLC systems are virtually the same as those used with GC systems but with a different interface. Understanding the different working principles and technical properties of the different MS instruments gives insight into the possibilities and limitations that do exist when coupling LC and MS. Five different mass spectrometers (analysers) have been successfully interfaced with LC systems, namely Quadrupole (also called single quadrupole or Q_1) analysers, Ion Traps (IT), Time-of-Flight (TOF) analysers, Sector Instruments (SI) and Fourier Transform Instruments (FTI). Various tandem instruments have also successfully been coupled to LC systems. These include Triple Quadrupole Instruments (Q_3), Quadrupole-TOF (Q-TOF) and various combinations of sector and quadrupole systems. The following discussion of some of the more commonly encountered systems is a brief summary of each type of detector with the aim of highlighting the basic theory of operation and the uniqueness of each system.

Quadrupole systems:

Both quadrupole and ion trap systems use radio frequency (rf) and direct current (dc) voltages (V_{rf} and V_{dc}, respectively) for the separation of ions. In a quadrupole instrument the rf and dc potentials are applied to four round (preferably hyperbolic cross-sectioned) rods arranged in a square layout. Opposite rods are electrically connected and the application of voltages to these pairs of rods creates a hyperbolic field within the rods. By careful manipulation and control of the V_{rf} and V_{dc} voltages a stability window can be created. Generally, the ratio V_{dc}/V_{rf} is chosen such that a 1Da window is selected, resulting in unit resolution over the entire mass range. Although modern quadrupole instruments have an upper mass limit between 3000 and 4000 Da, the typical benchtop instrument is limited to an effective 1000 Da upper limit. For quadrupole systems the energy and spatial distribution of ions produced in the source and entering the mass analyzer are not critical. This is very important for coupling to LC systems because LC-MS interfaces generally produce ions with a relatively wide energy and spatial distribution. These systems require relatively simple vacuum systems, are easy to use and are usually low in cost. The drawbacks of these systems are low mass resolution (1 Da) and limited mass range.

Ion Trap systems:

Quadrupole and ion trap instruments are the most abundant and widely used mass analysers because of their relatively low cost and benchtop design. The fundamental working principles of the ion trap are the same as described for quadrupole systems. Some describe an ion trap as a linear quadrupole bent to a closed loop [34]. The outer rod forms a ring, and the inner rod is reduced to a mathematical point in the centre of the trap. The top and bottom rods form end-

caps above and below the ring electrode. These end-caps are perforated to allow the injection and detection of ions. Within this three-dimensional quadrupole, the ions travel on a Lissajous figure-shaped path. Ions of different mass are stored together in the trap and released one at a time by scanning the applied voltages and ejecting the selected mass through the end-caps. By allowing a 10^{-3} torr pressure of helium into the trap, the motion of ions in the trap is dampened and the ions move closely around the centre of the trap. This prevents ion loss by collisions with the electrodes and improves the resolution due to limiting the spatial distribution of the ions. As for quadrupole systems, the energy and spatial distribution of ions produced in the source and entering the mass analyzer are not critical. These systems require relatively simple vacuum systems, are typically benchtop in design, low in cost and have inherent tandem MS capabilities. The major drawback of the system is the low resolution of typically 1 Da.

Time-of-Flight analysers:

The initial idea of measuring the mass of an ion by its flight time was put forward by Stephens in 1946 [35]. The very simple concept makes TOF an attractive method, but places high demands on the supporting electronics. The technique was improved by the introduction of electrostatic reflectors, enhanced ion optics to focus the ion beam towards the pusher and faster electronics. The basic working principle of a TOF mass spectrometer involves measuring the flight time of an ion through the mass spectrometer. Because of the known dimensions of the mass spectrometer and the energy of the ion, the *m/z* value of the ion can be calculated. The ion input is pulsed to provide a well defined start/stop signal that is essential for the time measurement. This also effectively converts the continuous flow of the LC system into a discrete series of ion packets. A new development was the focusing of the ion beam orthogonal to the flight tube and using a pulsed electric field to push the ions into the TOF analyzer. The addition of focusing optics to this type of arrangement creates a highly focused beam with minimal spatial distribution and a well-defined position. These parameters are crucial to ensure accurate mass assignment as any variation in the spatial distribution or position of an ion entering the TOF analyzer would directly affect this measurement and calculation.

Electronics play a vital role in TOF detectors as the whole mass range must be scanned within the flight time of the ions (typically 1 – 100 µs). Even with current state-of-the-art electronics with GHz sampling rates, the detectors of TOF systems are still the limiting factor in obtaining resolution higher than the typical 10 000 to 20 000. Well constructed TOF systems are capable of very high scan rates (up to 20 000 scans/s), have high resolution when using single or double reflectron designs and ultra-fast electronics, and have virtually no limit on the detectable mass range. The high resolution of TOF systems is vital in determining exact mass that would allow the accurate calculation of empirical formulae. This is vital if working with unknown compounds or confirming the structure of a known compound.

Sector Instruments:

The modern sector instruments are based on the first mass spectrometer (and the theory) used by Sir. J.J. Thomson who applied magnetic and electrical fields to a beam of electrons and obtained an estimate for the m/e value of the charged particles in the beam [36]. There are two types of sector instruments: magnetic and electric. If an ion moves through a magnetic field, the ion will move on a circular path with the centrifugal force equal to the force of the magnetic field on the ion. The magnetic field functions as a momentum separator and must be kept constant if ions of equal mass and charge must follow the same path through the magnetic field. The kinetic energy of the ions depends on their initial energy and spatial distribution prior to acceleration. The introduction of an electrostatic sector allows for the acceleration of ions with a known kinetic energy.

Double-focusing magnetic sector mass spectrometers make use of both a magnetic and electrostatic sector. These types of instruments typically produce high resolution mass spectra (> 10 000) and are capable of scanning large mass ranges – greater than 15 000 Da. Both the resolution and mass range can be increased but will lead to a loss in sensitivity due to instrumental parameters (reduced voltages and smaller slit openings. The ion source of a sector instrument is operated at very high voltages – as high as 10 kV, which can lead to problems when interfaced with a LC system due to arching. Although these systems are capable of high resolution and mass accuracy and able to do high energy CID experiments, the systems have limited sensitivity, are complex in design and are very expensive.

2.5 DEVELOPMENT OF EXTRACTION PROCEDURES

The success of any screening procedure is directly related to the efficacy of the extraction procedure for the compound(s) of interest. The ideal general screening procedures require that the extraction process extract all the compounds of interest present. The use of solvent extraction procedures typically target compounds of similar polarity and solubility, and multiple extractions with various solvents would have to be performed to cover all the compounds of interest. The dangers and costs associated with solvent extraction have limited the use thereof. Another extraction procedure that has come of age is solid phase extraction. The initial cartridges were available only with silica and C-18 functionality and functioned well in extracting all polar or apolar compounds. The introduction of the trifunctional ligands broadened the scope of the procedure by simultaneously trapping cationic, anionic and neutral (apolar) compounds. A few limitations of this technique are the pH stability of the silica stationary phase, and the almost complete loss of efficiency of these cartridges when allowed to run dry. Most of these limitations were overcome by the introduction of the hybrid polymeric silica columns (Waters Oasis HLB)

that do not suffer loss of efficiency when allowed to dry out, and have a wide pH stability for the hybrid polymeric silica (pH 1 - 12) stationary phase. Although polar compounds are retained on this stationary phase, the pH of the sample, wash solvent and eluding solvent must be carefully selected and controlled or will lead to low recoveries. Other positive aspect of SPE is low solvent consumption and the ease of automating the process.

2.6 DEVELOPMENT OF CHROMATOGRAPHIC SCREENING METHODS

The three primary tasks of a forensic analyst are to detect, identify and quantify potentially harmful substances in biological and other samples of forensic interest. The task of the unambiguous identification of a compound is the most challenging and crucial one, but most of the time neglected [37]. Often great emphasis is placed on the extraction and detection of a specific compound, but the identification is left to a single mass spectral analysis of the sample using SIM or MS-MS analysis techniques to differentiate the compound from the matrix and interfering compounds. According to de Zeeuw [37] the chromatographic separation of a sample prior to detection is essential – even when using a triple quadrupole mass detector (MS-MS experiments). The selection of the most suited analytical column is crucial and it must be able to separate most of the compounds detected. In HPLC-PDA analysis the choice of mobile phase and buffer is only limited to those that do not display detector response in the 200 to 600 nm range. The addition of a MS detector in tandem limits the choice of mobile phase and buffer to those compatible to the MS detector and necessitates the use of volatile buffers of low molecular mass.

The pH of the mobile phase plays a very important role in LC-MS analysis. The first effect is the stabilisation of the ionic state of the compounds to enable reproducible chromatography with good separation and peak shape. The second effect, which mainly affects API/APCI interfaces, is to ensure that the compounds of interest are ionised in solution (API) or that gas phase ionisation will occur due to the presence of suitable mobile phase components (APCI). The mobile phase can also have a very detrimental effect on the mass spectral detection of a compound if ion suppression occurs or if the mobile phase contributes to a high background signal. As a rule of thumb the mobile phase is kept as simple as possible with the addition of the lowest possible concentration of buffer.

2.7 SCREENING OF VISCERA AND *MUTI* SAMPLES OF FORENSIC INTEREST

Due to the diverse nature of the samples submitted to the FCL JHB (viscera, plants, environmental, crime scene related, pharmaceutical preparations, industrial formulations and *muti*), any screening procedure and instrument must be robust and capable of detecting a wide

range of compounds. The fact that any result produced by the forensic analyst might be used in a court of law necessitates the use of three-dimensional (3D) detectors to ensure correct identification and confirmation of compounds detected. Many forensic laboratories rely on primary screening procedures to indicate the presence/absence of classes of compounds. These techniques (immunoassay, TLC, etc.) are not capable of conclusively proving the absence or presence or identity of a specific compound. Any positive (as well as negative) result must be confirmed by another more selective test and was the reason why I did not implement these type of screening procedures.

The “ideal” screening method for this type of research must comply with the following criteria:

1. combine a high resolution chromatographic method with a 3D detector
2. combine multiple 3D detectors in series (destructive detectors placed last in line)
3. show good sensitivity for all compounds of interest
4. have archiving and data manipulation software
5. must be able to use commercial or user-created libraries to identify detected compounds
6. be able to function in an automated manner.

Chromatographic systems that would be able to function in this manner is HPLC, Ultra high performance liquid chromatography (UPLC), CE, GC and comprehensively coupled GC (GC x GC). The only chromatographic systems available to me at the FCL JHB are HPLC and GC. 3D Detector systems include scanning UV detectors like photodiode array (PDA), all the mass detectors described before, Fourier transform infra red (FTIR) detectors and nuclear magnetic resonance (NMR) detectors. The only detectors available to me at the FCL JHB are single quadrupole mass spectrometers for HPLC and GC and PDA detectors for the HPLC systems. The single quadrupole mass detectors include electron impact (EI) mass detectors for HPLC as well as GC, and atmospheric pressure ionisation (API) and atmospheric pressure chemical ionisation (APCI) detectors for HPLC. The only detectors that can be utilised in series are the PDA and HPLC-MS mass selective detectors (EI and API/APCI) with the PDA detector placed first in line.

The problems that might be encountered when analysing compounds of botanical origin by GC have been discussed before. Although certain botanically-derived molecules do survive the GC inlet and GC column temperatures, many degrade thermally or show too low volatility for analysis by GC or GC-MS systems. This effectively rules out the use of GC-MS systems as a routine screening instrument for the majority of botanical compounds of interest and leaves only the HPLC-MS systems to function as primary screening instruments. The fact that HPLC-MS systems are not suited for the detection of volatile compounds necessitates the use of the GC-

MS system as secondary screening instrument. Certain compounds of interest are typically present in low concentrations and may not show up on the primary and secondary screen, and would require the development of more specialised screening procedures on API/APCI instruments. These instruments are usually setup for the screening of classes of compounds (alkaloids, cardiac glycosides, coumarins, ephedrines, etc.) or for targeted analysis of a specific compound like atractyloside, aldicarb, strychnine/brucine, etc.

The ideal work flow diagram for this research, based on the current equipment and needs, is depicted in Figure 2.3. It must be noted that all the orange-coloured steps/instruments are currently not available, but should be added to the array of analytical instruments as soon as possible. At present the typical *muti*-related case would progress through the following analysis path:

- Screening by HPLC-EI-MS for non-volatile to semi-volatile compounds (PDA screen included)
- Screening by GC-MS for semi-volatile to volatile compounds
- Screening for selected classes of compounds, especially when indicated/requested in the case file. This is typically done on the ZMD or ZQ HPLC-MS systems.
- Quantification of detected compounds by:
  - GC (FID, NPD, ECD or FPD)
  - HPLC using a PDA detector (UV)
  - GC-MS
  - HPLC-EI-MS
  - HPLC-MS using an API/APCI ionisation source and a single quadrupole mass spectrometer (ZMD or ZQ).

2.8 IDENTIFICATION AND CONFIRMATION OF COMPOUNDS

It has been previously stated that the detection of a poison is the most difficult part of the analytical toxicology process (Chapter 1). This is true, but another aspect of the analytical toxicology process that is most of the time neglected and poorly addressed, is the unambiguous identification and confirmation of compounds. In a recent publication entitled “Substance identification: the weak link in analytical toxicology” [37], the author highlighted the need for standardised procedures and methodologies when identifying and confirming the identity of compounds. Various guidelines exist to evaluate any qualitative forensic identification procedure, namely those compiled by TIAFT, SOFT, AAFS and WADA.

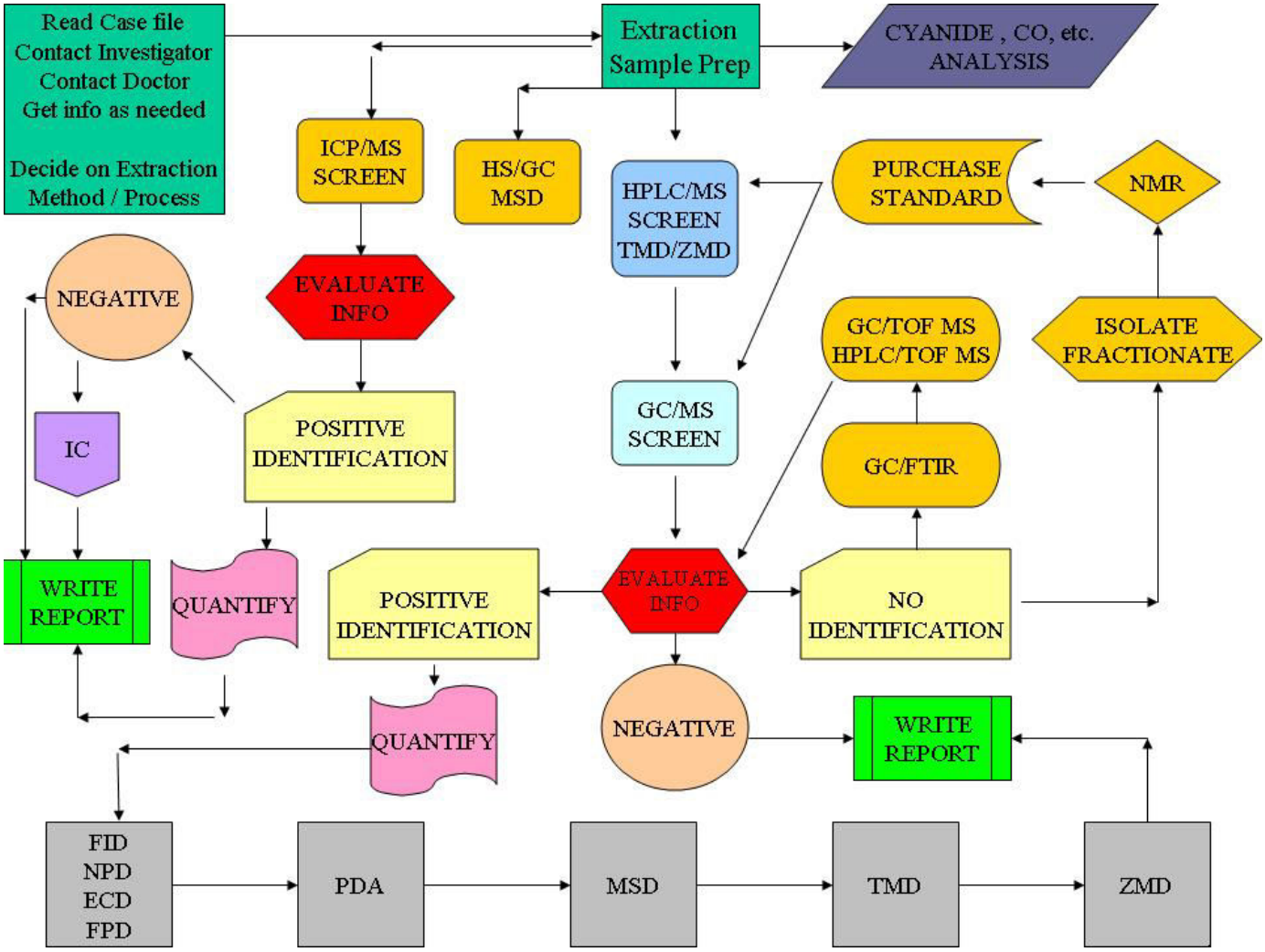


FIGURE 2.3

The ideal work flow diagram for forensic analysis at the Forensic Chemistry Laboratory of Johannesburg

The common elements can be summarised as follows [37]:

1. Confirmation must be based on mass spectrometry. MS-MS is also allowed.
2. The mass spectrum of the unknown compound must be compared with a reference standard analysed under the same chromatographic and mass spectral conditions.
3. SIM is preferable but full scan MS is also allowed.
4. Two to four diagnostic ions should be monitored with signal-to-noise ratio's > 3.
5. Relative abundances must be monitored and agree within permitted tolerance windows.
6. On-line chromatography (GC and LC) must be done prior to MS analysis.

These guidelines however differ substantially as to their criteria for confirmation and in accepting or rejecting the matching of analytical results [37].

For this study, the following criteria will be used:

a) On-line chromatography will be done prior to any detection technique (GC and LC).
b) Full scan MS will be preferred but SIM will be used in low concentration analysis.
c) The mass spectrum of the unknown compound must be compared with a reference standard analysed under the same chromatographic and mass spectral conditions and on the same day. If a reference standard is not available, a commercial mass spectral library (NIST 98) will be used as reference.
d) At least three diagnostic ions will be monitored with signal-to-noise ratio's > 3. Preference will be given to the monitoring of clusters of ions rather than three individual ions but more than one cluster of ions might be used.
e) Relative abundances must be monitored and agree within permitted tolerance windows.
f) A confirmation will be accepted as "positive" if any two of the following criteria are met:

    i. R_t and UV spectrum (PDA) if the compound is UV-active;
    ii. R_t and MS spectrum (EI) if the compound is stable during EI ionisation;
    iii. R_t and unfragmented MS spectrum (ESI);
    iv. R_t and fragmented MS spectrum (ESI using CID).

The confirmation will be considered as unambiguous if the two criteria are met on different chromatographic systems, e.g. R_t and MS (EI) spectrum on the TMD system and the R_t and unfragmented MS spectrum (ESI) on the ZQ system.

A major challenge is the procurement of reference standards. Many of the known compounds found in the medicinal and toxic plants of South Africa are not commercially available. This is mainly due to the low demand for these compounds internationally, but also due to the fact that many of the plants are indigenous to South Africa and not well characterised. The absence of a reference standard rules out quantification of the compound of interest and necessitates the use of a spectral reference library like NIST 98, assuming that the particular compound has been added to the library. If the compound has not yet been added to NIST 98, it must be isolated from the plant extract and characterised by means of ^{1}H and ^{13}C NMR and / or a TOF accurate mass determination and added to the user libraries.

2.9 SELECTION OF THE MEDICINAL AND POISONOUS PLANTS FOR THE STUDY

The most frequent request to the FCL JHB is to "Detect all toxic substances". This is often accompanied by requests like "Identify herbal toxin" or "Identify unknown exhibit" and usually state the cause of death as "Unknown", "Herbal medicine", "Alleged herbal ingestion" or "*Muti*".

A reterospective evaluation of the FCL JHB data base revealed that the following compounds were previously detected at the Laboratory:

- Anabasine
- Atropine
- Buphanidrin
- Buphanamine
- Ricinoleic acid
- Oleandrin / oleandrigenin

Reference to the following plants was found in the FCL JHB data base and forensic case files:

- *Ricinus communis*
- *Callilepis laureola*
- *Nerium oleander*
- *Abrus precatorius*

This study was initiated to develop the expertise to not only detect various compounds of botanical origin, but to be able to relate the compounds back to their botanical origin. This necessitated the inclusion of a few related plants and two plants that are widely used as *muti,* but that have not been detected before at the Laboratory. The following plants were selected for this study:

- *Nicotiana glauca*
- *Datura stramonium / Datura ferox*
- *Callilepis laureola*
- *Boophone disticha / Ammocharis coranica*
- *Abrus precatorius*
- *Ricinus communis*
- *Nerium oleander / Thevetia peruviana*
- *Bowiea volubilis.*

The availability of reference standards are usually one of the major stumbling blocks in developing or validating methods for plant-derived compounds. Although the active compounds are known for the group of plants mentioned above, only a few are commercially available. This will limit the extent to which some methods could be validated, as LOD and LOQ values as well as the quantification of compounds can only be accomplished with certified reference standards.

The thesis is written in monograph style whereby each plant monograph forms its own chapter. Certain methods were developed during the research that formed the backbone of the screening methodology used for *muti*-related forensic cases, and are included in most of the monographs. This duplication is unavoidable if the monographs are intended to be used as complete portions of research and to facilitate easy reference to the methodology used for each plant. Any adaptation or modification to these standard methods was clearly indicated in the relevant chapters.

CHAPTER 3

Nicotiana glauca

3.1 BOTANICAL DESCRIPTION

Nicotiana glauca R.A. Grah. (Family Solanaceae), is a virgate shrub or small tree (Figure 3.1) of up to five meters high with glaucous leaves and tubular yellow flowers (Figure 3.2). The fruit capsule with its numerous small, brown seeds develops within the persistent calyx [38]. It is commonly known as the wild tobacco, tree tobacco or *tabaka bume* (Southern Sotho).

Figure 3.1
Typical habit and habitat of *Nicotiana glauca*

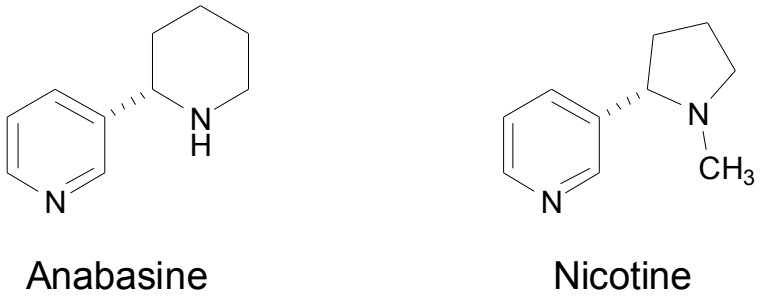
Figure 3.2
Flowers of *Nicotiana glauca*

3.2 ORIGIN AND DISTRIBUTION

Nicotiana glauca is indigenous to Argentina and was most likely introduced into Namibia in contaminated horse feed during the German occupation (1884 – 1914), from where it spread to South Africa. It is closely related to commercial tobacco, *Nicotiana tabacum* L., and is widespread throughout South Africa in disturbed places such as roadsides and riverbanks [39].

3.3 TYPE OF TOXIN

Nicotiana glauca contains a pyridine-type alkaloid anabasine (Figure 3.3) also called neonicotine, with a molecular mass of 162.23. Anabasine is closely related to nicotine (Figure 3.3) and shares the same molecular mass of 162.23. Both anabasine and nicotine have teratogenic properties and exposure to it may cause reproductive defects. The LD_{50} (gpg, scu) of anabasine is listed as 22 mg/kg, while the LD_{50} (rat, orl) of nicotine is given as 50 mg/kg [40].



Anabasine Nicotine

Figure 3.3

Chemical structures of anabasine and nicotine

3.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

Ingestion of sufficient amounts of plant material containing anabasine will result in increased salivation, vertigo, confusion, disturbed vision and hearing, photophobia, nausea, vomiting, diarrhoea, respiratory depression, spasms, neuromuscular blockage and finally death [41, 42]. These effects are result of blocking of the autonomic ganglia, neuromuscular junctions and acting as a cholinesterase inhibitor. Nicotine has a similar effect and causes irreversible cholinesterase inhibition whereas anabasine displays reversible cholinesterase inhibition [43, 44].

3.5 ETHNOPHARMACOLOGICAL IMPORTANCE

The smoking of *N. glauca* has been reported [45, 46, 47] and the plant has also been used medicinally [45] and in ethnoveterinary medicine [48]. Warmed leaves are applied to the head to relieve headache, on the throat to relieve pain and put in shoes for painful feet [49]. Animal deaths, mainly of ostriches, have also been reported [45]. It has been used as an insecticide [40, 41, 50], but its use has been discontinued due to the development of more specific and less toxic insecticides. The medicinal use of *N. glauca* has also been reported, mainly administered to people, in all the provinces of South Africa and neighbouring states [51]. The magical use of *N. glauca* has been reported in Namibia, the Limpopo Province, as well as Eastern and Western Cape [51].

3.6 POISONING

3.6.1 Accidental

In humans, accidental ingestion of *N. glauca* does occur [42, 52, 53, 54, 55] and usually results in death of the unsuspected user if sufficient plant material is ingested. In southern Africa a traditional meal is prepared consisting of porridge and marog (*Amaranthus hybridus*, Amaranthaceae), but juvenile *N. glauca* plants are sometimes inadvertantly collected with the marog (or as marog), resulting in poisoning (Figures 3.4 and 3.5). Three confirmed cases of accidental poisoning have been submitted to the FCL JHB since 2001, resulting in the death of at least 15 people. One of these cases will be discussed in section 3.7.

FIGURE 3.4

Juvenile *Nicotiana glauca* plants (Fig. 3.4) and *Amaranthus hybridus* plant (Fig. 3.5) showing the marked visual similarity between the two plants

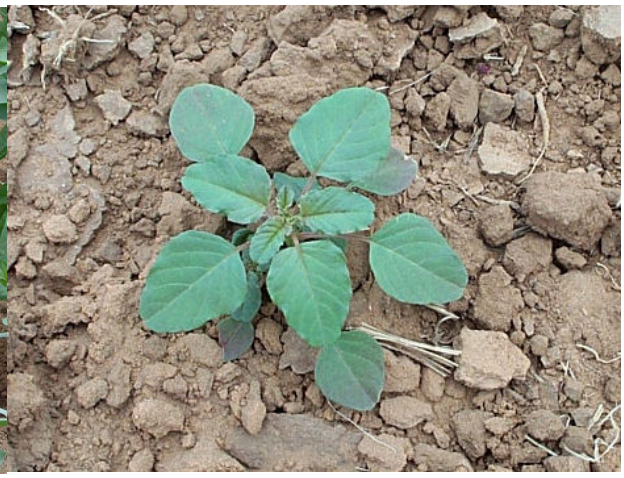


FIGURE 3.5

Juvenile *A. hybridus* plant

3.6.2 Deliberate

No reference could be found to indicate deliberate poisoning with *N. glauca*.

3.7 FCL JHB INVESTIGATION

Nicotiana glauca is one of the plants that were found in the FCL JHB database. Anabasine has also been detected in a plant sample using GC-MS (EI). As mentioned in 3.6.1, *N. glauca* has been implicated in the mortality of people due to accidental exposure. In the Limpopo Province of South Africa some communities have approached their local health inspectors and complained about “food poisoning” after preparing their traditional meal. In all the reported cases poisoning were caused by misidentification – juvenile *N. glauca* plants were collected instead of *A. hybrida*.

In one of the tragic cases submitted to the FCL JHB, a mother prepared a traditional meal for her and her son after collecting marog. After the meal, both began to vomit, became confused, collapsed and died soon there after. The viscera and a botanical sample found at the scene were submitted to the FCL JHB for chemical analysis.

3.8 EXPERIMENTAL PROCEDURE

3.8.1 Standards and reagents

(±)-Anabasine (90%), potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (> 99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%) was obtained from BDH Laboratory Supplies, England. Sulfuric acid (Suprapur 96%) and ammonia (GR 25%) Pro Analisi was obtained from E-Merck, Darmstadt, Germany. Acetonitrile (gradient quality 190 nm UV cut-off) and methanol (gradient quality 205 nm UV cut-off) were obtained from Romil, England. HPLC grade water (20 MΩ) was obtained from a Milli-Q / reversed osmosis system (Millipore, USA). Oasis HLB solid phase extraction (SPE) cartridges (60 mg) were obtained from Waters Corporation, Milford, USA. The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [56].

3.8.2 Instruments and conditions

A Waters 2690 HPLC system equipped with both a 996 photodiode array (PDA) detector and a Termabeam mass selective detector (TMD) [electron impact (EI) mode; maximum mass 1000 *m/z*] from Waters was used for initial screening and high concentration studies. A Waters 2690 HPLC system equipped with a 996 PDA detector and a Z spray mass selective detector (ZMD) (Micromass, UK) (electrospray mode; maximum mass 2000 *m/z*) was used for low concentration studies. A Waters Xterra RP C18 HPLC column (150 mm x 2 mm, 5 µm) was used for all experiments. A Harvard syringe pump (Model 11) was obtained from Harvard Apparatus, Holliston, USA and was used for all direct injection experiments on the ZMD detector.

To ensure the maximum retention of basic alkaloids, the initial chromatographic conditions were 100% water containing 10 mM ammonium acetate, the pH adjusted to 9.5 with ammonia (25%). After injection, the chromatographic conditions were gradually changed through a linear gradient profile to 90% acetonitrile and 10% of the original aqueous mobile phase in 40 minutes. These conditions were kept stable for five minutes where after the HPLC system re-equilibrated the analytical column to the initial conditions. The flow rate was kept constant at 0.2 ml/min. The nebuliser of the TMD detector was optimised with a caffeine test solution (100 µg/ml) to ensure efficient nebulisation and droplet formation. The electrospray ionization (ESI) probe of the ZMD detector was optimised by direct injection via a syringe pump using an anabasine test solution (10 µg/ml).

3.8.3 Chemical fingerprinting of *N. glauca* trees

To investigate the alkaloid variation in *N. glauca*, materials from three isolated trees were collected in Gauteng Province (South Africa). The first tree was found near the Kingsway Campus of the University of Johannesburg in Hursthill, Johannesburg (called UJHB plant); the second tree in an industrial site in Meadowbrook Extension 8, Germiston (called Germiston plant), and the third tree in an industrial area in Witfield, Boksburg (called Witfield plant). Leaves and flowers were collected from each tree and dried separately at 40 ^{o}C to constant weight. The leaves and flowers were finely ground using an Ika Werk A10 water-cooled mill (Janke & Kunkel, Straufen, Germany) and 0.5 g of each specimen was mixed with 25 ml 0.02 M sulfuric acid. The acidified plant material was extracted at 40 ^{o}C for 30 minutes using a shaking water bath. The plant material was filtered off and re-extracted with 25 ml 0.02 M sulfuric acid. The aqueous extracts were combined and the pH adjusted to approximately 9.5 with ammonia solution (25%). The aqueous extract of each plant was passed through an Oasis HLB SPE cartridge and the absorbed material stripped from the solid phase material with 1.5 ml methanol. The methanol extracts were injected into each HPLC system (2 µl into TMD; 1 µl into ZMD) using the optimised HPLC method described above.

3.8.4 Chemical extraction and analysis of viscera and case exhibit

As two of the fatalities under investigation were submitted to our Laboratory as a possible food poisoning case, a general extraction procedure was followed. An aliquot of liquefied stomach wall and contents (3 g) was mixed with 20 ml of PBS solution and sonicated for thirty minutes. After filtration through filter paper (Whatman No 41), the aqueous phase was passed through an Oasis HLB cartridge and the absorbed compounds stripped from the solid phase material with 0.4 ml methanol. The methanol solution was injected (10 µl) directly into each HPLC system and analysed using the optimised HPLC method described.

A sample of the flowers (3 g) submitted with the case, was treated as described for the viscera samples. The methanol solution, obtained by stripping the bound compounds from the Oasis cartridge, was injected (1 µl) directly into each HPLC system and analysed using the optimised HPLC method described above.

3.9 RESULTS AND DISCUSSION

3.9.1 Chromatography and detection of anabasine

Anabasine is a diprotic base with a pK_{a2} of 3.21 [40]. At a neutral pH, more than 75% of the anabasine will be in a protonated state, resulting in strong interaction with residual silanol groups that will result in poor peak shape and severe peak tailing. The chromatography can be improved by lowering the pH to 2, but this again will lead to protonation of anabasine, and various other

basic alkaloids, thereby becoming unretained on the HPLC column. Alternatively, a base deactivated reversed-phase column can be used with the addition of triethylamine (TEA) to the mobile phase [57], but this will lead to unnecessary complications when interfacing the HPLC system to a mass selective detector, especially the ZMD detector. Ion-pairing reagents will also improve the peak shape, reduce the tailing and increase the retention of anabasine and other basic alkaloids [58], but commonly used ion-pairing reagents such as hexanesulfonic acid or octanesulfonic acid will render any interfaced mass selective detector inoperative due to the involatile nature of the eluent components. Ammonium acetate displays ion suppressing characteristics and is also volatile, and can therefore be included in an eluent to be introduced into a mass spectrometer.

A concentration of 10 mM ammonium acetate, adjusted to a pH of 9.5 with ammonia, was found to be sufficient to affect reproducible retention times - 14.93 ± 0.03 (TMD system), 14.72 ± 0.03 (ZMD system) - but low enough not to interfere with ion formation during nebulisation into the mass spectrometers. A typical chromatogram of an anabasine standard (1000 µg/ml) on the TMD/PDA HPLC system (Figure 3.6) shows anabasine with a retention time of 14.92 minutes and characteristic UV and mass spectra. Likewise, a typical single ion monitor (SIM) chromatogram of an anabasine standard (10 µg/ml) on the ZMD HPLC system (ESI^+ mode) (Figure 3.7) shows anabasine with a retention time of 14.77 minutes resulting in an ESI^+ "mass spectrum". The mass ion observed at 163 m/z relates to the protonated species $[M+H]^+$ that normally form under these conditions.

On the TMD HPLC system a calibration curve was prepared covering the 100 - 1000 µg/ml concentration range. A linear curve-fitting algorithm that produced a 0.997 coefficient of determination ($r = 0.998$; $r^2 = 0.997$) was applied. A second calibration curve, covering the 500 ng/ml – 10 µg/ml concentration range, produced identical results to the calibration curve at the higher concentration range. On the ZMD HPLC system a calibration curve was prepared covering the 100 - 10 000 ng/ml concentration range. The coefficient of determination was 0.9997 and was obtained using a second order curve fitting algorithm ($r = 0.9997$; $r^2 = 0.9994$). The limit of detection (LOD) and the limit of quantitation (LOQ) for the three detectors used are summarized in Table 3.1. The LOD was determined experimentally - the LOD was taken as the concentration that produced a detector signal that could be clearly distinguished from the baseline noise (±3 times baseline noise), and the LOQ was taken as the concentration that produced a detector signal ± 10 times greater than the LOD signal.

3.9.2 Chemical fingerprinting of *N. glauca* trees

The extraction procedure used was developed to extract alkaloids selectively from plant material. The chromatograms obtained from the flower and leaf extracts of each plant (Fig. 3.8) clearly

show that anabasine is the main alkaloid extracted from the flowers and leaves of each plant. Some authors [59, 60, 61] have reported the presence of nicotine in *N. glauca*, but nicotine could not be detected in the samples. It has also been reported in literature [60] that only the leaves contain anabasine, but it was found that both leaves and flowers contained anabasine, although the alkaloid concentration were higher in leaves that in flowers (Table 3.2). The three trees studied were sufficiently uniform (both qualitatively and quantitatively) in their alkaloid contents to allow a reliable characterization of the species (Fig. 3.8).

TABLE 3.1

The LOD and LOQ for anabasine with the PDA, TMD and ZMD detectors

DETECTOR	LOD	LOQ
PDA (200 - 600 nm)	250 ng/ml	500 µg/ml
TMD (50 - 550 amu)	10 µg/ml	50 µg/ml
ZMD (100 - 300 amu)	1 ng/ml	50 ng/ml

To ensure that nicotine was not overlooked in the extracts analysed, or that anabasine and nicotine co-eluted, an alkaloid cocktail containing anabasine, nicotine, scopolamine and atropine was injected into the TMD HPLC system. From the chromatogram obtained (Figure 3.9) it was clear that anabasine [R_t = 15.52; (1)] was well resolved from nicotine [R_t = 16.72; (2)], scopolamine [R_t = 18.89; (3)] and atropine [R_t = 20.25; (4)]. The optimised electrospray conditions for anabasine also produced distinctively different fragmentation / adduct spectra for anabasine and nicotine.

3.9.3 Evaluation of SPE extraction and analysis of viscera and exhibit

The PDA/TMD chromatogram obtained from the 10 µl injections of each stomach homogenate extraction, as well as the 1 µl injection of the extract of the flower sample is depicted in Figure 3.10. Small amounts of anabasine were detected in both viscera samples and a large amount of anabasine was detected in the flower exhibit (Table 3.2). The exhibit also displayed an alkaloid profile similar to those obtained from the flowers of the Witfield, Germiston and UJHB plants (a major anabasine peak with trace levels of other components).

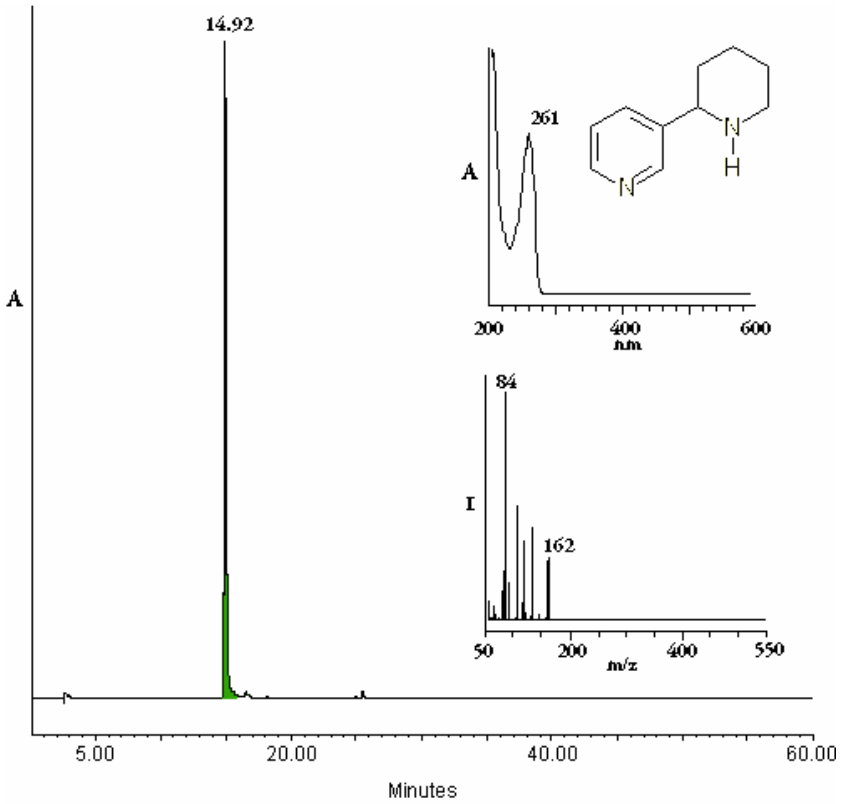


FIGURE 3.6

Chromatogram, UV and EI spectrum of a 1000 µg/ml anabasine standard (TMD system)

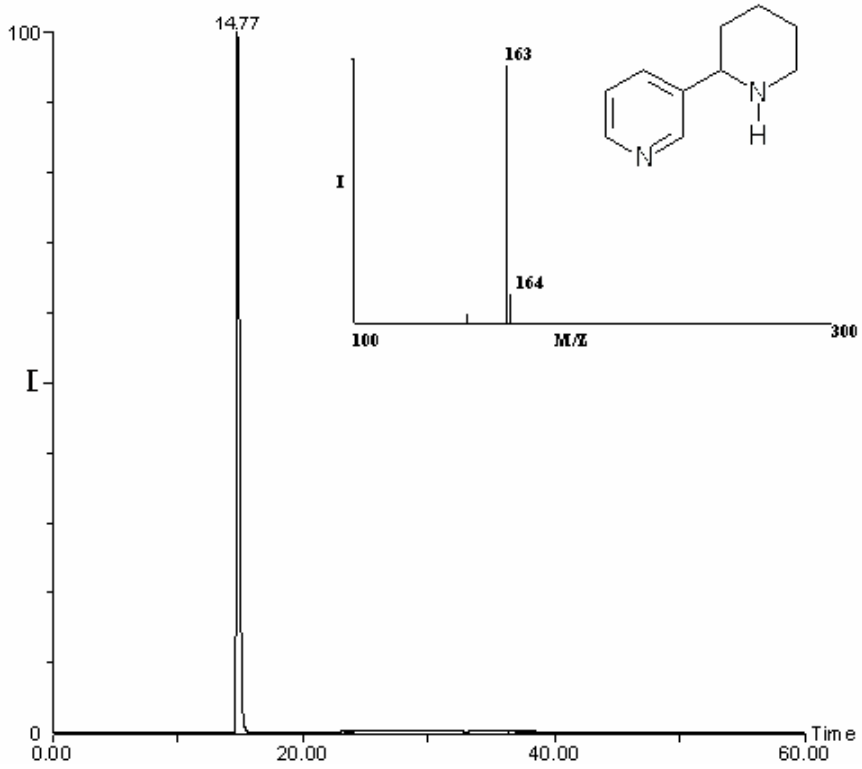


FIGURE 3.7

Chromatogram and ESI mass spectrum of a 10 µg/ml anabasine standard (ZMD system)

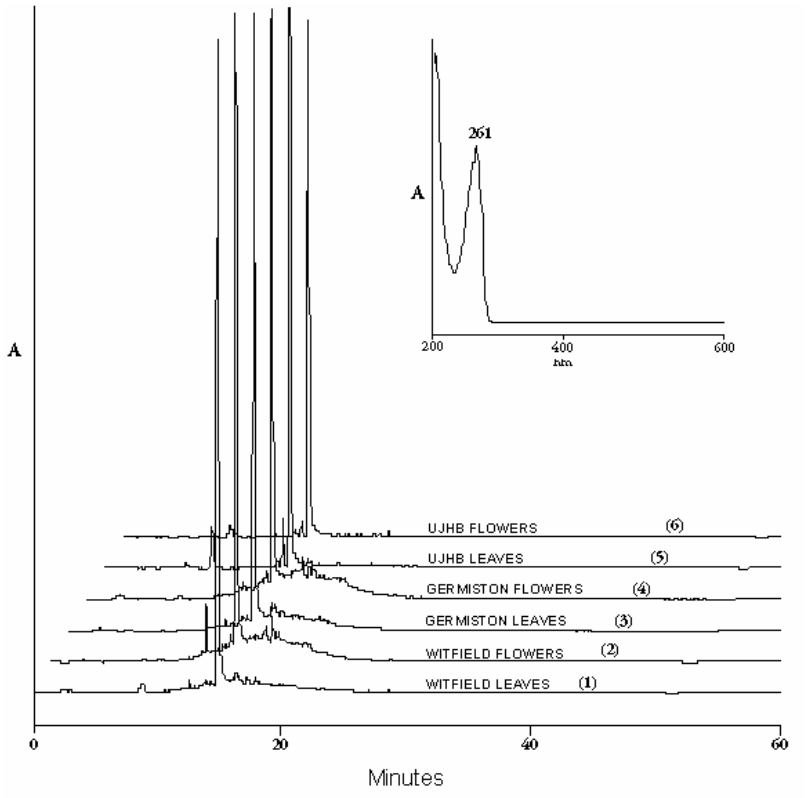


FIGURE 3.8

Comparison of the UV chromatograms of three geographically isolated *N. glauca* trees on the TMD system (leaves and flowers analysed – see Table 3.2)

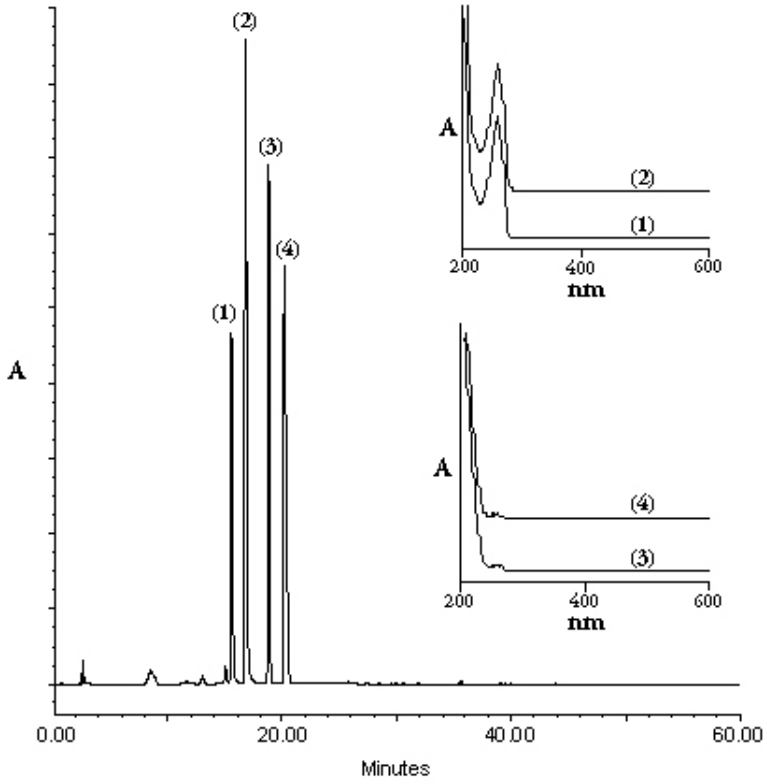


FIGURE 3.9

HPLC-UV analysis of anabasine (1), nicotine (2), scopolamine (3) and atropine (4)

TABLE 3.2

Anabasine contents of viscera and plant samples analysed on the TMD and ZMD systems

SAMPLE (n=3)	R_t (min) (Anabasine) PDA/TMD	R_t (min) (Anabasine) PDA/ZMD	% Match TMD NIST Library	Anabasine concentration PDA detector (µg/ml) [µg/g wet weight] {µg/g dry weight}	Anabasine concentration ZMD detector (µg/ml) [µg/g wet weight] {µg/g dry weight}
Stomach - Mother	15.20 ± 0.49	14.67 ± 0.06	NM (No Match)	17.4 ± 0.5 [2.3]	19.1 ± 0.6 [2.6]
Stomach - Son	15.11 ± 0.52	14.87 ± 0.08	NM	5.1 ± 0.4 [0.8]	9.3 ± 0.3 [1.0]
Flower Exhibit	15.02 ± 0.31	14.76 ± 0.05	85 ± 2	{1840 ± 60}	{1883 ± 95}
Witfield tree					
Leaves (1)	14.95 ± 0.11	14.73 ± 0.09	91 ± 1	{1991 ± 75}	NQ (Not Quantified)
Flowers (2)	14.99 ± 0.09	14.72 ± 0.08	86 ± 2	{1467 ± 58}	
Germiston tree					
Leaves (3)	15.05 ± 0.16	15.62 ± 0.14	83 ± 6	{1851 ± 82}	NQ
Flowers (4)	15.01 ± 0.14	15.68 ± 0.11	79 ± 7	{1408 ± 67}	NQ
UJHB tree					
Leaves (5)	14.98 ± 0.08	14.71 ± 0.08	90 ± 1	{1924 ± 71}	NQ
Flowers (6)	15.11 ± 0.11	14.74 ± 0.06	89 ± 2	{1517 ± 51}	NQ

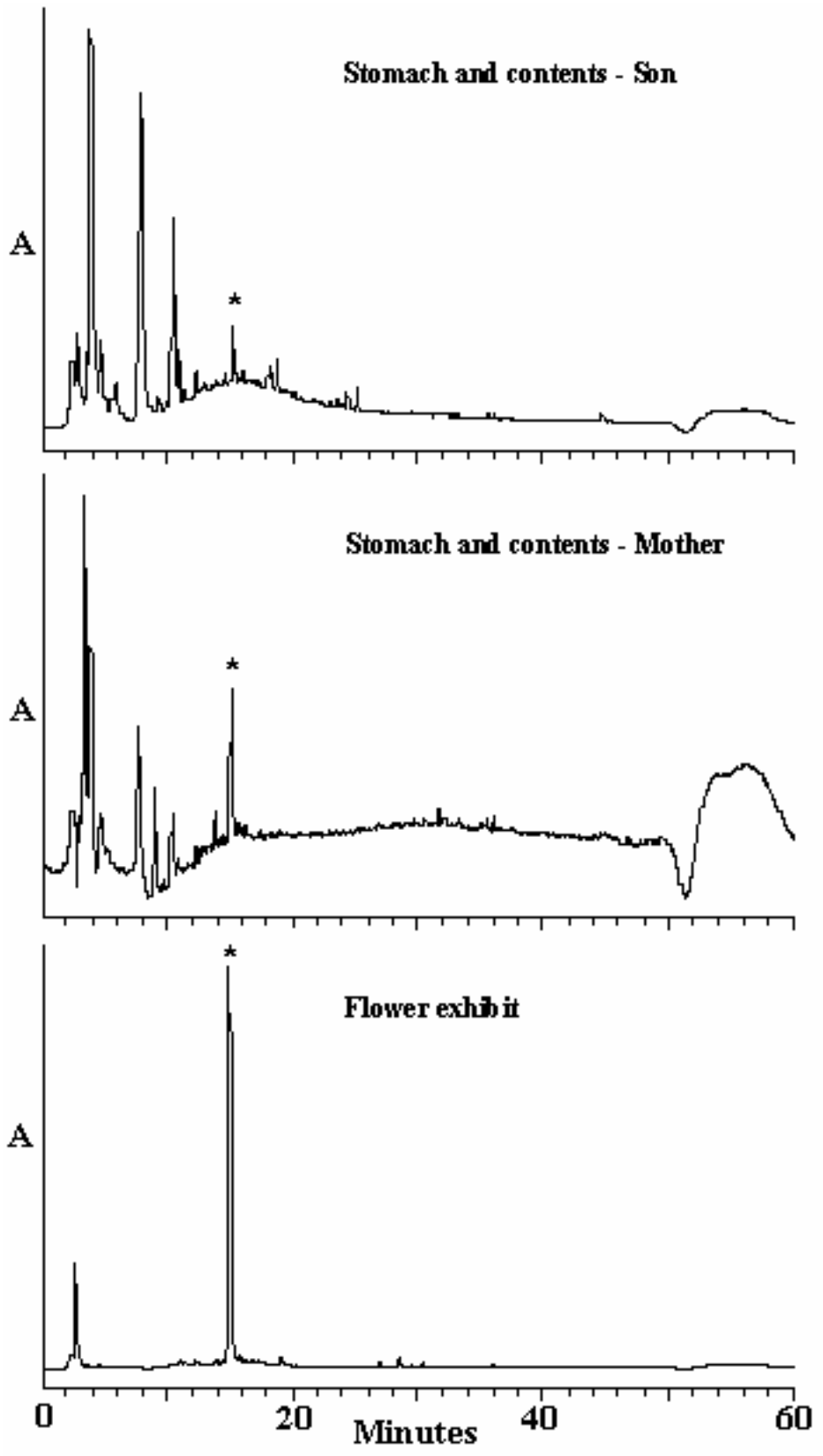


FIGURE 3.10

HPLC-UV analysis of the stomach contents of mother, child and the flower sample. Anabasine indicated with an astrix (*)

The PDA/ZMD chromatograms obtained from 10 µl injections of each stomach homogenate extraction and the 1 µl injection of the flower exhibit extract are given by Figure 3.11. The results obtained correlated very well with those from the PDA/TMD HPLC system as anabasine was detected in both viscera samples and the flower exhibit. The characteristic ESI^+ spectrum of anabasine was observed in all samples analysed.

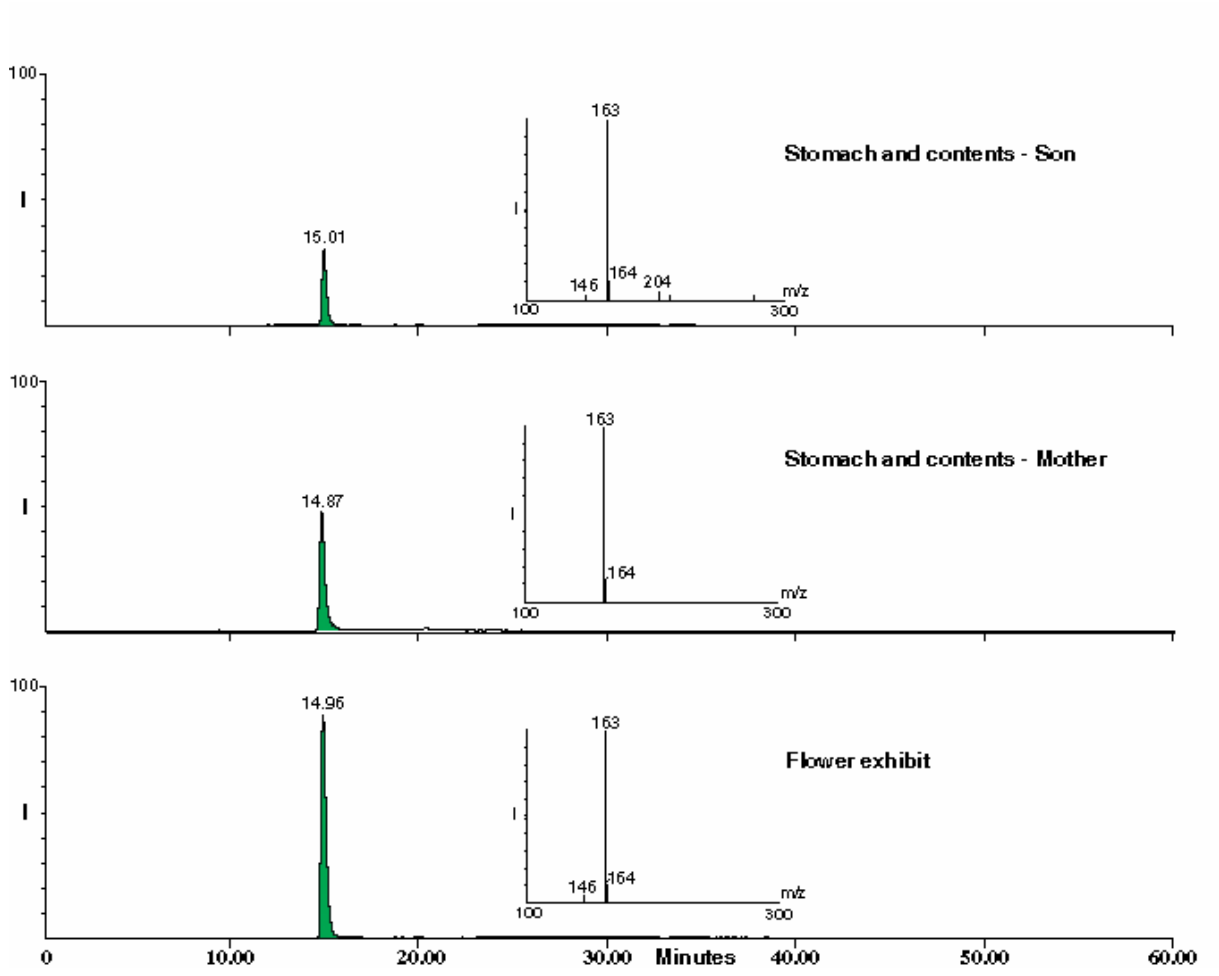


FIGURE 3.11

HPLC-ESI^+ analysis of the stomach contents of mother, child and the flower exhibit

3.10 FORENSIC APPLICABILITY

Anabasine was successfully detected and quantified by means of the HPLC – PDA or HPLC-MS system. The compound was successfully ionised in electron impact mode (TMD system) or API mode (ZMD system: single quadrupole operated in ESI^+ mode). However, the API mode produced a much stronger signal and facilitated the detection of anabasine at the low ng/ml

level. The low molecular mass of anabasine made the use of ISCID impractical as no significant fragmentation was observed above 100 amu.

Although the anabasine concentration in the stomach (and contents) of both the mother and son appeared to be too low for a fatal poisoning, it must be taken into consideration that both the mother and son vomited extensively before they died from a neuromuscular blockade resulting in respiratory suppression. Orfila's motto could not be fully satisfied in this case as no blood samples were submitted for toxicological evaluation.

This method is a good alternative to GC-MS for the detection, identification and quantification of anabasine in biological samples in a single analysis run, but will outperform most GC-MS (single quadrupole in EI mode) systems in the ng/ml concentration range when using electrospray technology.

CHAPTER 4

Datura stramonium / Datura ferox

4.1 BOTANICAL DESCRIPTION

Datura stramonium L. (Family Solanaceae), also called Apple of Peru, Devil's Apple, Devil's Trumpet, Jamestown Weed, Jimson weed, Mad-apple, Stinkweed, Thorn apple, Moon flower, Stinkblaar, Olieboom, *ijoyi* (Xhosa) or *iloyi* (Zulu) is an erect, subherbaceous annual up to 1.5 m high with dark green or purplish leaves which are usually paler below. Flowers are white, mauve or purplish with a narrow funnel shape (Figure 4.1). The erect fruit capsules are green but turning brown in colour, 50 mm long and covered with slender spikes up to 10 mm long [62] (Figure 4.2). These capsules are filled with numerous brown to black, kidney-shaped seeds of approximately 3 mm in length with a pitted exterior [63] (Figures 4.3 and 4.4). *Datura ferox* is closely related to *D. stramonium* and is often mistaken for it, but the capsules have fewer, larger and much thicker spines [64]. The flowers of *D. ferox* are also narrowly funnel-shaped but are only white in colour.

Figure 4.1
Typical *Datura ferox* plant

FIGURE 4.2
Developing unripe fruit of *Datura stramonium*

4.2 ORIGIN AND DISTRIBUTION

Certain authors reported the origin of *D. stramonium* as uncertain [62], but most agreed that this plant originated from the tropical areas of Central and South America [62, 63, 64] and it is now a cosmopolitan weed in temperate regions. It is commonly found along riverbanks, roadsides and disturbed sites and is widely distributed through South Africa [62, 63, 64] with its first documented appearance in 1714 [65].

FIGURE 4.3
Ripe fruit of *Datura ferox*

FIGURE 4.4
Seeds of *Datura stramonium*

4.3 TYPE OF TOXIN

The toxicity of *Datura* species is well known and has been linked to deaths and poisonings for centuries; some authors consider it responsible for the losses suffered by the army of Mark Anthony in 36 B.C. [65]. The main toxic principles are tropane alkaloids: hyoscyamine, which forms a diastereomeric (epimeric) mixture known as atropine upon isolation, and scopolamine [66]. Atropine (Figure 4.5) has a molecular mass of 289.37. Scopolamine (also called hyoscine, Figure 4.5) has a molecular mass of 303.36.

4.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

Atropine has anticholinergic activity and causes blurred vision, suppressed salivation, vasodilation, a relaxation of the bronchial smooth muscles, increased heart rate and delirium [63, 64, 67, 68, 69]. It also reduces rigidity in Parkinsonism and is used as an antidote to poisoning with parasympathomimetic agents, e.g. nerve gases and organophosphorus insecticides [69].

Scopolamine is an antimuscarinic agent and a smooth muscle relaxant. It is used as an antispasmodic agent with antinauseant properties, and is extensively used for the prevention of motion sickness and in pre-operative medication [63, 64, 69].

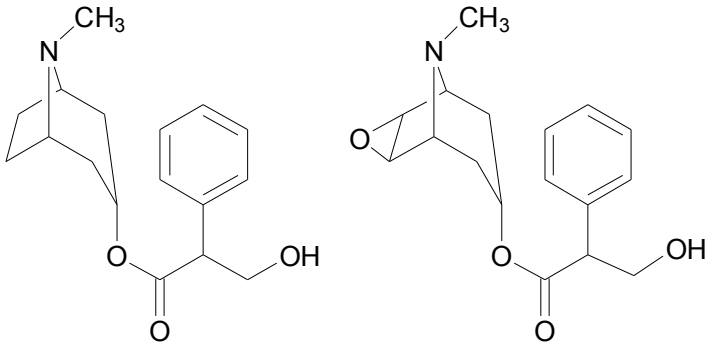


FIGURE 4.5

Chemical structures of atropine and scopolamine

4.5 ETHNOPHARMACOLOGICAL IMPORTANCE

Although *D. stramonium* and *D. ferox* are listed in South Africa as declared weeds [62], they are widely used as medicinal plants for the treatment of asthma and to reduce pain. Weak infusions are used as hypnotics by the elderly and as aphrodisiacs by adults. Other medicinal uses include the treatment of gout, boils, abscesses and wounds [63]. *Datura* species have also been used as medicinal plants in Argentina and by the American Indians. The native populations of the western Amazon, Andes, Colombia, Ecuador, Bolivia and Chile have used various *Datura* species as hallucinogens [65, 70]. *Datura stramonium* is also widely used in the mythical context in South Africa and its neighbouring states [71]. *Datura* species have also been used in criminal activities [67, 72]. In Europe, the seeds and plant extracts of *D. stramonium* were used in the treatment of mania, epilepsy, melancholy, rheumatism and convulsions [70].

4.6 POISONING

4.6.1 Accidental

Accidental *Datura* poisoning is quite common and various cases have been reported: *Datura* poisoning from eating a hamburger [73], scopolamine poisoning from homemade “moon flower” wine [74], atropine poisoning after drinking contaminated Indian tonic water [75], atropine poisoning after eating porridge contaminated with *D. stramonium* [76], atropine poisoning after drinking tainted comfrey tea [77], drinking of a tea blend containing *D. stramonium* leaves [78] and atropine poisoning after eating contaminated honey [79]. In Botswana, a large number of

people were affected after consuming sorghum flour contaminated with *Datura* seeds [80]. Many fatalities have occurred, especially among children who are attracted to the capsules and seeds [70].

4.6.2 Deliberate

Intentional poisoning with *D. stramonium* has also been reported in several cases, namely a fatal poisoning with *D. stramonium* [81] and *D. ferox* [82], the smoking and ingestion of *D. stramonium* for its mind-altering properties [83] and the eating and chewing of *Datura* seeds in a suicide attempt [84].

4.7 FCL JHB INVESTIGATION

Atropine is one of the compounds regularly detected at the FCL JHB, but has not been linked to an exposure to atropine-containing plants. Scopolamine has not been successfully detected probably due to its relatively poor UV characteristics and instability when analysed by GC or GC-MS. Although *D. stramonium* and *D. ferox* are listed in the FCL JHB database, they have not been positively linked to any poisoning or death due to exposure to botanically derived products (*muti)*. A good method that would detect atropine and scopolamine in a single run and at the ng/ml level was dearly needed and prompted the development of this method.

The developed method was successfully applied to a suspected heart attack case. A Caucasian male was rushed to hospital after apparently suffering of a mild heart attack. He was stabilised and sent home only to be rushed back a couple of days later after another heart attack. He could not be stabilised and died the same day. An anonymous phone call to the Police warned of the possibility that the deceased was murdered and a post mortem was done and the samples submitted to the FCL JHB for chemical analysis.

4.8 EXPERIMENTAL PROCEDURE

4.8.1 Standards and reagents

(±)-Atropine (99%), (-)-scopolamine hydrochloride (98%), potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (> 99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%) was obtained from BDH Laboratory Supplies, England. Sulfuric acid (Suprapur 96%), hydrochloric acid (Suprapur 96%) and ammonia (GR 25%) Pro Analisi was obtained from E-Merck, Darmstadt, Germany. Acetonitrile (gradient quality 200 nm UV cut-off) and methanol (gradient quality 205 nm UV cut-off) were obtained from Romil, England. HPLC grade water (20 MΩ) was obtained from a Milli-Q / reversed osmosis system (Millipore, USA). Oasis HLB solid phase extraction (SPE) cartridges

(60 mg) were obtained from Waters Corporation, Milford, USA. The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [56]. According to this method, PBS is prepared by adding the anhydrous salts of KCl (200 mg), NaCl (8000 mg), KH_2PO_4 (200 mg) and Na_2HPO_4 (1150 mg) to a 1-liter flask. One liter of deionized water is added and stirred to dissolve. Finally the pH was adjusted to 7.0 with 10% phosphoric acid. HCL-PBS was prepared as described above, but the pH was lowered to 5 with hydrochloric acid.

4.8.2 Instruments and conditions

A Waters 2690 HPLC system equipped with a 996 PDA detector and a Z spray mass selective detector (ZMD) (Micromass, UK) [electrospray mode (ESI); maximum mass 2000 m/z] was used in the ESI^+ mode. Various Waters Xterra HPLC columns were evaluated (RP-C18, MS-C18 and Phenyl), but the Waters Xterra Phenyl HPLC column (150 mm x 2.1 mm, 5 µm) performed the best and was used for all experiments. A Harvard syringe pump (Model 11) was obtained from Harvard Apparatus, Holliston, USA and was used for all direct injection experiments on the ZMD detector. Sample mixing was done on a Heidolph rotating sample mixer, and sample cleanup was done by centrifuging all samples at 4000 rpm using a Centronic S-577 centrifuge.

To ensure the maximum retention of basic alkaloids, the initial chromatographic conditions were 10% acetonitrile and 90% water containing 10 mM ammonium acetate, the pH adjusted to 10.5 with ammonia (25%). After injection, the chromatographic conditions were gradually changed through a linear gradient profile to 80% acetonitrile and 20% of the original aqueous mobile phase in 20 minutes. These conditions were kept stable for four minutes where after the HPLC system re-equilibrated the analytical column to the initial conditions. The flow rate was kept constant at 0.2 ml/min. The electrospray ionization (ESI) probe of the ZMD detector was optimised by direct injection via a syringe pump using an atropine test solution (10 µg/ml). The desolvation temperature was set to 400 ^oC and the source block temperature to 120 ^oC. The capillary voltage was set to 0.4 V, the extractor to 2.0 V, the RF lens to 0.5 V and the cone voltage to 25 V.

4.8.3 Chemical fingerprinting of *D. stramonium* and *D. ferox* plants

To evaluate the variation in alkaloid profile of seeds from the two *Datura* species, four plants of each species were collected from geographically isolated areas. For *D. stramonium*, four plants from each colour variety (white flowers and purple flowers) were collected. Table 4.2 gives sample details of the 12 *Datura* plants evaluated. Five seeds from the dry capsules of each plant were dried at 40 ^oC for 48 hours. The seeds were ground with a pestle and mortar and the pulp was extracted with three 5 ml aliquots of HCl-PBS buffer for 15 minutes. The HCl-PBS buffer extracts of each plant were combined and were eluted through an Oasis HLB (60 mg) solid

phase extraction cartridge and washed with 5 ml de-ionized water. The SPE cartridges were dried using vacuum and the alkaloids eluted with 5 ml methanol. The methanol fractions were evaporated under reduced pressure and reconstituted in 1 ml of methanol.

4.8.4 Chemical extraction and analysis of viscera and case exhibit

As the fatality under investigation was submitted to the Forensic Chemistry Laboratory as a suspected heart attack case, a general extraction procedure was initially followed. An aliquot of liquefied stomach (including contents), liver and kidney contents (3 g each) were mixed with 20 ml of PBS solution and shaken for thirty minutes on the rotating sample mixer. After centrifugation for 15 minutes the aqueous phase was passed through an Oasis HLB cartridge and the absorbed compounds stripped from the solid phase material with 5 ml methanol. The methanol was evaporated under reduced pressure and reconstituted in 0.5 ml of methanol. The sample (in methanol) was injected (10 µl) directly into the HPLC system and analysed using the optimised HPLC method described.

4.9 RESULTS AND DISCUSSION

4.9.1 Chromatography and detection of atropine and scopolamine

Due to the unstable nature of scopolamine [40, 41], atropine was used in the initial chromatographic studies. The high pK_a value (10.2) for atropine posed some problems as most analytical columns can not function at pH values higher than 9 due to degradation of the silica support structure of the reversed phase analytical columns. Lowering the pH will reduce column bleed, but results in poor peak shape and markedly reduced sensitivity. To enable direct coupling of the HPLC to the ZMD detector, a mobile phase containing ammonium acetate in the aqueous portion was evaluated. The Xterra range of columns from Waters, USA, (RP C18, MS C18 and Phenyl), which can be used at a pH higher than 9, were evaluated and found to be suitable for the chromatography of atropine. The introduction of scopolamine to the test mixture resulted in increased tailing of the atropine peak. This hampered the baseline resolution between atropine and scopolamine as scopolamine eluted just after the atropine peak. The Xterra Phenyl stationary phase did not suffer from this problem as the elution order reversed – scopolamine eluted first followed by atropine with a much improved peak shape.

A mobile phase containing 10 mM ammonium acetate, adjusted to a pH of 10.5 with ammonia, was found to result in reproducible chromatography without reduction in sensitivity. A typical chromatogram of an atropine/scopolamine test mixture (50 ng/ml of each analyte) (Figure 4.6) shows scopolamine with a retention time of 20.02 minutes and atropine with a retention time of 21.63 minutes. Under these chromatographic and mass spectrometric conditions, atropine produced an ESI^+ mass spectrum with a $(M+H)^+$ = 290.4 amu (which is also the base peak),

while scopolamine produced an ESI^+ mass spectrum with a $(M+H)^+$ = 304.4 amu (also the base peak) (Figure 4.7). Atropine displayed very little dissociation under these conditions, while scopolamine displayed minor in-source collision-induced dissociation (mass fragments at 138.3 and 156.4 amu). In-source collision-induced dissociation (ISCID) can be induced by increasing the cone voltage from 25 V to 50 V. This resulted in a characteristic fragmentation pattern for each alkaloid that can serve as a second confirmation of the analyte.

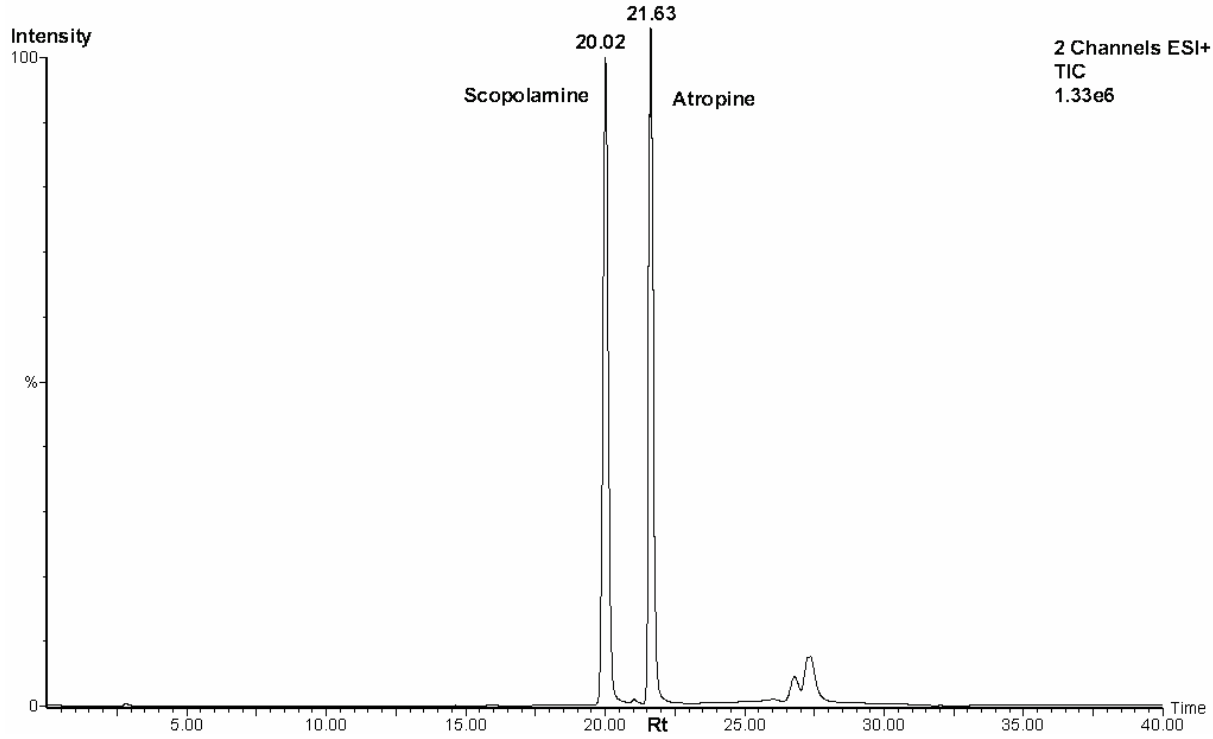


FIGURE 4.6

A typical ESI^+ chromatogram of 50 ng/ml atropine/scopolamine test mixture (ZMD system)

The limit of detection (LOD) and the limit of quantitation (LOQ) for the two detectors used are summarized in Table 4.1. The LOD was determined experimentally, and was taken as the concentration that produced a detector signal that could be clearly distinguished from the baseline noise (±3 times baseline noise). The LOQ was taken as the concentration that produced a detector signal ±10 times greater than the LOD signal. The poor LOD and LOQ values of the PDA detector can be ascribed to the poor chromophoric properties of atropine and scopolamine (Figure 4.8), which rendered UV detection unusable, and this detection technique was not further utilized. A calibration curve was prepared covering the 100 – 10 000 ng/ml concentration range. The coefficient of determination was 0.9987 and was obtained using a second order curve fitting algorithm ($r = 0.9987$; $r^2 = 0.9982$).

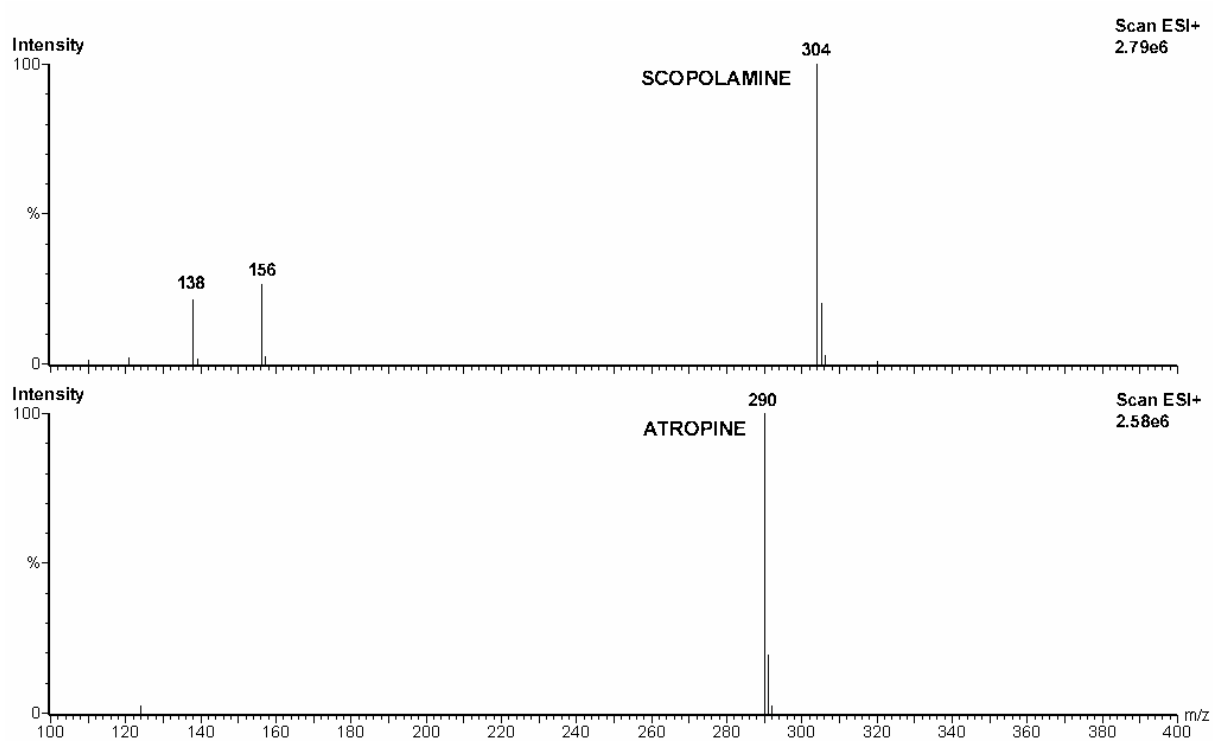


FIGURE 4.7

ESI$^+$ spectra of atropine and scopolamine as analysed on the ZMD system

FIGURE 4.8

UV spectra of atropine and scopolamine as analysed on the ZMD system

TABLE 4.1 LOD and LOQ results for atropine and scopolamine on the PDA and ZMD detectors.

DETECTOR	LOD Scopolamine	LOD Atropine	LOQ Scopolamine	LOQ Atropine
PDA (200 - 600 nm)	1 µg/ml	1 µg/ml	10 µg/ml	10 µg/ml
ZMD (100 - 400 amu)	100 pg/ml	10 pg/ml	100 ng/ml	1 ng/ml

4.9.2. Chemical fingerprinting of *D. stramonium* and *D. ferox* populations

The extracts of the seeds from the eight *D. stramonium* plants (Table 4.2) produced similar chromatograms with atropine as main component and scopolamine as minor component (Figure 4.9). The ratio between scopolamine and atropine was on average 1:7 for both colour varieties, but it must be noted that the ratio varied between populations and between colour varieties (1:8 average for purple and 1:6 average for white) (Table 4.1). The results differ from published results (plant material was basified with sodium hydroxide prior to extraction with dichloromethane) that stated that the scopolamine-atropine ratio is 1:2 [66]. The extracts of the seeds from the four *D. ferox* plants (WFDFW, ZRDFW, EBDFW and EDVFW) produced similar chromatograms, but with scopolamine as the main component and atropine as the minor component. The ratio between scopolamine and atropine was on average 7:1, but it must be noted that the ratio varied between populations (Table 4.2).

Visual differentiation between the seeds of *D. ferox* and *D. stramonium* was hampered by the large variation in size and colour between the species as well as within species. The chemical profile, however, clearly distinguished between the two species and was sufficiently uniform (both qualitatively and semi-quantitatively) in their alkaloid contents to allow a reliable characterization of the species.

4.9.3 Evaluation of SPE extraction and analysis of viscera samples

Due to the fact that the forensic samples were submitted to the Forensic Chemistry Laboratory as an unknown case, a general extraction of the viscera was performed. Traces of atropine was detected on the LC-MS system in the liquefied stomach sample, but could not be confirmed on the GC-MS system. A closer inspection of the minced stomach revealed several brown to black kidney shaped seeds, which was removed from the sample for separate analysis. A methanol extract of the intact seeds produced no significant results, but an extract of the ground seeds tested positive for atropine and scopolamine as the major and minor components, respectively (Figure 4.10).

TABLE 4.2. *Datura* species used, their geographical locations and the alkaloid ratio of the seeds.

Species	Collection site	Reference in text	Atropine : scopolamine Ratio in seeds
Datura stramonium (White flowers)	Witfield, Boksburg, Gauteng Province	WFDSW	6:1
	Mellville Koppies, Johannesburg, GP	MVDSW	4.6:1
	East Rand Mall, Boksburg, GP	ERMDSW	4.8:1
	Zeerust, North-West Province	ZRDSW	6.9:1
Datura stramonium (Purple flowers)	Elsburg, Germiston, GP	EBDSP	8.4:1
	Witfield, Boksburg, GP	WFDSP	8.6:1
	Edenvale, Kempton Park, GP	EDVDSP	6.9:1
	Zeerust, NWP	ZRDSP	7.6:1
Datura ferox (White flowers)	Witfield, Boksburg, GP	WFDFW	1:8.4
	Zeerust, NWP	ZRDFW	1:6.9
	Elsburg, Germiston, GP	EBDFW	1:5.6
	Edenvale, Kempton Park, GP	EDVDFW	1:7.2

The chromatographic and mass spectral profiles obtained were identical to that of the *D. ferox* seed extracts. The liquefied stomach sample, without any visible seeds present, as well as the liquefied liver and kidney samples were extracted utilizing the HCl-PBS buffer method. Atropine (major component) and scopolamine (minor component) were detected in the stomach sample (Figure 4.11) but not in the liver or kidney sample. These results were initially confusing as the same alkaloid profile as the intact seeds from the stomach (typical *D. ferox* alkaloid profile) was expected (scopolamine: atropine = 7:1). The obtained profile was more comparable with a *D. stramonium* alkaloid profile (scopolamine: atropine = 1:7). In an attempt to mimic the conditions in the stomach, a few *D. ferox* seeds were ground as before, but the HCl-PBS buffer was acidified to pH 1.5 with hydrochloric acid. The extract obtained from this experiment was analysed with the optimised HPLC-ZMD method and a very good match was obtained with the liquefied stomach sample that contained no seeds.

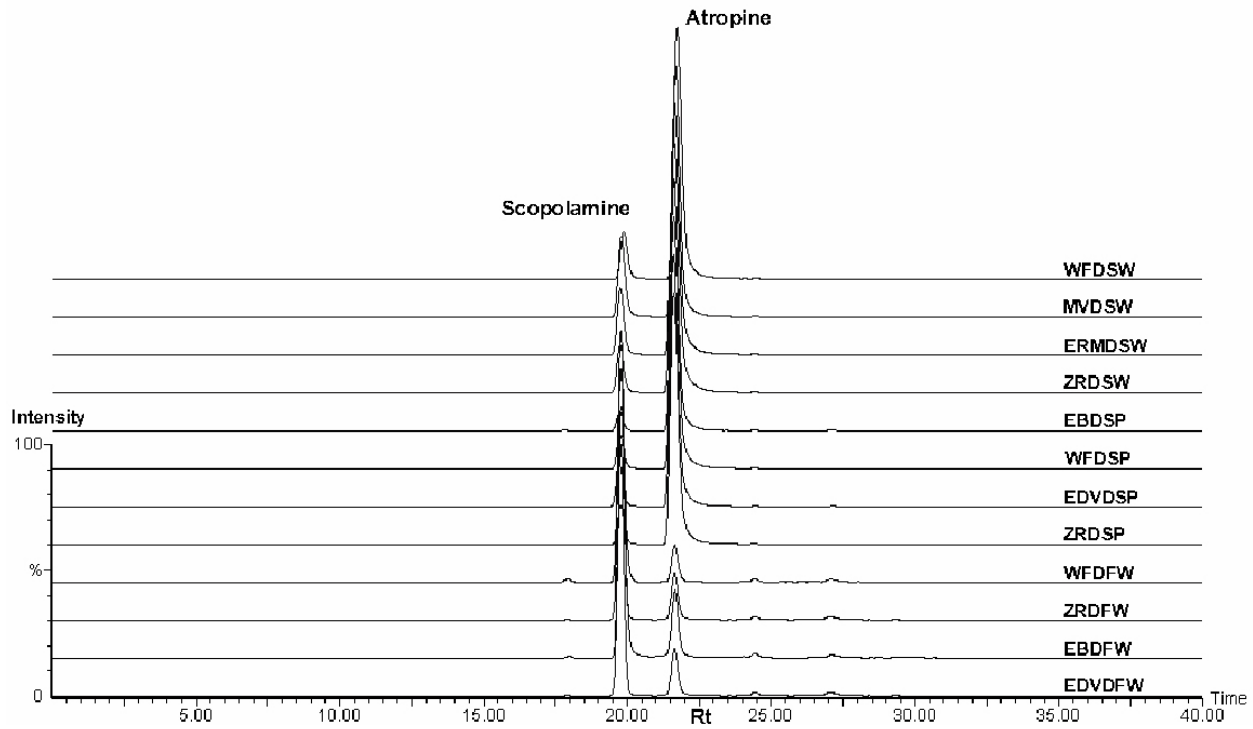


FIGURE 4.9

The chromatograms of the seed extracts from the twelve *Datura* plants (ZMD system)

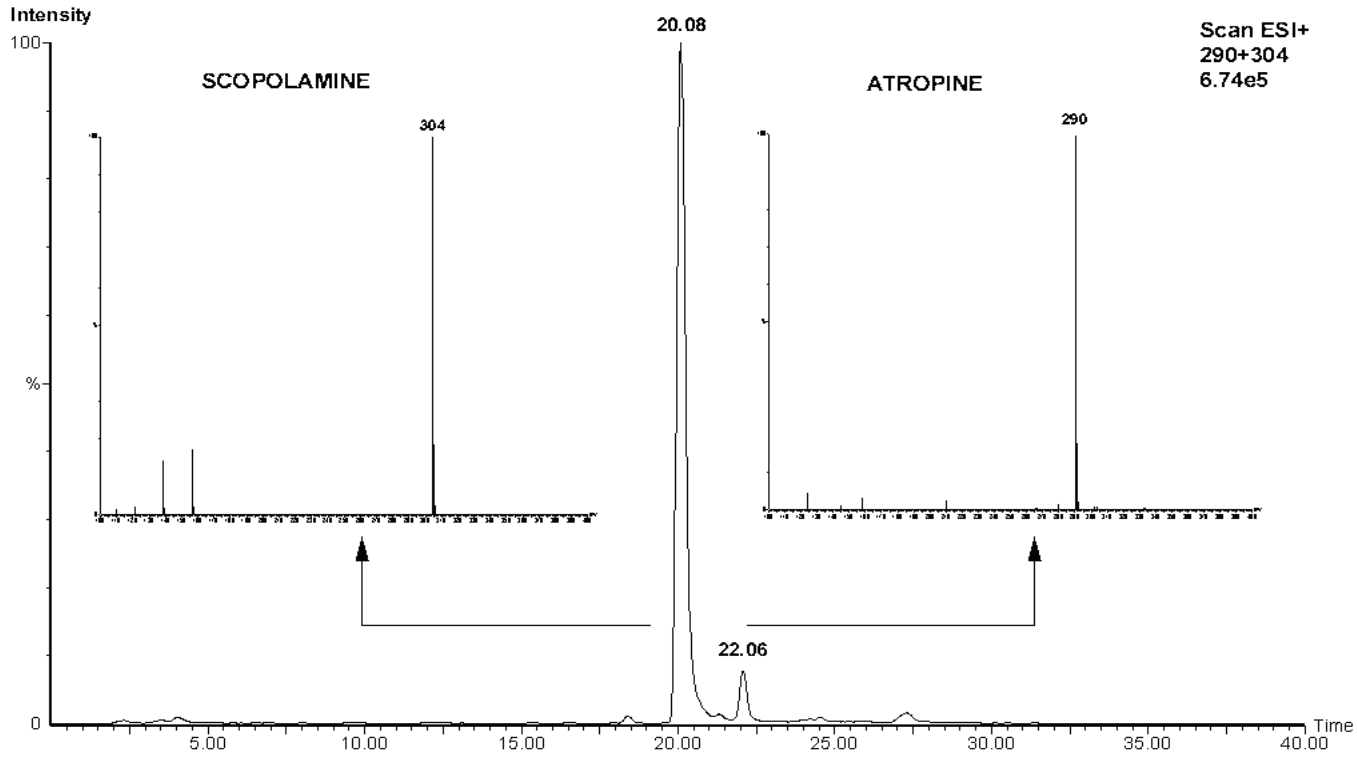


FIGURE 4.10

HPLC-ESI$^+$-MS analysis, after grinding, of the intact seeds found in the stomach (ZMD system)

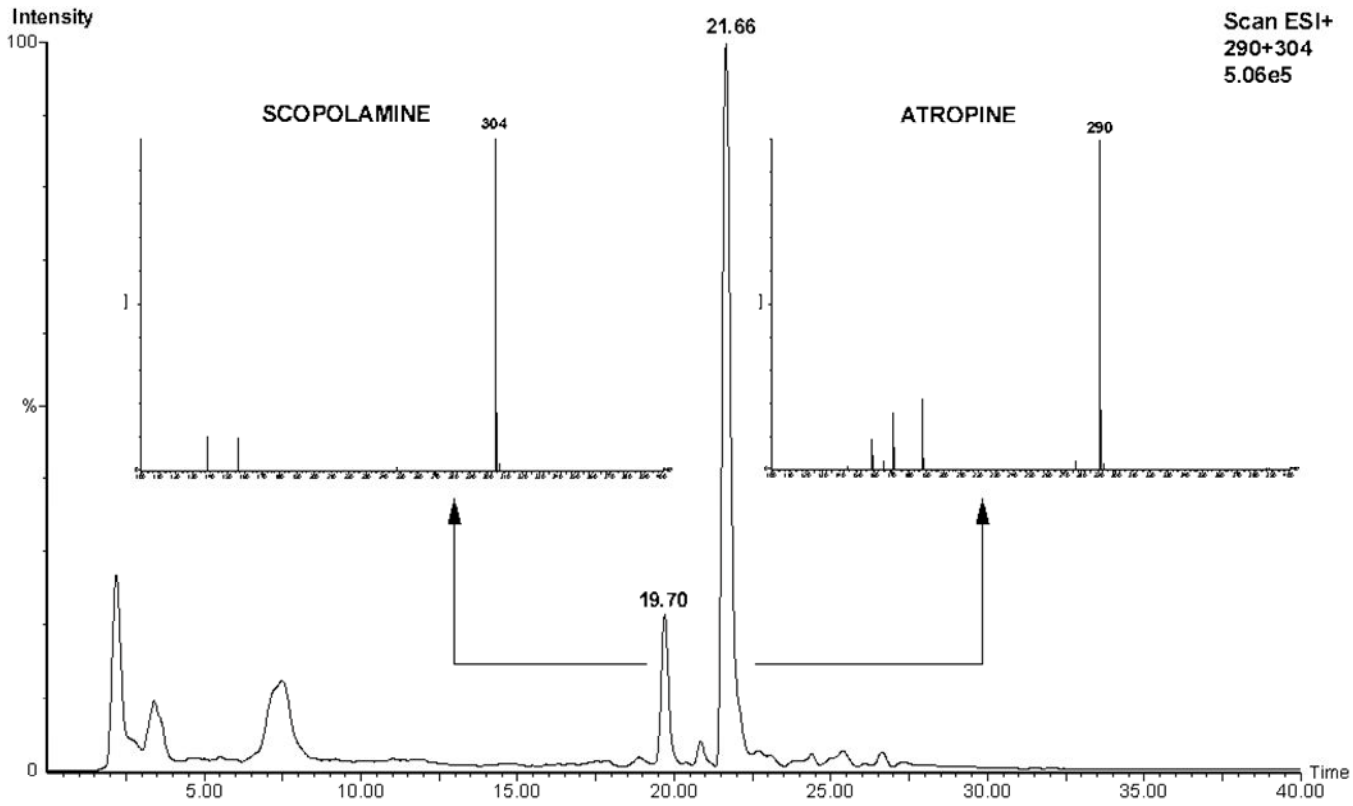


FIGURE 4.11

HPLC-ESI$^+$-MS analysis of the liquified stomach sample without visual seeds present (ZMD system)

This confirmed the hypothesis that *D. ferox* seeds were administered to the patient, and that the scopolamine released from the macerated seeds was hydrolyzed at strong acidic pH, but that the more stable atropine remained intact.

As a final confirmation of atropine and scopolamine in the seeds and liquefied stomach sample, the samples were re-analysed by utilizing a dual function MS method: Function 1 used a cone voltage of 25 V while Function 2 used a cone voltage of 50 V to induce ISCID. The results clearly confirmed the presence of atropine and scopolamine in the stomach and its contents (Figure 4.12).

4.10 FORENSIC APPLICABILITY

Atropine and scopolamine were successfully ionised in electron impact mode (TMD system) and API mode (ZMD system: single quadrupole operated in ESI$^+$ mode), but both compounds displayed superior sensitivity when using the API interface. Both compounds could also be confirmed on the ZMD system by ISCID and produced characteristic fragmentation mass spectra.

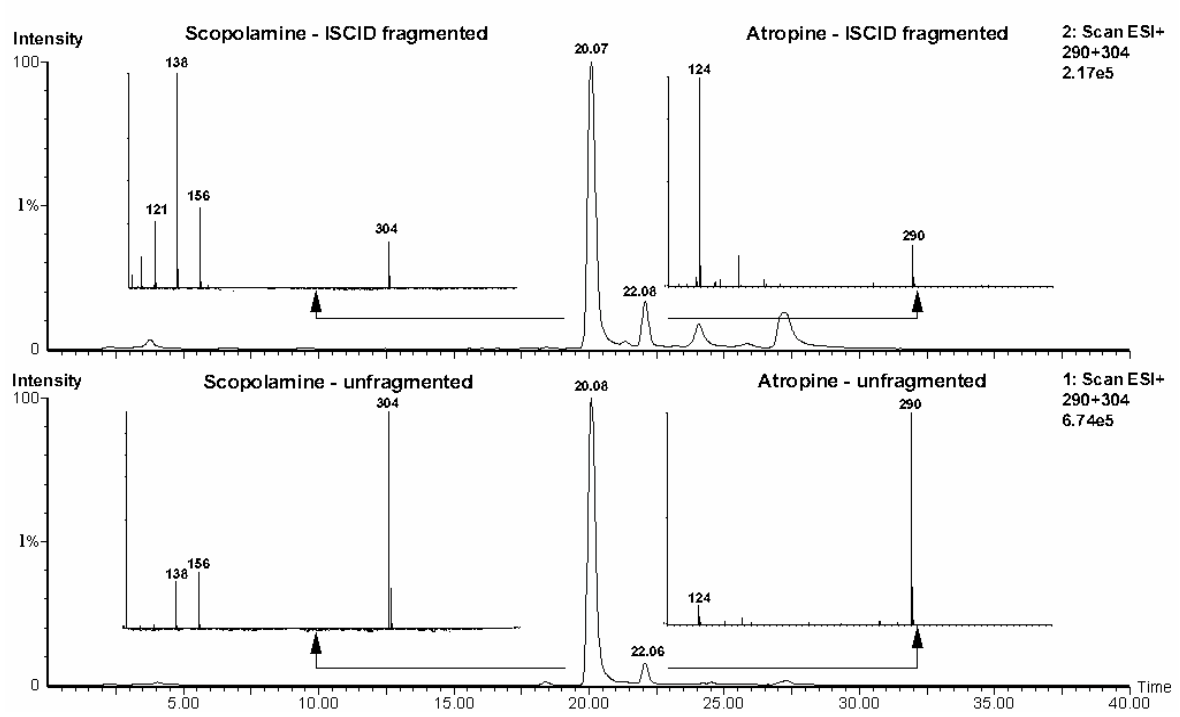


FIGURE 4.12

HPLC-ESI$^+$-MS analysis of the liquefied stomach contents (with seeds) using a dual scan function and ISCID (ZMD system)

The detection of these compounds by means of a HPLC-PDA system is not advised as both compounds displayed relatively poor UV characteristics. It is clear that HPLC coupled to an ESI mass selective detector offers a reliable and very sensitive method for the identification and confirmation of atropine and scopolamine in biological samples. By using the optimised technique described above, the seeds of *D. stramonium* can be distinguished from those of *D. ferox* by comparing their atropine-scopolamine ratios.

The absence of atropine/scopolamine (page 54) in the liver and kidney samples (the organs of metabolism and/or excretion) were puzzling. This might be due to the fact that the person died soon after the ingestion of the *D. ferox* seeds, resulting in minimal metabolism and/or excretion. Another explanation might be found in the sampling technique used at the mortuary – only a small portion of the liver and one kidney was removed and submitted for analysis.

CHAPTER 5

Callilepis laureola

5.1 BOTANICAL DESCRIPTION

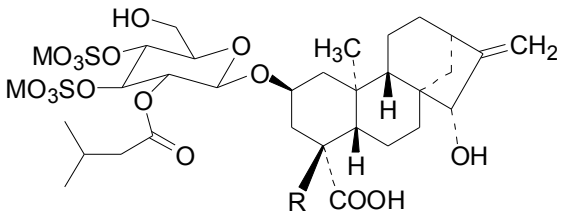
Callilepis laureola DC. (Asteraceae) is an attractive suffrutex of approximately 60 cm in height with erect, leafy annual branches arising from a permanent woody base [85, 86] (Figure 5.1). The flowers are borne in large heads comprising blackish disc florets surrounded by a single series of white ray florets. The plant is well known by the Zulu name *impila*, which means 'health' [85], but is also called the "Ox-eye daisy", "Wildemagriet" or *ihlmvu* (Zulu).

5.2 ORIGIN AND DISTRIBUTION

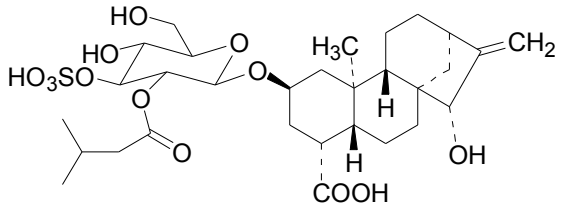
Callilepis laureola is widely distributed through the eastern part of South Africa, Zimbabwe and eastern Africa. There are five species of *Callilepis* which are all indigenous to southern Africa.

Figure 5.1

Typical habit of *Callilepis laureola*



Atractyloside (KATR): M=K, R=H
Protonated atractyloside (ATR): M=H, R=H
Carboxyatractyloside (CATR): M=H, R=COOH



Atractyloside monodesulfate

FIGURE 5.2

Chemical structures of KATR, ATR and [ATR-SO_3]

5.3 TYPE OF TOXIN

The toxic principles of *C. laureola* are atractyloside (KATR) (Figure 5.2), the dipotassium salt of a sulfonated kaurene glycoside, and carboxyatractyloside (CATR) (Figure 5.2), the carboxylated analogue of atractyloside [87, 88]. Carboxyatractyloside was also identified as the toxic principle of *Atractylis gummifera* L. (Asteraceae), a plant linked to human fatalities in Mediterranean countries since ancient times [89, 90, 91, 92]

5.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

The active components are atractyloside and carboxyatractyloside. Atractyloside has a strychnine-like action, producing convulsions of a hypoglycaemic type [93]. It acts as a specific inhibitor of oxidative phosphorylation by blocking ADP transport at the mitochondrial membrane and as such it is often used as a biochemical tool [91, 94]. No LD_{50} could be obtained from the manufacturers of KATR (Calbiochem, USA), but the calculated LD_{50} (orl) of carboxyatractyloside in rats is 13.5 mg/kg [95]. The onset of the first symptoms begins after 6 to 36 hours and includes nausea, vomiting and epigastric pain. This is very rapidly followed by a coma that deepens with time and may coinside with convulsions. The presence of a nephrotoxin and/or hepatotoxin has been frequently suggested by the pathologists of the Johannesburg mortuary due to simultaneous detection of severe liver and kidney damage during autopsies (personal communications with the pathologists of the Johannesburg mortuary).

5.5 ETHNOPHARMACOLOGICAL IMPORTANCE

The tuberous root of the plant (Figure 5.3) is used in traditional medicine by the Zulu and Xhosa people of South Africa [85, 96, 97, 98]. The application of *impila* range from alleviating stomach complaints, tapeworm infestations, it is utilised as a vermifuge, used for purgative applications, as a cough expectorant, for the treatment of whooping cough, for enemas and as an emetic. It is also used in spiritual context to ward off evil spirits [97, 99] and its spiritual use in people have been reported in Swaziland, Mpumalanga, Free State, KwaZulu-Natal and the Eastern Cape [100]. *Atractylis gummifera* is also used as a traditional medicine and topical use as a caustic on abscesses and boils has been reported [101].

5.6 POISONING

5.6.1 Accidental

Callilepis laureola has been implicated in the death of numerous patients who used medication (*muti*) prepared from *impila* [85, 96, 98, 102, 103, 104, 105, 106].

5.6.2 Deliberate

Deliberate poisoning with *C. laureola* has not been reported in literature, but the criminal addition of *A. gummifera* root, with the intention to kill, has been reported [90].

FIGURE 5.3

Tuberous roots of *Callilepis laureola*

5.7 FCL JHB INVESTIGATION

Callilepis laureola is one of the plants sometimes mentioned in case files submitted to the FCL JHB and reference to it was also found in the FCL JHB database. According to some authors *C. laureola* is one of the more commonly used medicinal plants in South Africa (see §5.5). Numerous forensic case files studied at the FCL JHB indicated the possibility that a nephrotoxin and/or a hepatotoxin was responsible for the death of the person, but chemical analysis did not reveal any toxic substances.

No reliable method existed for the analysis of the intact atractyloside using mass spectrometry, and the FCL JHB did not have any method for the extraction or detection of the compound.

5.8 EXPERIMENTAL PROCEDURE

5.8.1 Standards and reagents

Atractyloside (>99% by TLC), potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (> 99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%) (NH_4OAc) and acetic acid (99%) were obtained from BDH Laboratory Supplies, England. Sulfuric acid (Suprapur 96%), hydrochloric acid (Suprapur 96%), phosphoric acid (Suprapur 96%), formic acid (98 - 100%) and ammonia

(GR 25%) were obtained from Merck, Darmstadt, Germany. Acetonitrile (gradient quality, 200 nm UV cut-off) and methanol (gradient quality, 205 nm UV cut-off) were obtained from Romil, England. HPLC grade water (20 $M\Omega cm^{-1}$) was obtained from a Milli-Q / reversed osmosis system (Millipore, USA). The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [56]. The anhydrous salts of KCl (0.20 g), NaCl (8.00 g), KH_2PO_4 (0.20 g) and Na_2HPO_4 (1.15 g) were dissolved in 1 L deionised water and the pH adjusted to 7.0 with 10% phosphoric acid. HCl-PBS and NH_3-PBS were prepared as described above, but the pH was adjusted to 4.5 with hydrochloric acid and to 10.0 with ammonia, respectively.

Oasis HLB solid phase extraction (SPE) cartridges (3 ml with 60 mg of sorbent) were obtained from Waters Corporation, Milford, USA. A 12-port vacuum manifold was used for all extractions.

5.8.2 Instruments and conditions

5.8.2.1 LC – MS conditions

A Waters 2695 Separations Module (SM) equipped with a 996 photodiode array (PDA) detector and a Z spray mass selective detector (ZQ) (Micromass, UK) [Multi-mode ESCi, maximum mass 4000 *m/z*, ESCi (+/-) mode] was used. The ESCi interface was only used in the negative-ion electrospray mode. Various reversed phase HPLC columns from Phenomenex (Luna C18, Max-RP, Polar-RP), Thermo Hypersil-Keystone (Hypercarb, Duet), and Waters (Xterra RP-C18, MS-C18 and Phenyl) were evaluated. All the column dimensions were 250 x 2.1 mm except for the Xterra Phenyl column that was 150 x 2.1 mm. The built-in syringe pump was used for all flow-injection and optimisation experiments on the ZQ detector. For optimal chromatography, the following conditions were used: a linear gradient mobile phase consisting of buffer (25 mM NH_4OAc, pH 6.7): acetonitrile, starting at 90% buffer: 10% acetonitrile and ending at 10% buffer: 90% acetonitrile after 20 minutes. The column was allowed to flush at the high organic mobile phase for 5 minutes where after the mobile phase composition was changed to initial conditions and allowed to equilibrate for 10 minutes. The flow rate was 0.2 ml/min without splitting. However, these conditions were not optimal for the mass spectral detection of the atractyloside standard or the separation and detection of atractyloside in a biological matrix. The best-compromised conditions for the detection of atractyloside by LC-MS are summarised in Table 5.1. The HPLC column was kept at 40 °C for all experiments. Preliminary MS optimisation was performed using an APCI interface in ESI^+, ESI^-, $APCI^+$ and $APCI^-$ modes. High purity nitrogen gas (Air Products) was used as nebulising gas at a flow rate of 450 L/h while cone gas was utilised at a flow rate of 30 L/h. In later experiments, the cone gas was found to reduce the efficiency of the ionisation of atractyloside and was deactivated. The optimised MS conditions are collated in Table 5.2. Optimisation experiments were done in scan mode (*m/z* 200 – 900) and quantitative work was done in single ion monitoring (SIM) mode with a dwell time of 0.6 s

and a span of 0.4 Dalton. The ions used for SIM experiments were the deprotonated molecule ion at *m/z 725* and the main fragment ion at *m/z* 645.

TABLE 5.1 Optimised HPLC conditions for the detection of atractyloside (ZQ system)

VARIABLE	OPTIMISED
Column	Waters Xterra Phenyl 300 x 2.1 mm (3 µm) (2 x 150 mm columns in series)
Column temperature (°C)	40 ± 1
Mobile phase components	Methanol Acetonitrile H_2O (10 mM NH_4OAc pH 4.5)
Gradient profile	Initial conditions of 90:10 H_2O:MeOH for 2 minutes Linear gradient to 10:90 H_2O:ACN for 18 minutes Isocratic conditions of 10:90 H_2O:ACN for 5 minutes Return to initial conditions within 5 minutes Equilibrate for 5 minutes
Mobile phase flow rate	0.2 ml/min
Degassing	In-line (normal mode)
Sample temperature (°C)	5 ± 1

TABLE 5.2 Optimised ESI-MS conditions for the detection of atractyloside (ZQ system)

CONDITION	ATR	ATR-SO_3
Mode and polarity	ESI negative	ESI negative
Capillary voltage (KV)	3.00	3.50
Cone voltage (V)	27	65
Desolvation temperature (°C)	400	450
Extractor voltage (V)	1.0	1.0
RF lens (V)	1.0	3.5
Source temperature (°C)	120	120
Cone gas flow (L/h)	0	0
Desolvation gas flow (L/h)	450	450
Ion Energy	0.5	0.5
Multiplier (V)	-650	-650
Resolution (LMR & HMR)	15.8	15.8

5.8.2.2 Flow-injection analysis experiments

Flow-injection analysis (FIA) experiments were initially done by infusing 10 μl/min of 10 μg/ml atractyloside standard directly into the MS interface. This however, produced a very unstable MS signal. Subsequent FIA experiments were done by infusing the atractyloside standard into the HPLC mobile phase of 0.2 ml/min 50:50 buffer: methanol. This greatly improved the MS signal strength as well as the stability of the signal. However, in the presence of methanol, atractyloside was poorly ionised and in later experiments, the mobile phase was changed to 50:50 buffer (10mM ammonium acetate, pH 4.5 with acetic acid): acetonitrile.

5.8.2.3 Evaluation of the analytical procedure

The limit of detection (LOD) for the standard was determined experimentally, and was taken as the concentration of the standard that produced a detector signal that could be clearly distinguished from the baseline noise (±3 times baseline noise). The limit of quantitation (LOQ) was taken as the concentration of the standard that produced a detector signal ±10 times greater than the LOD signal. The practical limit of quantification was determined by spiking old impila tuber and extracting the spiked sample as described. A calibration curve was prepared covering the 1 ng/ml – 160 μg/ml concentration range. The precision of the instrumental method was determined by multiple injections (n=10) of the atractyloside standard at three concentration levels covering the analytical range of the method.

5.8.3 Extraction of *Callilepis laureola* tubers and unknown powdered samples

Tubers of *C. laureola* were obtained from the local traditional healer's market in Johannesburg. A second, fresh sample came from the *muti* market in Durban, South Africa. The unknown powdered samples were submitted to the Forensic Chemistry Laboratory as part of Police Forensic exhibits linked to ten cases submitted to the Laboratory for toxicological screening and possible identification. The tubers were gently cleaned with a brush prior to extraction to remove soil and bulk surface contaminants and were then grated by hand and thoroughly mixed to provide a homogeneous sample.

Five 1 g aliquots of the ground tuber were placed in separate 50 ml polypropylene screw-top centrifuge sample tubes. Thirty milliliters (30 ml) of the three PBS buffer mixtures (phosphate, HCl and ammonia), de-ionised water and methanol, respectively, were added to the sample tubes. Samples were mixed on a Heidolph rotating sample mixer for 30 minutes, and the supernatant recovered by centrifugation at 4000 rpm using a Hermle Z 300 centrifuge for 15 minutes. Five SPE cartridges were prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used. The supernatants were applied to the SPE cartridges by hand and drawn through the cartridges at approximately 1 ml/min by applying a mild vacuum.

The cartridges were washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. They were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates of each cartridge combined into one collection tube. The collection tubes were placed into a Zymark Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of a 50:50 methanol: water mixture. The powdered samples, of unknown origin, were extracted as described above, but by using the standard Waters SPE method (PBS buffer) [56] and adjusting the pH of the buffer to 4.5 with phosphoric acid.

5.8.4 Chemical extraction and analysis of viscera and case exhibit

An aliquot of each liquefied viscera sample [stomach (including contents), liver and kidney] (3 g each) were placed in separate 50 ml polypropylene screw-top centrifuge sample tubes. Thirty milliliters (30 ml) of PBS-HCl buffer was added to each sample and the tubes shaken on a Heidolph rotating sample mixer for thirty minutes. After centrifugation at 4000 rpm using a Hermle Z 300 centrifuge for 15 minutes the aqueous phase of each sample was passed through an Oasis HLB cartridge and the absorbed compounds stripped from the solid phase material with 5 ml methanol. The SPE cartridges were prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used. The cartridges were washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. They were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates of each cartridge combined into one collection tube. The collection tubes were placed into a Zymark Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of a 50:50 methanol: water mixture.

5.9 RESULTS AND DISCUSSION

5.9.1 Chromatography and detection of atractyloside

Atractyloside (KATR, Figure 5.2), first isolated by M. Lefranc in 1868 [14], has an empirical formula of $C_{30}H_{44}K_2O_{16}S_2$ and a molecular mass of 802.13 [protonated form ATR (Figure 5.2): $C_{30}H_{46}O_{16}S_2$, molecular mass 726.22]. The compound contains multiple polar groups and is soluble in water but has relatively poor solubility in organic solvents. Under the normal reversed-phase chromatographic conditions, the acidic form of atractyloside (ATR) is virtually unretained on all the Phenomenex, Waters and Hypersil-Keystone columns we tested, except for the Waters Xterra Phenyl and Hypersil Hypercarb columns. The Hypercarb column showed excessive retention, as ATR is strongly attracted to the graphite surface and displayed pronounced tailing. Retention on the Waters Xterra Phenyl column (150 mm x 2.1 mm, 5 µm)

depended on the buffer concentration and pH. This column outperformed the other HPLC columns evaluated and was used for all experiments. The best chromatographic effects were observed using an eluent containing ammonium acetate buffer (25 mM at pH 6.7) and acetonitrile. These conditions however, were not conducive to the MS detection of ATR due to ionisation suppression. By lowering the buffer concentration to 10 mM and adjusting the pH to 4.5 with acetic acid, stable chromatography and ionisation were observed for ATR.

ATR was poorly detected on the UV (PDA) detector due to the chromophoric properties of the compound, and this detection mode was discontinued in subsequent experiments. To obtain optimal MS conditions, flow-injection analysis experiments were performed. The results obtained from FIA experiments differed, as expected, from those obtained by injecting the atractyloside standard with the 2695 SM. Therefore, only those results that had an impact on the LC-MS separation and detection of ATR, were further investigated and discussed. ATR was only observed in the ESI negative (ESI^-) mode and all other detection modes were deactivated. It was found that a cone voltage of 27 V was optimal for the detection of the unfragmented ATR $[ATR-H]^-$ (*m/z* 725). Increasing the cone voltage to 65 V resulted in the fragmentation of ATR and the formation of a desulfated species $[ATR-SO_3-H]^-$ (*m/z* 645). Under chromatographic conditions, at a low cone voltage (27 V), the full scan spectrum of ATR (Fig. 5.4) was dominated by the deprotonated ion $[ATR-H]^-$ (*m/z* 725). Other ions observed were $[ATR-2H+Na]^-$ at *m/z* 747*,* a fragment due to the loss of SO_3 $[ATR-SO_3-H]^-$ at *m/z* 645, as well as two doubly charged ions, $[ATR-2H]^{2-}$ and $[ATR-SO_3-2H]^{2-}$, at *m/z* 362 and 322, respectively.

As mentioned before, ATR was only observed in the ESI negative mode, and was very susceptible to changes in the eluent composition, MS parameters and nebulisation process. In FIA experiments, a 100 μg/ml atractyloside standard produced poor detector response on the ZQ detector. The use of a 1000 μg/ml atractyloside standard for experimental work led to excessive contamination of the MS inlet. Deactivation of the cone gas resulted in a dramatic improvement in detection and allowed the use of a 10 μg/ml standard. The eluent composition also affected the ionisation of ATR. In FIA experiments, methanol: buffer was used as eluent, but the presence of methanol in the eluent suppressed the ionisation of ATR and acetonitrile was used in subsequent experiments. This change in chromatographic conditions not only enhanced the detection of ATR in LC-MS analysis, but also improved peak shape and shortened, as expected, the retention time of ATR. However, the presence of acetonitrile in the initial mobile phase resulted in co-elution of the various compounds detected in the tuber extracts. A starting mobile phase of buffer: methanol, followed by a replacement of the methanol with acetonitrile after two minutes, restored the resolution needed without any compromise in the ionisation of ATR. Under these conditions, ATR produced a single chromatographic peak at R_t 18.18 minutes (Figure 5.4) displaying the characteristic fragmentation described above (Figure 5.4).

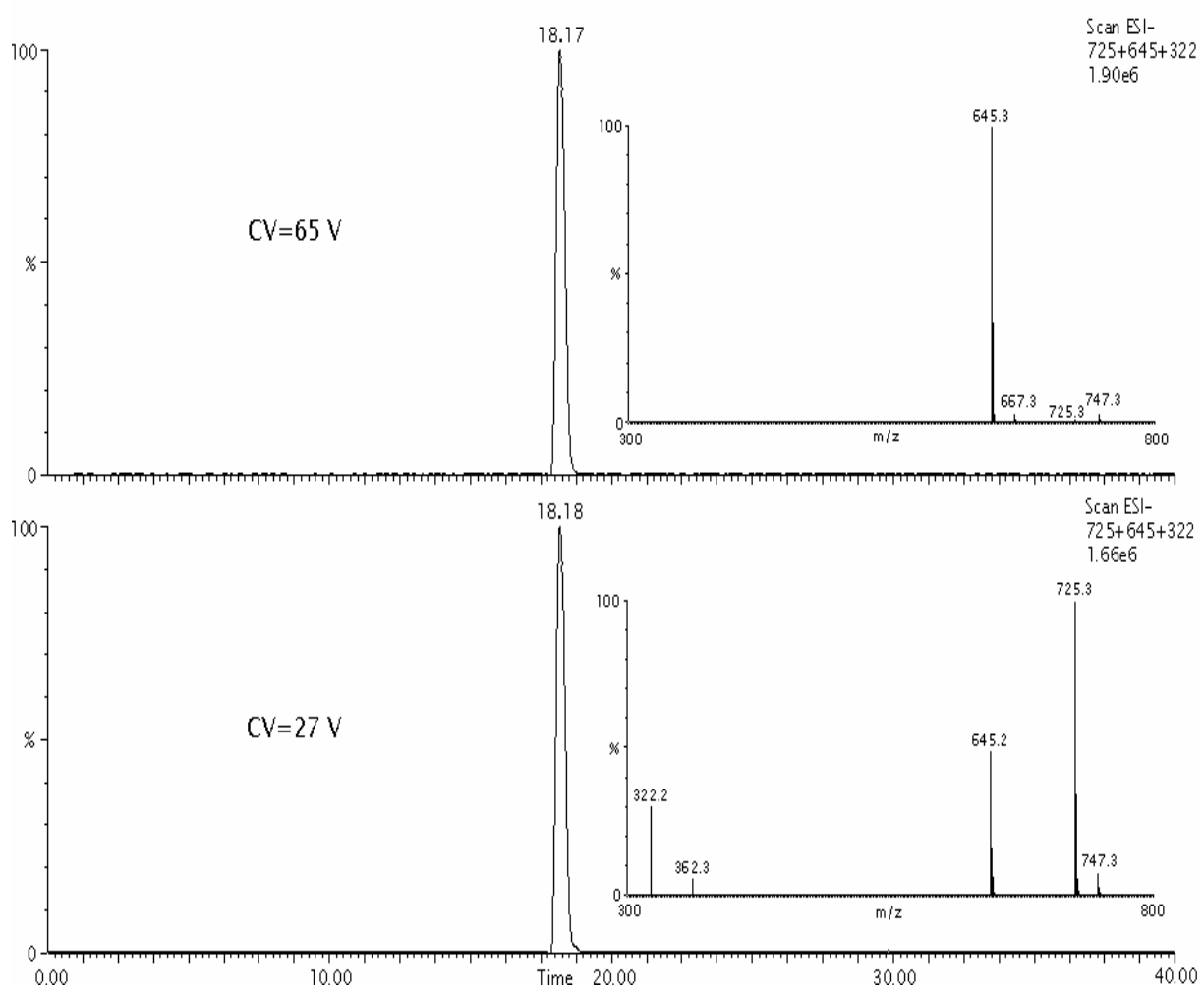


FIGURE 5.4

ESI$^-$ chromatograms and spectra of a 10 µg/ml ATR standard obtained under optimised conditions (ZQ system)

5.9.2 Evaluation of the analytical method

The LOD was determined by using various dilutions of the atractyloside standard. The LOD of ATR in SIM mode, using the main fragmentation ion (*m/z* 645) was found to be 100 pg/ml and the unfragmented ion (*m/z* 725) was used as confirmation. The calibration curve covering the 1 ng/ml – 160 µg/ml range, was best described by a second order polynomial function (r^2 = 0.998). For the range 100 – 1000 ng/ml, the calibration curve was linear with a coefficient of determination of 0.999. The precision of the method was determined and the results are summarised in Table 5.3. The RSD's of the peak areas for the standards were on average below 3 % (n=10), while the RSD's in the biological matrices increased to 3.5 % (n = 8) in the fresh *impila* tuber and 5.2 % in the unknown powdered sample # 207.

TABLE 5.3 Evaluation of RSD values in various matrices for ATR analysis (ZQ system)

SAMPLE	CONCENTRATION	N	% RSD ON PEAK AREA OF ATR
ATR Std	10 ng/ml	10	2.9
	1 μg/ml	10	2.2
	100 μg/ml	10	3.8
Fresh Tuber	N/A	8	3.5
Unknown powder # 207	N/A	8	5.2

5.9.3 Solid phase extraction of ATR, *C. laureola* tuber and powdered herbal samples

Initial SPE experiments with an atractyloside standard indicated that the compound could be successfully retained and eluted from the Oasis HLB cartridges with any of the PBS buffers as well as water. However, extraction of atractyloside from the tuber produced mixed results. Of the five extraction solvents evaluated (PBS-Phosphate, PBS-HCl, PBS-NH_3, MeOH and H_2O), the MeOH extracted the least atractyloside, followed by H_2O, PBS-Phosphate and PBS-NH_3. Although the PBS-HCl yielded the most atractyloside, the PBS-NH_3 produced the cleanest extract but failed to extract all the compounds of interest (CATR was not detected in this extract). The PBS-HCl buffer was selected as the buffer of choice for all further extractions as it extracted all the compounds of interest.

Unknown powdered samples are regularly sent to our laboratory as part of forensic investigations. These samples are usually of botanical origin, sometimes fortified with metal salts to create a "stronger *muti*", or might even contain human or animal remains. Ten cases involving unknown powdered samples were previously extracted according to our systematic toxicological screening process and found to contain no toxic substances. As the original samples were no longer available, the original extracts of the powdered samples were used.

5.9.4 Analysis of *C. laureola* tuber and unknown powdered samples

The extract of the *impila* tuber obtained from the local traditional healer's market was analysed with the method described above. The masses of interest, namely 725, 645 and 322 were monitored simultaneously. In initial experiments with a 150 mm column, it was clear that co-elution prevented obtaining a clean spectrum for each compound eluted. The column length was doubled (two columns used in series) and the mobile phase changed to afford better separation without drastically altering the retention times of those compounds detected (see 5.8.1). The optimised HPLC conditions are summarised in Table 5.1 and the optimised MS conditions are listed in Table 5.2. These conditions were found to give near-baseline resolution of the

compounds detected. The *impila* tuber extract was re-analysed with the optimised HPLC conditions (Figure 5.5). From the results, it was clear that ATR was not present, but that the main constituent was a monodesulfated derivative of carboxyatractyloside (R_t 17.44). Also detected was the monodesulfated derivative of atractyloside (R_t 21.16), a compound that could also be produced by in-source collision-induced dissociation (ISCID) of atractyloside. The absence of atractyloside was most likely caused by ageing, as these tubers were stored at room temperature for two years.

Fresh *C. laureola* tubers were obtained and sample preparation was done as discussed above. The extract was analysed with the optimised HPLC method. The results (Fig. 5.6) were similar to those obtained with the old tubers. Although the monodesulfated derivatives of atractyloside and carboxyatractyloside were still present, atractyloside (R_t 18.25) was detected as the major component in the tuber extract (8.8 ± 0.4 µg/g; n = 5). It is possible that in the plant material atractyloside and carboxyatractyloside loses one sulfate group with ageing. As tubers used in traditional medicine may be harvested and stored for extended times before utilisation, the possible degradation of atractyloside should always be taken into account in forensic cases. Therefore, screening methods should be developed that do not only screen for ATR and CATR, but also for their monodesulfated derivatives.

The ten cases involving unknown powdered samples were analysed with the optimised method. ATR was detected in four of the cases in trace levels, but case # 207 contained sufficient ATR levels to enable quantification (10.1 ± 0.6 ng/g; n = 5) (Figure 5.7). The low ATR levels may be due to degradation of the sample (assuming the whole sample was *impila* tuber), or might indicate that the powder was formulated from more than one plant source and that *impila* was but one of a few plants used to formulate the *muti*.

5.9.5 Solid phase extraction and analysis of viscera samples

Due to a lack of a suitable chromatographic separation and mass spectral detection technique for ATR and CATR in the FCL JHB, extracted viscera were not screened for the presence of these two compounds prior to the development of the method described here. A selection of 70 cases, previously analysed and found to be negative (no toxic substances detected), were analysed with the full scan method. The results were disappointing as no ATR, CATR or any of the monodesulfated compounds could be detected. The samples were re-analysed with the more sensitive SIM method by using the main fragmentation ion (*m/z* 645). The results indicated that 65% of the cases contained compounds with the *m/z* 645 mass ion. The samples were then re-analysed with a dual-function method using both the SIM masses (*m/z* 725 and 645). The new set of results indicated that only 45% of the cases contained compounds with both mass ions, the correct mass ion ratios and within the retention time window.

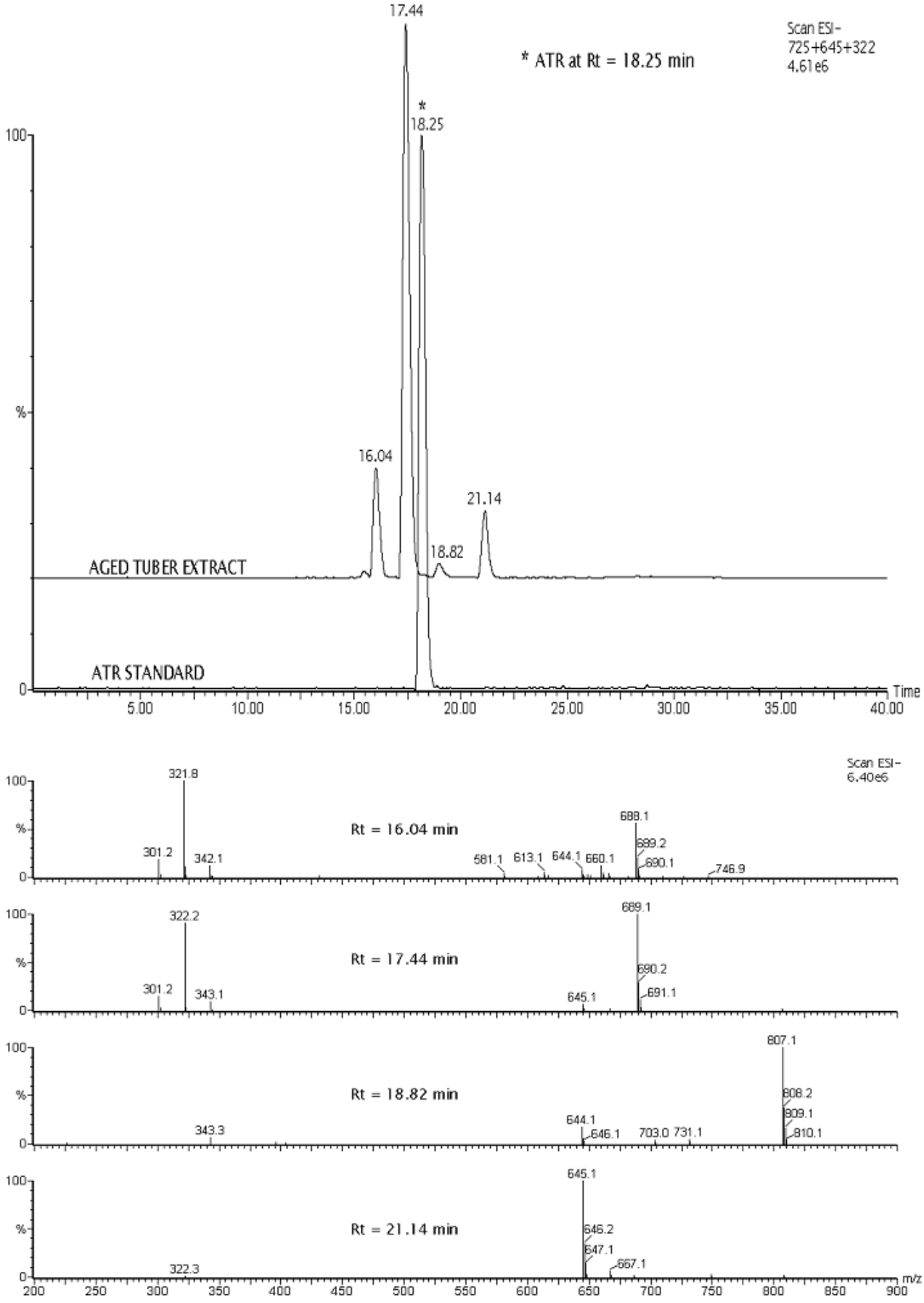


FIGURE 5.5

ESI$^-$ chromatogram and spectra of the main compounds detected in the aged tuber extract. Overlaid the chromatogram of a 10 μg/ml ATR standard (ZQ system)

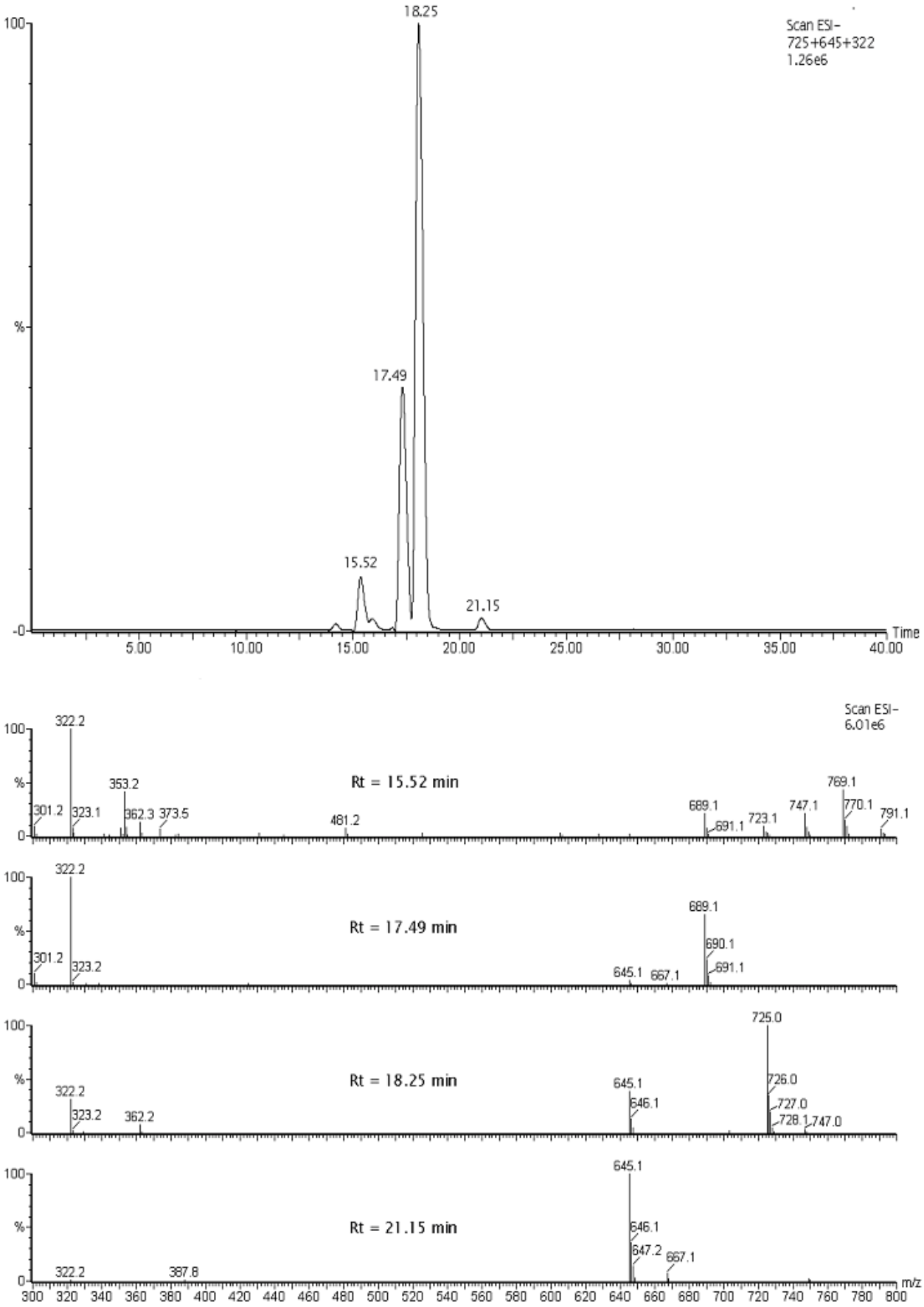


FIGURE 5.6

ESI$^-$ chromatogram and spectra of the main compounds detected in the fresh tuber extract (ZQ system)

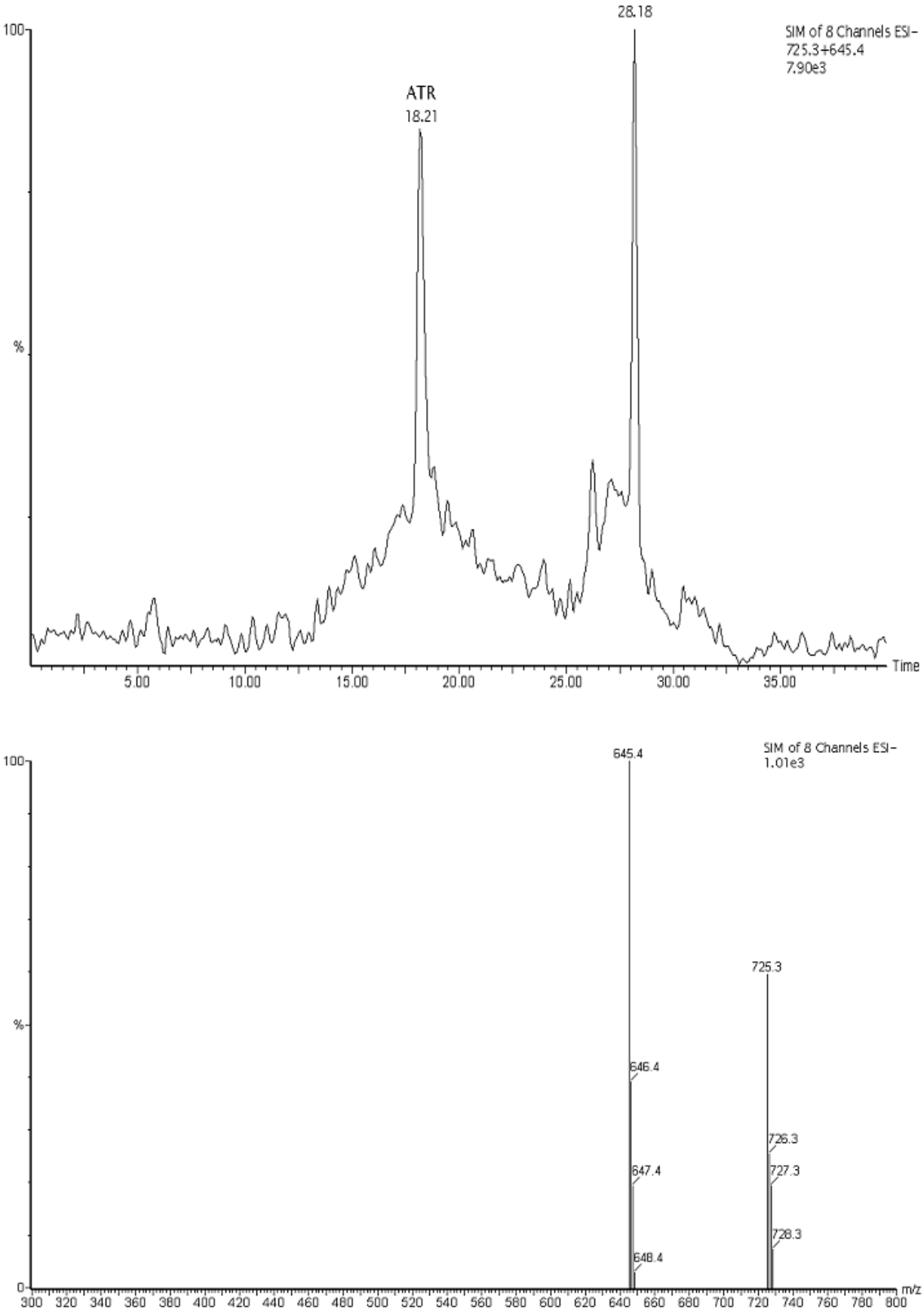


FIGURE 5.7

ESI$^-$ chromatogram and spectra of the analysis of the unknown powdered sample of Case 207 (ZQ system)

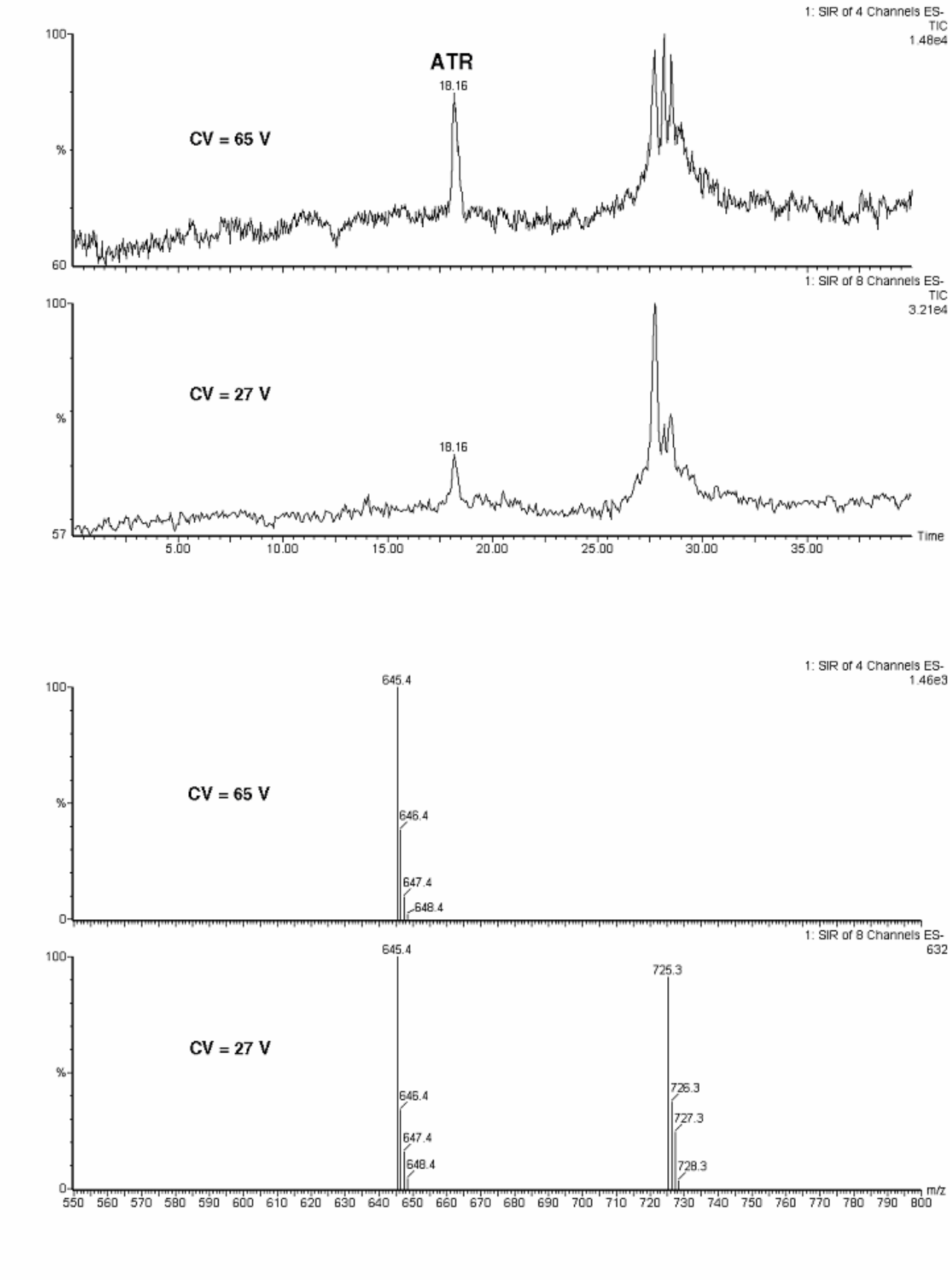


FIGURE 5.8

ESI$^-$ SIM analysis of the unknown powered sample of Case 1294 (ZQ system)

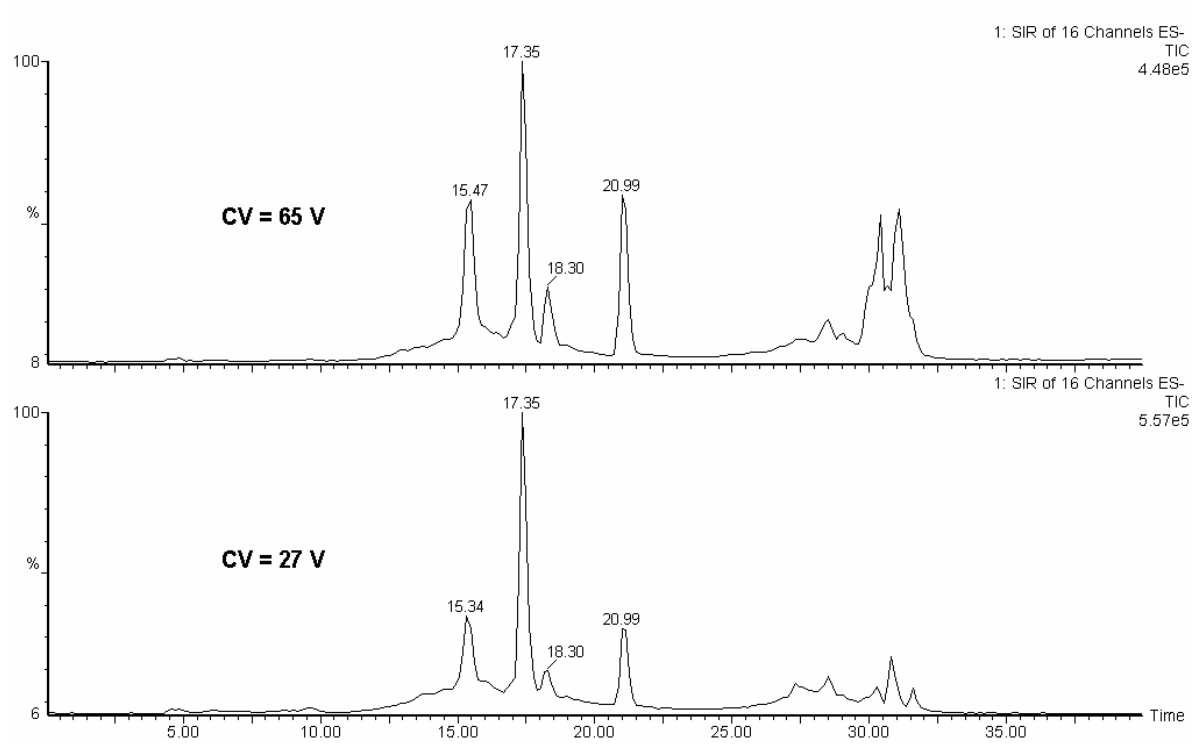


FIGURE 5.9

ESI$^-$ chromatograms of the SIM analysis of the stomach and contents of Case 283 (ZQ system)

5.9.6 Optimisation of mass spectral conditions for trace analysis

If SIM results must be presented and defended in a court of law, it is the excepted norm to use three to four unique mass ions to monitor. It is also preferable to use collision-induced dissociation, as mass ions produced under those conditions can serve as a confirmation of the initial results. Due to the fact that the results were not sufficiently conclusive, a new MS method was developed utilising the two scan functions at different cone voltages (27 V and 65 V) but also monitoring the 4 mass ions in each of the isotope clusters at *m/z* 725 and 645. The 70 cases were re-analysed with the new method resulting in 30% producing positive results, of which 6% were conclusively positive for the presence of ATR and / or ATR-SO_3. The results from one of the positive cases (unknown powdered sample of Case # 1294) are depicted in Figure 5.8 and clearly show the typical isotope clusters at *m/z* 725 and 645 within the expected retention time window. The newly developed method was expanded even further to enable the detection of CATR and CATR-SO_3 by also monitoring the four mass ions in each of the isotope clusters at *m/z* 769 and 689, respectively. This adaptation of the MS method did not improve the results of the 70 "historic" cases evaluated as no CATR or CATR-SO_3 could be detected. Although the new MS method is very specific and versatile in detecting the main toxic principles of *impila* and their monodesulfated derivatives, it showed a decrease in sensitivity due to the long cycling times necessary to monitor the 16 masses in each of the scan functions.

Table 5.4. Analysis of forensic samples using the optimised MS conditions for the determination of ATR, CATR and their monodesulfated derivatives. FS % and RS % refers to the Forward Search % and Reverse Search % done against the compounds detected in the *impila* tuber. (DNM = Did not match)

	Scan Function 1 16 Masses CV = 27								Scan Function 2 16 Masses CV = 65							
	CATR		$CATR\text{-}SO_3$		ATR		$ATR\text{-}SO_3$		CATR		$CATR\text{-}SO_3$		ATR		$ATR\text{-}SO_3$	
	FS %	RS %	FS %	RS %	FS %	RS %	FS %	RS %	FS %	RS %	FS %	RS %	FS %	RS %	FS %	RS %
STOMACH	91.6	98.6	99.5	100	98.3	99.5	99.8	100	85.4	DNM	98.3	100	97.4	99.8	99.7	100
LIVER	97.5	98.5	95.8	100	ND	ND	94.9	99.9	95.8	DNM	99.3	100	ND	ND	99.8	99.9
KIDNEY	97.3	DNM	94.0	100	ND	ND	94.8	100	97.2	DNM	99.6	100	ND	ND	96.9	99.6
BILE	49.9	94.6	97.6	98.9	ND	ND	97.5	97.5	94.7	DNM	98.3	DNM	ND	ND	96.7	100
BLOOD	96.2	97.1	96.8	98.5	92.1	DNM	92.9	99.9	94.8	DNM	95.8	100	95.0	99.8	97.0	99.9

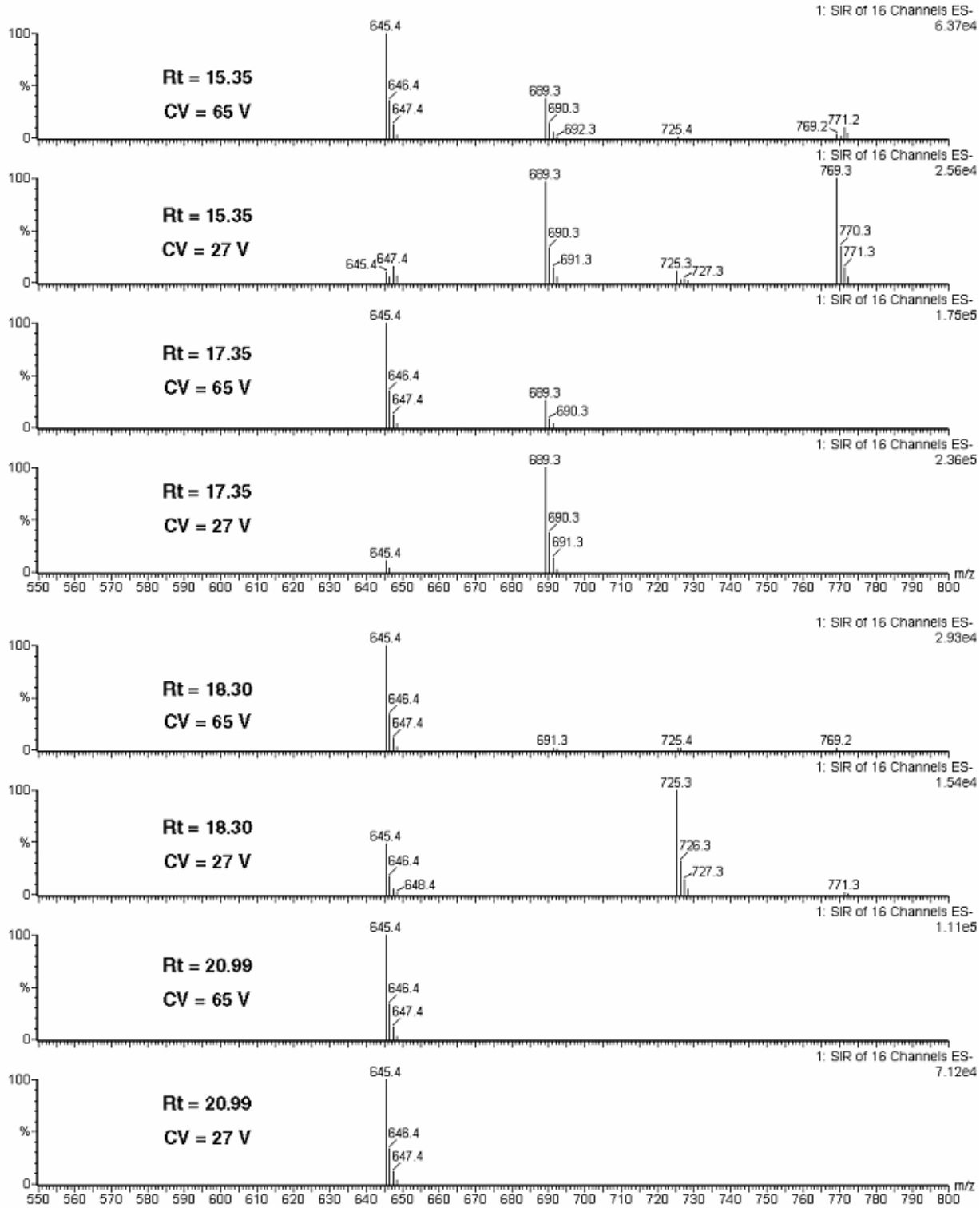


FIGURE 5.10

ESI$^-$ spectra obtained from the 16 ion SIM analysis of the stomach and contents of Case 283 (ZQ system)

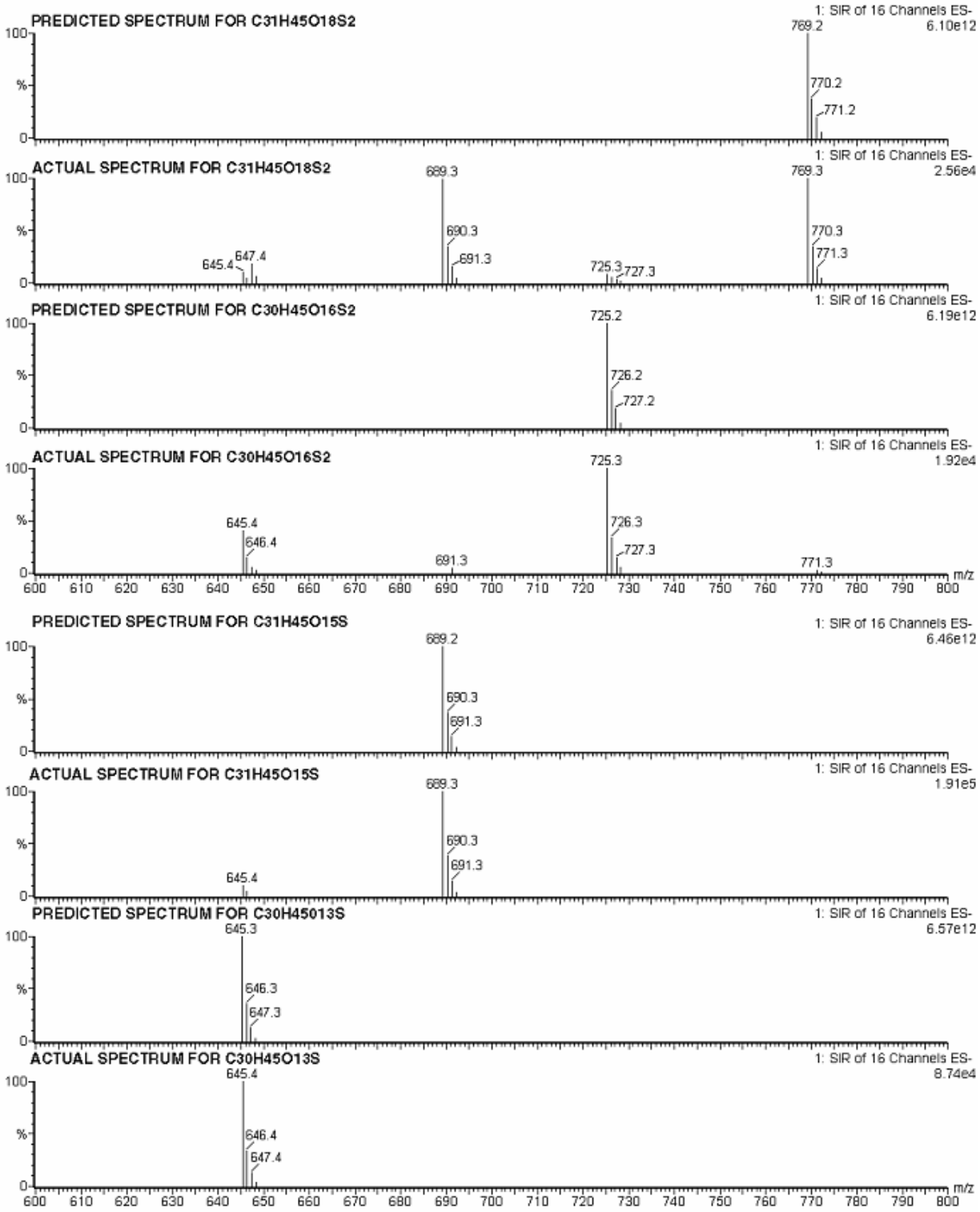


FIGURE 5.11

Predicted and actual ESI$^-$ spectra of the compounds identified in the stomach and contents of Case 283 (ZQ system)

A recently received forensic case (# 283) comprising of various viscera samples, namely stomach and its contents, liver, kidney, bile and blood, was investigated. The samples were extracted with the PBS-HCl method and analysed with the new MS method. All the forensic exhibits submitted for analysis were found to contain ATR, ATR-SO_3, CATR and CATR-SO_3. The detected compounds were matched using the Forward Search (pure compound evaluation) and Reverse Search (impure compound evaluation) search routines of MassLynx software (Table 5.4). The results obtained from the analysis of the stomach and its contents (Figure 5.9 and Figure 5.10) clearly indicated the excessive use of a herbal preparation containing *impila* tuber or the direct ingestion of the tuber.

5.10 FORENSIC APPLICABILITY

The analysis of atractyloside in plant material has always been problematic. The detection and positive identification of intact ATR and CATR in human viscera has in the past been a major challenge and is reported here for the first time. From the results of this study it seems that atractyloside poisoning is as frequent as reported [1, 102, 106]. The lack of screening procedures for atractyloside and carboxyatractyloside resulted in many forensic cases not being linked to exposure to or abuse of *impila* or products derived from the *impila* tuber.

The HPLC-ESI-MS method described here allows for the analysis of atractyloside and carboxyatractyloside in *C. laureola*, unknown powder samples and human viscera, and is capable of detecting various other atractyloside derivatives present in the tuber. With the proposed method, linearity was observed in the range of 100 – 1000 ng/ml but the working range covered the 1 ng/ml – 160 µg/ml concentration range with RSD values < 3% (n = 10). The method facilitated the quantification of ATR in fresh *impila* tuber (8.8 ± 0.4 µg/g; n = 5), an unknown powdered exhibit of case # 1294 (10.1 ± 0.6 ng/g; n = 5) and the viscera samples of case # 283 [280.1 ± 16.5 ng/g (averaged); n = 8]. Due to the simplicity of the method, it is ideally suited as a general forensic screening method for samples to detect the presence of atractyloside and carboxyatractyloside, and can be used to confirm the identity of tubers collected for medicinal use and to determine the ATR levels of collected plant material.

The method described is robust and can be adjusted to do full or SIR MS scans. Therefore, it is ideally suited for either high concentration or trace level analysis of ATR and CATR in various matrices. By using a dual MS scan function approach, the compounds of interest can be detected and confirmed using a single quadrupole mass spectrometer without any derivatisation or lengthy sample preparation steps. This method will be useful to any laboratory tasked with the screening of biological samples for the presence of atractyloside, carboxyatractyloside or their monodesulfated derivatives.

CHAPTER 6

Boophone disticha

6.1 BOTANICAL DESCRIPTION

Boophone disticha L. Herb (Amaryllidaceae), previously known as *Boophane disticha* L. Herb [107], is a very distinctive bulbous plant growing from a large, partially submerged bulb (Figure 6.1). The bulb is easily recognised by the thick layers of papery scales (Figure 6.2) surrounding the fleshy inner part (Figures 6.3). The symmetrically arranged strap-like leaves emerge after flowering. Attractive pink to red flowers are borne in a rounded inflorescence in early spring [108, 109] (Figure 6.4). This plant does not flower every year and when relocated will not flower for a couple of years. It is also known as bushman poison bulb, *incwadi* (Xhosa) and *incotha* (Zulu) [64].

6.2 ORIGIN AND DISTRIBUTION

Boophone disticha is widely distributed throughout the southern and eastern parts of South Africa and may be found further north into tropical Africa [109]. It is usually found in open grassland but will grow in most places provided its given well-drained soil and adequate sunlight.

Figure 6.1
Typical habitat of *Boophone disticha*

Figure 6.2
Papery bulb scales of *Boophone disticha*

Figure 6.3

Cross-section of a *Boophone disticha* bulb

Figure 6.4

Inflorescence of *Boophone disticha*

6.3 TYPE OF TOXIN

The toxic principles of *B. disticha* are a group of isoquinoline alkaloids of the Amaryllidaceae type [108]. Numerous alkaloids have been isolated from the bulb with buphanidrine one of the main compounds (Fig. 6.5). Other alkaloids include buphanisine, buphanamine, *3-O*-methyl-rinamidine (originally named undulatine, but the name was revised because another, unrelated compound was also called undulatine) and acetylnerbowdine. Table 6.1 and Figure 6.6 are summaries of all the known (characterised) compounds of *B. disticha* [110].

TABLE 6.1 Known alkaloids from the bulb of *Boophone disticha*

ALKALOID	ALTERNATIVE NAMES	EMPERICAL FORMULA	ACCURATE MASS
BUPHANISINE (1)		$C_{17}H_{19}NO_4$	285.1365
LYCORINE (2)		$C_{16}H_{17}NO_4$	287.1158
BUPHANAMINE (3)		$C_{17}H_{19}NO_4$	301.1314
POWELLINE (4)		$C_{17}H_{19}NO_4$	301.1314
BUPHANIDRINE (5)	DISTICHINE	$C_{17}H_{21}NO_4$	315.1471
BUPHANITINE (6)	NERBOWDINE	$C_{17}H_{21}NO_5$	319.1420
DISTICHAMINE (7)		$C_{18}H_{19}NO_5$	329.1263
3-O-METHYLCRINAMIDINE (8)	UNDULATINE	$C_{18}H_{21}NO_5$	331.1420

6.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

Buphanidrine (**5**) is a powerful analgesic, hallucinogen and neurotoxin with a LD_{50} (s.c., mice) of less than 9 mg/kg [110]. Symptoms of poisoning are well documented [45] and include dizziness, restlessness, impaired vision, unsteady gait, intense visual hallucinations, coma and finally death. Large doses of the bulb extract will also induce diarrhoea, vomiting and a general weakness, which will rapidly lead to death. The toxicity of this plant is well known and is reflected in the name “poison bulb” or “gifbol” and its earlier use as an arrow poison [111, 112].

6.5 ETHNOPHARMACOLOGICAL IMPORTANCE

Boophone disticha is one of the most important medicinal bulbs of southern Africa but its extremely high toxicity has led to several human fatalities. The highly toxic nature of the bulb scales might be the reason for its earlier use as a preservative. A discovery of 2000-year-old human (Khoi-San) remains mummified with *Boophone* scales is a testimony to the historical and cultural importance of this plant [113]. One of the main medicinal uses is the treatment of painful wounds and as an outer dressing after circumcision. It is also applied to boils and septic wounds and may be used as a bandage or mixed with water, milk or oil [45, 114, 115]. Weak decoctions of the bulb scales are administered orally or rectally (as an enema) for various complaints such as headaches, chest pain, abdominal pain, weakness and eye conditions [45]. Very weak decoctions of the bulb scales are used as an effective sedative, but higher doses will induce vivid visual hallucinations which are sometimes used for divination, initiation rites and the initiation of diviners [109, 111, 116]. Although the dried bulb scales are most commonly used, the fresh scales are often applied to burns and used to treat rashes and skin disorders, including eczema. It is also used to relieve rheumatic pains, arthritic swelling, sprains, muscular strains and other inflammatory conditions [117]. An unusual practice is the ingestion of the flowers in gruel to alleviate the sensation of dizziness [118]. The plant is also cultivated as a protective charm [119] and used in magical applications [120].

6.6 POISONING

6.6.1 Accidental

Poisoning with *B. disticha* has been reported by many authors [45, 108, 109, 111, 117, 118, 121, 122] and is usually accidental. This may be due to the underestimation of the toxicity of the particular bulb used to prepare the *muti*, or the administration of too large a dose of the decoction. Mild poisoning will produce temporary symptoms that would diminish within 24 hours, but acute poisoning usually results in severe vomiting, weakness, coma and death.

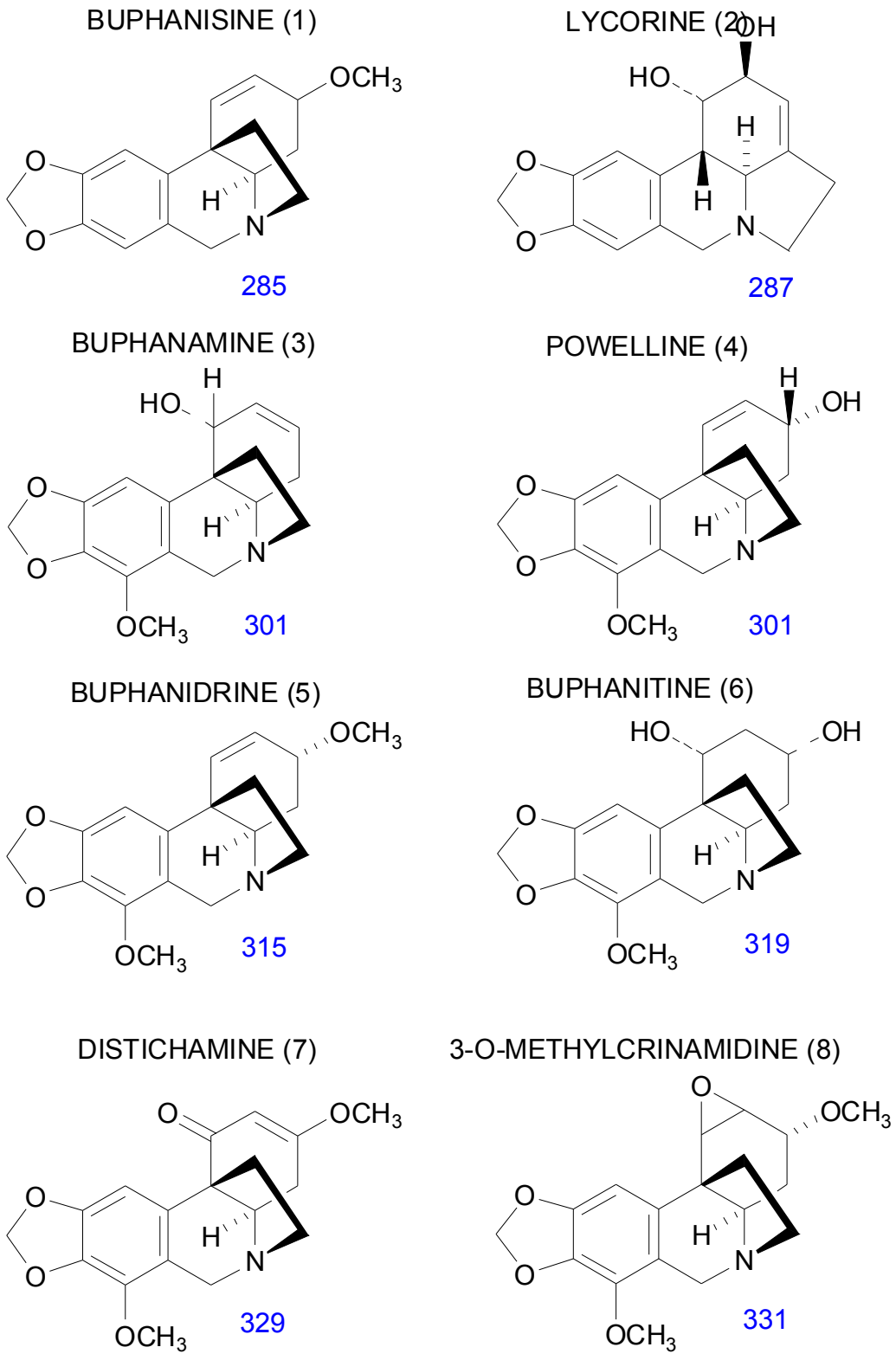


Figure 6.5

Known alkaloids found in the bulb of *B. disticha.* The nominal mass of each structure is shown beneath each structure (Blue number)

6.6.2 Deliberate

Decoctions of the bulb and bulb scales have been used to commit murder and suicide. In recorded suicide attempts, the decoction is used as an enema which can be administered via the rectum or vagina [45].

6.7 FCL JHB INVESTIGATION

Buphanidrine and buphanamine have been detected at the FCL JHB and were identified using commercial spectral libraries like NIST on the GC-MS (EI) and HPLC-MS (EI) systems. The detection of the alkaloids of *B. disticha* in viscera samples was prior to this research only successful in cases where the person overdosed on a *muti* prepared from the bulb of *B. disticha*. If sufficient time lapsed from exposure to sampling, the levels of alkaloids dropped too low for detection by the screening methods used in GC-EI-MS and HPLC-EI-MS (TMD). The fact that standards are not commercially available also hampered the development of screening methods and sensitive and selective detection methods for targeted analysis.

The aims of this investigation were:

- Aim 1: To extract the shredded bulbs of *B. disticha* with suitable organic solvent(s) to determine the alkaloid profile of the major components.
- Aim 2: To determine if the standard SPE extraction method could extract the major alkaloids from these shredded bulbs.
- Aim 3: Since some of the major alkaloids have not been characterised properly, the third aim was to extract sufficient amounts of alkaloids to enable purification and characterisation by LC-MS and NMR.
- Aim 4: To develop a standard extraction and screening method for the alkaloids in human viscera.
- Aim 5: To compare the alkaloid profiles of *Ammocharis coranica* and *B. disticha* in order to be able to rule out the presence of *A. coranica* in *muti* samples by pattern recognition. This would be accomplished by comparing the mass spectral BPI or TIC patterns of unknown samples with the mass spectral BPI or TIC patterns of *A. coranica* and *B. disticha*.

6.8 EXPERIMENTAL PROCEDURE

6.8.1 Standards and reagents

No standard are commercially available for the alkaloids of *B. disticha*. Potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (> 99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%)

(NH_4OAc) and acetic acid (99%) were obtained from BDH Laboratory Supplies, England. Sulfuric acid (Suprapur 96%), hydrochloric acid (Suprapur 96%), phosphoric acid (Suprapur 96%), formic acid (98 - 100%) and ammonia (GR 25%) were obtained from Merck, Darmstadt, Germany. Acetonitrile (gradient quality, 200 nm UV cut-off) and methanol (gradient quality, 205 nm UV cut-off) were obtained from Romil, England. Chloroform, cyclohexane, dichloromethane (spectrophotometric grade) were obtained from Sigma Chemical Company, USA. HPLC grade water (20 $M\Omega cm^{-1}$) was obtained from a Milli-Q / reversed osmosis system (Millipore, USA). The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [56]. The anhydrous salts of KCl (0.20 g), NaCl (8.00 g), KH_2PO_4 (0.20 g) and Na_2HPO_4 (1.15 g) were dissolved in 1 L deionised water and the pH adjusted to 5.0 with 10% hydrochloric acid.

Oasis HLB solid phase extraction (SPE) cartridges (3 ml with 60 mg of sorbent) were obtained from Waters Corporation, Milford, USA. A 12-port vacuum manifold was used for all extractions.

6.8.2 Instruments and conditions

A Waters 2690 HPLC system equipped with both a 996 photodiode array (PDA) detector and a Termabeam mass selective detector (TMD) (electron impact (EI) mode; maximum mass 1000 *m/z* from Waters) was used as initial screening instrument and high concentration studies. A Waters 2690 HPLC system equipped with a 996 PDA detector and a Z spray mass selective detector (ZMD) (Micromass, UK) (electrospray mode; maximum mass 2000 *m/z*) was used for low concentration studies and as confirmation. A Phenomenex Aqua C18 (250 x 2.1mm, 5 µm) HPLC column was used on the TMD system while a Waters Xterra MS C18 HPLC column (250 mm x 2 mm, 5 µm) was used on the ZMD system. A Harvard syringe pump (Model 11) was obtained from Harvard Apparatus, Holliston, USA and was used for all direct injection experiments on the ZMD detector.

The Phenomenex Aqua column (TMD system) was used with an acidic mobile phase of 1% formic acid in the water, starting at 95% water: 5% acetonitrile and slowly ramping the organic component to 50% methanol: 50% acetonitrile in 40 minutes. These conditions were kept for 5 minutes where after the HPLC column was re-equilibrated with the initial conditions. The flow rate was 0.2 ml/min and the column temperature 40 ^oC.

To ensure the maximum retention of basic alkaloids on the Xterra MS C18 column, the initial chromatographic conditions were 90% water containing 10 mM ammonium acetate, the pH adjusted to 10 with ammonia (25%) and acetonitrile. After injection, the chromatographic conditions were gradually changed through a linear gradient profile to 90% acetonitrile and 10% of the original aqueous mobile phase in 20 minutes. These conditions were kept stable for 5

minutes where after the HPLC system re-equilibrated the analytical column to the initial conditions. The flow rate was kept constant at 0.2 ml/min while the column temperature was kept constant at 40 ^{o}C. The nebuliser of the TMD detector was optimised with a caffeine test solution (100 µg/ml) to ensure efficient nebulisation and droplet formation. The electrospray ionization (ESI) probe of the ZMD detector was optimised by direct injection via a syringe pump using a cocaine test solution (10 µg/ml). The optimised conditions for the TMD detector are listed in Table 6.2 and those for the ZMD detector in Table 6.3. The optimised chromatographic conditions for the Xterra MS C18 column are listed in Table 6.4. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian Inova 300 MHz NMR spectrometer.

Table 6.2 HPLC gradient profile of the general screening method (TMD system)

	TIME min	FLOW ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		0.20	0	0	95	5	6
2	1.00	0.20	0	0	95	5	4
3	10.00	0.20	0	0	80	20	6
4	12.00	0.20	0	0	80	20	4
5	20.00	0.20	0	30	50	20	5
6	35.00	0.20	0	10	0	90	6
7	37.00	0.20	0	10	0	90	6
8	40.00	0.25	0	10	0	90	6
9	45.00	0.25	0	10	0	90	6
10	50.00	0.20	0	0	95	5	5

6.8.3 Chemical extraction of *B. disticha* bulb and *A. coranica* scales

Boophone bulbs were purchased from the Soms Traditional Healer's market in Johannesburg. *Ammocharis coranica* bulb scales were obtained from Mr. P. Winter (collected at Haenertsburg, Limpopo Province). The *Boophone* bulbs were cut into slices and dried at 40 ^{o}C to constant weight while the *Ammocharis* bulb scales were only dried at 40 ^{o}C to constant weight. The bulb slices and bulb scales were finely ground using an Ika Werk A10 water-cooled mill (Janke & Kunkel, Straufen, Germany). Three 5 g portions of shredded *Boophone* bulb were placed in three separate 50 ml polypropylene screw-top centrifuge sample tubes. PBS-HCl buffer (pH 5) (30 ml) was added to one tube, PBS-HCl buffer (pH 7) (30 ml) was added to the second tube while PBS-NH_3 buffer (pH 9) (30 ml) was added to the third tube.

The tubes were capped and shaken on a Heidolph rotating sample mixer for thirty minutes. Oasis HLB SPE cartridges were prepared by washing consecutively with 6 ml of methanol, water

and the extracting buffer used. After centrifugation on a Hermle Z 300 centrifuge at 4000 rpm for 15 minutes, the aqueous phases were passed through the conditioned Oasis HLB cartridges, washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. The cartridges were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates combined into a single collection tube. The collection tube was placed into a Zymark Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residue was reconstituted with 1 ml methanol.

The shredded bulb scales of *A. coranica* were extracted as described for *B. disticha*.

TABLE 6.3 Optimised MS conditions for the detection of *Boophone* alkaloids on the TMD system

TMD CONDITIONS	*BOOPHONE* ALKALOIDS
Mode	EI positive
Ionisation energy (eV)	70
Gain	10
Nebuliser temperature (°C)	80
Expansion temperature (°C)	90
Source temperature (°C)	225
Capillary tip temperature (°C)	160
Desolvation gas flow (L/h)	30
Sampling rate	1.0
Scan range (amu)	50 - 550
Ion Energy	1.0
Multiplier gain (V)	1275

6.8.4 Isolation of the alkaloids from the bulb of *B. disticha*

A dormant *B. disticha* bulb was prepared as described in §6.8.3. Due to the fact that large volumes of organic solvent can be more readily reduced in volume than similar volumes of aqueous buffer, it was decided to do a large-scale organic solvent extract. The shredded bulb was placed in a 5 liter Schott bottle, covered with cyclohexane, a magnetic stirrer bar added and

mixed on an Ikamag RH (Janke & Kunkel, Straufen, Germany) stirrer for 1 hour. The cyclohexane was filtered off through a Buchner funnel packed with celite 545 (SMM chemicals, South Africa), the bulb residues returned to the 5 liter Schott bottle and re-extracted with cyclohexane (CHEX). This procedure was repeated with chloroform and methanol as extracting solvent. The chemically similar organic filtrates were pooled and evaporated under reduced pressure at 50 ^{o}C on a Büchi RE 111 Rotovapor equipped with a Büchi 461 water bath (Büchi, Switzerland). The dried residues of each solvent extraction were re-dissolved in the minimum amount of similar solvent.

Table 6.4 Gradient profile of the alkaloid screening method (ZMD system)

	TIME min	FLOW Ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		0.20	0	0	90	10	1
2	2.00	0.20	0	0	90	10	1
3	25.00	0.20	0	0	10	90	6
4	25.00	0.20	0	0	10	90	6
5	34.00	0.20	0	0	90	10	4

The crude extracts were evaluated on aluminium TLC plates (Merck Silica gel 60 F_{254}) using a mobile phase of cyclohexane: chloroform: dietylamine (5:4:1). The plates were evaluated under UV_{254} and displayed complex mixtures of numerous compounds. Analysis on the ZMD system (ESI^+) confirmed the TLC results and indicated that certain compounds were found in one of the fractions only, but that other compounds were found in more than one fraction. The crude extracts were combined and fractionated by hand on a Supelco Versa Flash system (Sigma Aldrich) under the conditions summarised in Table 6.5. The samples were collected and spotted onto TLC plates and developed in the same eluent as used on the Versa Flash system. An evaluation of the TLC plates indicated that various compounds still co-eluted – even under the chromatographic conditions used on the preparative LC system. Each fraction was analysed on the ZMD system (ESI^+) and those fractions of similar composition were pooled and evaporated under reduced pressure at 40 ^{o}C on the Zymark Turbovap system.

The chromatographic conditions used on the Waters Xterra MS C18 analytical column was fed into the Prep Calculator software of the Waters Corporation to recalculate the conditions that must be used for a Waters Xterra MS C18 column (300 mm x 7.8 mm, 10 µm). The same gradient profile as described for the analytical column did not give sufficient resolution and was changed to the conditions listed in Table 6.6. These conditions were used to fractionate the

alkaloids from 100 µl injections of the re-dissolved residue with a LKB 2211 Superrac fraction collector by monitoring the UV spectra with the PDA detector between 200 and 600 nm.

6.8.5 Chemical extraction and analysis of viscera and case exhibits

An aliquot of each liquefied viscera sample [stomach (including contents), liver and, kidney (3 g each)] were placed in separate 50 ml polypropylene screw-top centrifuge sample tubes. Thirty milliliters (30 ml) of PBS-HCl buffer (pH 5) was added to each sample and the tubes capped and shaken on a Heidolph rotating sample mixer for thirty minutes. Oasis HLB SPE cartridges (3 ml – 60 mg) were prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used. After centrifugation of the sample tubes at 4500 rpm (Hermle Z 300 centrifuge) for 15 minutes, the aqueous phase of each sample was passed through a conditioned Oasis HLB cartridge and the cartridges were washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. The cartridges were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates of each cartridge combined into one collection tube. The collection tubes were placed into a Zymark Turbovap and evaporated at 40 ^{o}C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of methanol. The solid case exhibits [suspected *muti* bottle and rubber bulb (used to administer an enema)], were carefully washed down or rinsed out with methanol. The solvent was evaporated on the Zymark Turbovap system as described above. The piece of plant bulb that was submitted with one of the cases (Case 1175) was prepared as described in §6.8.4.

6.9 RESULTS AND DISCUSSION

6.9.1 Chromatography and detection of Amaryllidaceae alkaloids

The bulbs of *B. disticha* contains numerous alkaloids of the crinine-type and at least eight alkaloids have been characterised and reported in literature [40, 41] (Figure 6.5). Analysis of the crude extract, using the standard acidic screening method on the TMD system (Figures 6.6 and 6.7), displayed a complex mixture that could not be separated fully using the PDA detector. The TMD detector was more successful in detecting some of the compounds, but displayed, as expected, extensive tailing.

A HPLC-ESI-MS method was previously developed for the separation of various basic drugs of abuse and this method was used to separate the various alkaloids found in the crude extract of the bulb (Figure 6.8). The ESI^{+} TIC and BPI chromatograms were complex with various compounds co-eluding or only partially separated. A SIM analysis of the full scan data however, produced a distinctive pattern of *Boophone* compounds (Figure 6.9).

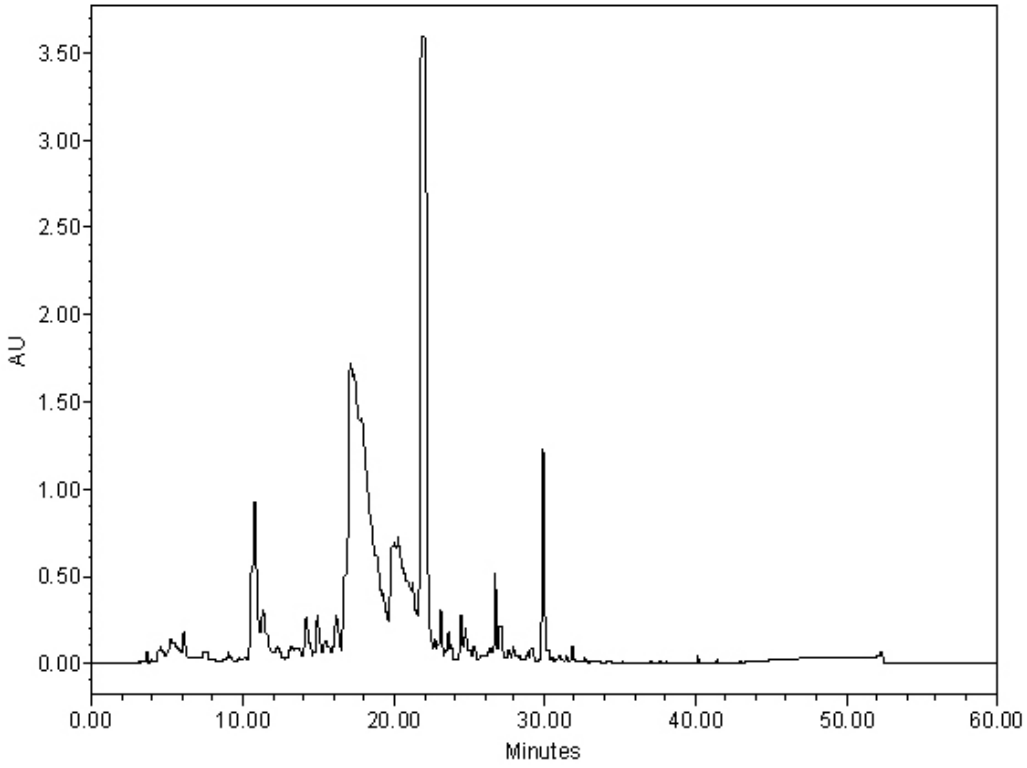


Figure 6.6

PDA chromatogram of a crude bulb extract of *Boopone disticha* on the TMD system

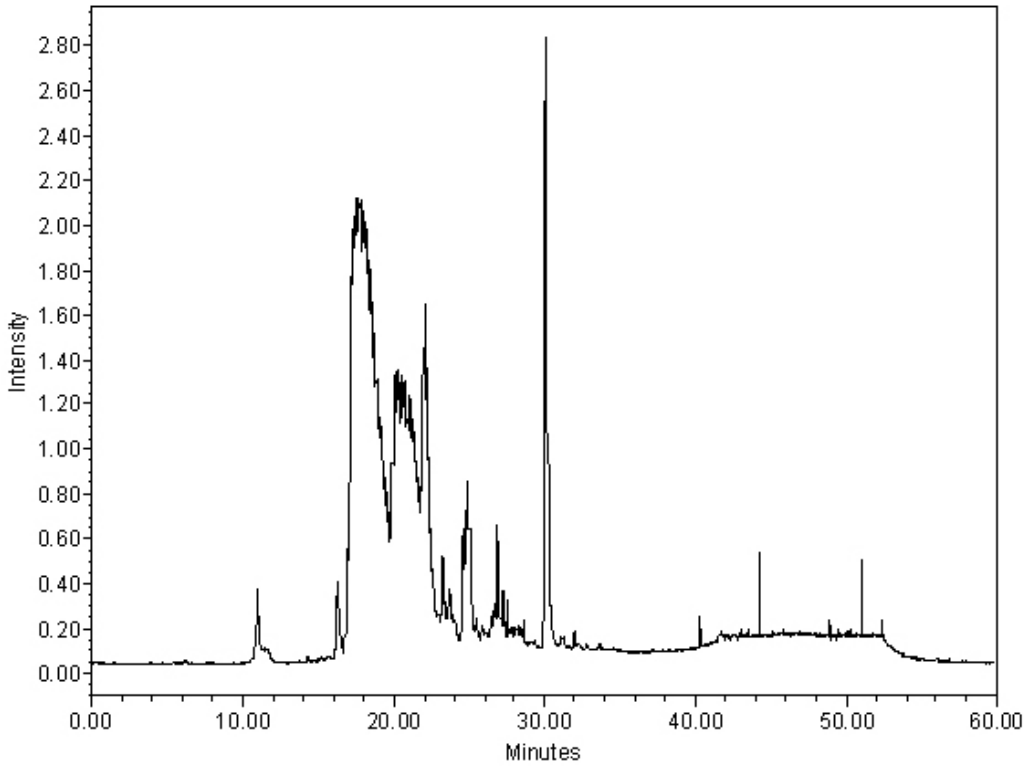


Figure 6.7

EI chromatogram of a crude bulb extract of *Boopone disticha* on the TMD system

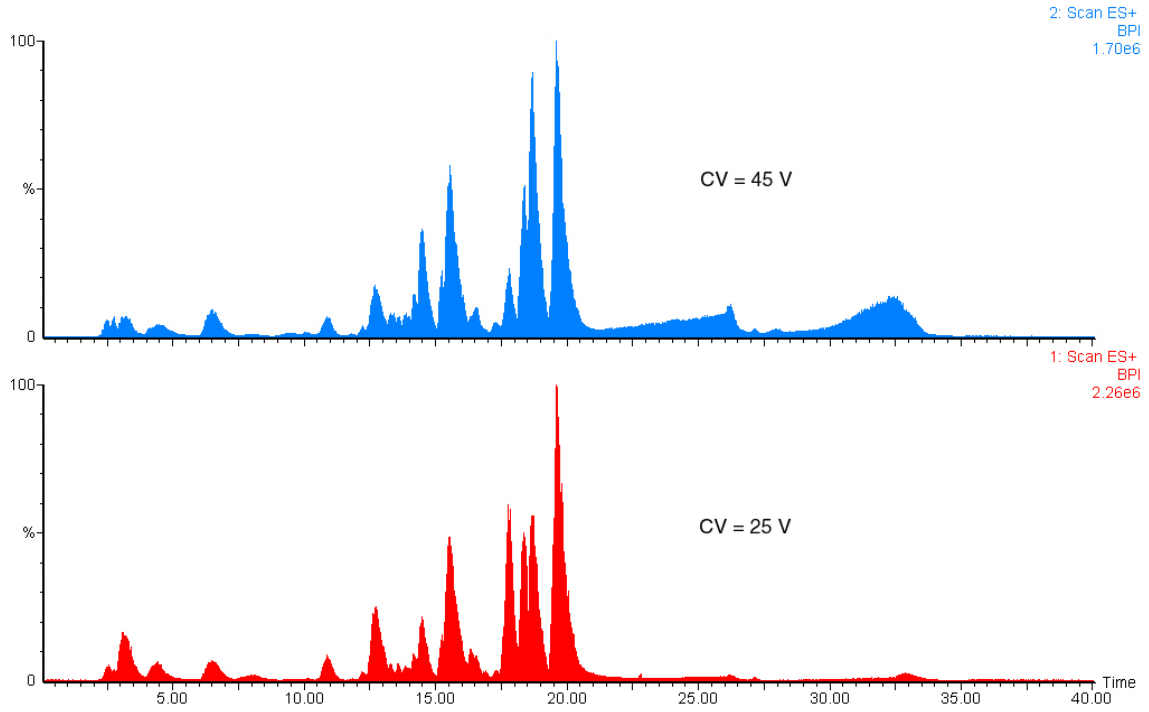


Figure 6.8

ESI$^+$ BPI chromatograms of a crude bulb extract of *Boopone disticha* on the ZMD system at two cone voltages (25 V and 45 V)

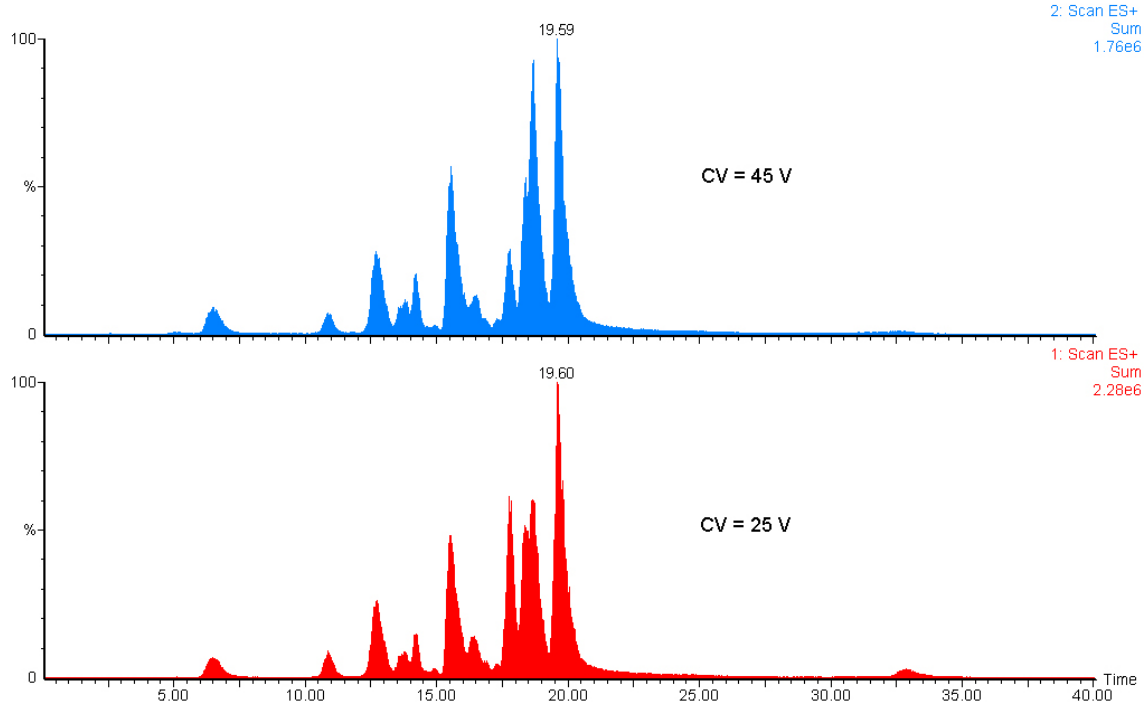


Figure 6.9

ESI$^+$ Chromatograms of a crude bulb extract of *Boopone disticha* on the ZMD system obtained by monitoring selected masses (286, 288, 302, 316, 320, 330 & 332 amu) at two cone voltages (25 V and 45 V)

Table 6.5 Preparative LC conditions used on the Versa Flash system for the separation of *Boophone* alkaloids

PARAMETER	SETTING / CONDITION
Column	Sigma Aldrich Versa Flash 150 x 40 mm x 10 µm
Pump System	Accu^{Tm} SciLog
Pump Head	RHOCTC (Tefzel)
Flow Rate (ml / min)	10
Eluent Composition 1	CHEX:$CHCl_3$:DEA (5:4:1)
Eluent Composition 2	CHEX:$CHCl_3$:DEA:MeOH (4:4:1:1)
Eluent Composition 3	CHEX:$CHCl_3$:DEA:MeOH (2:4:1:3)
Eluent Composition 4	$CHCl_3$:DEA:MeOH (4:1:5)
Fraction size (ml) and collection mode	15 ml manually by hand

Table 6.6 Gradient profile of the preparative HPLC alkaloid fractionation method as used on the ZMD system

	TIME min	FLOW ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		2.76	0	60	40	0	1
2	5.0	2.76	0	60	40	0	6
3	15.0	2.76	0	70	30	0	6
4	20.0	3.00	0	90	10	0	6
5	22.0	3.00	0	90	10	0	4
6	25.0	3.00	0	60	40	0	6
7	30.0	2.76	0	60	40	0	4

The ZMD detector has the distinct advantage of being selective due to the fact that ionisation is highly dependant on the eluent chemistry. It can even be made even more selective by only monitoring those masses common to the alkaloids of *B. disticha*.

6.9.2 Chemical extraction and purification of *B. disticha* bulb compounds

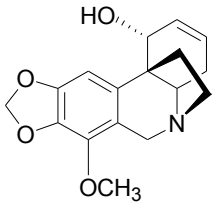
As mentioned before in §6.8.4 the solvent extracts of the shredded bulb was analysed by LC-ESI-MS and displayed complex chromatograms with some compounds co-eluding. An evaluation of the various extracts at a few selected masses (*m/z* 286, 288, 302, 316, 320, 330 and 332) indicated that some compounds were not only found in one of the extracts, but were

extracted by all the solvents while other compounds were only found in one of the solvent extracts. For this reason the crude solvent extracts were pooled and evaporated to an oily residue. The residue was re-dissolved in a methanol: chloroform (1:1) mixture but required the addition of a small amount of hexane to remain a clear solution.

This mixture was not suitable for loading directly onto the Versa Flash column as it hampered the separation of the most apolar compounds. The crude sample was dissolved in 250 ml of cyclohexane: chloroform: methanol (4:3:3) mixture in a round bottom flask and 30 g of silica gel (Merck Silica gel 60) was slowly added while stirring the mixture. The flask was placed on the Büchi Rotovapor and evaporated to dryness under reduced pressure at 50 ^{o}C. The coated silica gel was packed into a 40 mm x 40 mm precolumn and placed in front of the main Versa Flash column. The main Versa Flash column was already conditioned with eluent composition 1 (Table 6.5). The pump was started and the compounds eluted from the precolumn and separated on the main column. The fractions were monitored by TLC (UV 254 nm) and the solvent composition changed to a more polar composition when no more compounds were eluted with the less polar solvent composition. None of the fractions contained a single pure compound and those that only displayed one major and a few minor spots on TLC contained multiple compounds when analysed on the ZMD system. The fractions with similar TLC profiles were combined and two of these combined samples were fractionated on the preparative HPLC column. The UV spectrum was monitored and the main compounds fractionated on the LKB 2211 Superrac fraction collector.

Six compounds were isolated and the structures confirmed by NMR spectroscopy:

Buphanamine



Buphanamine

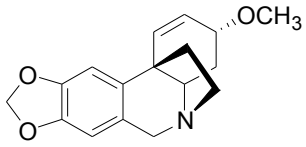
C17H19NO4
Exact Mass: 301.13141

δ_H ($CDCl_3$, 300 MHz) 6.55 (1H, s, H-10), 5.95 (1H, dddd, *J* 10.0, 5.6, 2.8 & 1.9 Hz, H-2), 5.84, 5.82 (2H, 2 x d, *J* 1.5 Hz, OCH_2O), 5.78 (1H, ddd, *J* 10.0, 4.6, 2.9, H-3), 4.70 (d, *J* 5.6 Hz, H-1), 4.11(1H, d, *J* 16.7 Hz, H-6α), 3.92 (3H, s, OMe), 3.78 (1H, d, *J* 16.7 Hz, H-6β), 3.35 (2H, m, H-4a, 12*exo*), 2.70 (1H, ddd, *J* 13.2, 8.2 & 8.7, H-12*endo*), 2.52 (1H, dddd, *J* 19.6, 5.6, 4.6 & 1.9, H-4α), 1.95 (1H, m, H-4β), 1.85 (2H, m, H-11).

δ_C ($CDCl_3$, 75 MHz) 148.5 (C-9), 140.6 (C-7), 133.5 (C-8), 128.6 (C-3), 125.6 (C-2), 117.7 (C-6a), 100.6 (OCH_2O), 98.2 (C-10), 64.3 (C-1), 59.1 (C-4a), 59.0 (OMe), 56.8 (C-6), 51.3 (C-12), 48.2 (C-10b), 38.6 (C-11), 28.1 (C-4).

Until recently, only the ^{13}C NMR of buphanamine was published [123].However, a recent paper [124] also included the ^{1}H NMR data. Our NMR data differ slightly from those reported, but this is an indication that our spectrum was obtained on the salt form of buphanamine.

Buphanisine



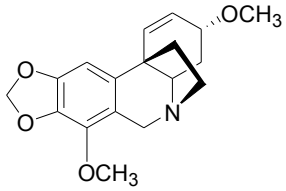
Buphanisine
C17H19NO3
Exact Mass: 285.13649

δ_H ($CDCl_3$, 300 MHz) 6.81 (1H, s, H-10), 6.56 (1H, d, *J* 10.2, H-1), 6.46 (1H, s, H-7), 5.95 (1H, dd, *J* 9.9 and 5.1, H-2), 5.86, 5.85 (1H, d, *J* 1.4, OCH_2O), 4.40 (1H, d, *J* 16.9, H-6α), 3.79 (1H, m, H-3β), 3.78 (1H, d, J 16.9, H-6β), 3.40 (2H, m, H-4a, 12α), 3.33 (3H, s, OMe) 2.89 (1H, ddd, J 13.5, 9.0, 6.3, H-12β), 2.16 (2H, m, H-4α, 11β), 1.92 (1H, ddd, J 12.3, 10.8, 6.0, H-11α), 1.58 (1H, td, J 13.5 and 4.2, H-4β).

δ_C ($CDCl_3$, 75 MHz) 146.2 (C-9), 145.8 (C-8), 138.0 (C-10a), 132.5 (C-1), 125.5 (C-2), 125.5 (C-6a), 106.9 (C-7), 103.0 (C-10), 100.8 (OCH_2O), 72.4 (C-3), 63.0 (C-4a), 61.8 (C-6), 56.5 (OMe), 53.1 (C-12), 44.4 (C-10b), 43.8 (C-11), 28.3 (C-4).

The ^{13}C NMR data was in agreement with literature data [125].

Buphanidrine



Buphanidrine
C18H21NO4
Exact Mass: 315.14706

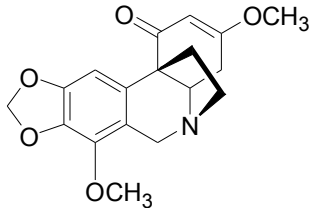
δ_H ($CDCl_3$, 300 MHz) 6.51 (1H, s, H-10), 6.47 (1H, d, *J* 10.0 Hz, H-1), 5.93 (1H, dd, *J* 10.0 & 5.2 Hz, H-2), 5.81 – 5.82 (2H, 2d, *J* 1.5 Hz, OCH_2O), 4.27 (1H, d, *J* 17.2 Hz, H-6α), 3.92 (s, 7-OMe), 3.85 (1H, d, *J* 17.2 Hz, H-6β), 3.77 (1H, m, H-3), 3.51 (1H, m, H-12*exo*), 3.39 (1H, dd, *J* 10.0 &

4.1 Hz, H-4a), 3.30 (3H, s, 3-OMe), 2,24 (1H, m, H-12*endo*), 2.20 (2H, m, H-11), 1.9 (1H, m, H-4β), 1.57 (1H, td, *J* 12.0 & 3.9, H-4α).

δ_C ($CDCl_3$, 75 MHz) 148. 4 (C-9), 140.8 (C-7), 138.1 (C-10a), 133.5 (C-8), 131.6 (C-2), 125.6 (C-1), 115.1 (C-6a), 100.6 (OCH_2O), 98.8 (C-10), 71.8 (C-3), 62.5 (C-4a), 59.0 (C-6), 57.4 (OMe), 56.5 (C-6), 52.6 (C-12), 44.5 (C-10b), 42.9 (C-11), 27.3 (C-4).

The ^{13}C NMR data agree with those reported in the literature [126], but there are differences in the ^{1}H NMR spectrum, which may indicate that our spectrum was obtained from the acetic acid salt of buphanidrine (acetate was observed in the NMR spectrum).

Distichamine



Distichamine

C18H19NO5
Exact Mass: 329.12632

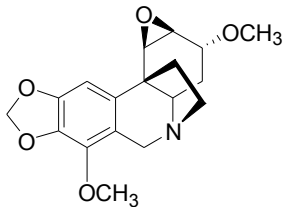
δ_H ($CDCl_3$, 300 MHz) 7.17 (1H, s, H-10), 5.85, 5.83 (2H, 2 x d, *J* 1.2 Hz, OCH_2O), 5.36 (1H, d, *J* 1.8 Hz, H-2), 4.21 (1H, d, *J* 17.4 Hz, H-6α), 3.94 (3H, s, OMe), 3.77 (1H, d, *J* 17.4 Hz, H-6α), 3.69 (3H, s, OMe), 3.67 (1H, dd, *J* 11,1 & 6.6 Hz, H-4a), 2.91 (1H, ddd, *J* ??, 8.7 & 6.6 Hz, H-12*endo*), 2.56 (1H, dd, *J* 17.4 & 6.6 Hz, H-4α), 2.42 (1H, ddd, *J* 17.4, 11.1 & 1.5 Hz, H-4β), 2.35 (1H, ddd, *J* 12.6, 9.3 and 3.6 Hz, H-11*endo*), 2.20 (1H, ddd, *J* 12.6, 10.2 & 6.3 Hz, H-11*exo*).

δ_C ($CDCl_3$, 75 MHz) 198.5 (C-1), 173.2 (C-3), 147.9 (C-9), 140.1 (C-7), 135.3 (C-10a), 133.8 (C-8), 116.3 (C-6a), 102.3 (C-2), 100.6 (C-10), 100.6 (OCH_2O), 66.2 (C-4a), 59.1 (7-OCH_3), 57.6 (C-6), 55.9 (3-OCH_3), 52.4 (C-12), 50.4 (C-10b), 41.4 (C-11), 30.2 (C-4).

The isolation of this compound was described in the older literature [127], but this is the first time that the structure is reported.

***3-O*-Methylcrinamidine**

(Formely called undulatin. However, there is also a different compound called undulatine and to prevent confusion, we will use the name 3-*O*-Methylcrinamidine).



3-O-Methylcrinamidine

C18H21NO5
Exact Mass: 331.14197

δ_H ($CDCl_3$, 300 MHz) 6.60 (1H, s, H-10), 5.85, 5.84 (2H, 2 x d, *J* 1.5 Hz, OCH_2O), 4.18 (1H, d, *J* 17.4 Hz, H-6α), 3.95 (3H, s, 7-OMe), 3.74 (1H, d, *J* 3 Hz, H-1), 3.69 (1H, d, *J* 17.7 Hz, H-6β), 3.41 (3H, s, 3-OMe), 3.30 (1H, m, H-2), 3.15 (1H, ddd, m, H-12α), 3.0 (1H, m, H-4a), 2.7 (1H, m, H-12β), 2.3 (1H, m, H-11α), 1.9 (1H, m, H-11β) 1.73 (1H, m, H-4α), 1.38 (1H, td, *J* 13.2 & 2.7 Hz, H-4β).

δ_C ($CDCl_3$, 75 MHz) 141.1 (C-7), 138.9 (C-10a), 133.3 (C-8), 117.8 (C-6a), 100.6 (OCH_2O), 96.4 (C-10), 74.9 (C-3), 61.2 (C-4a), 59.1 (7-OMe), 58.7 (C-6), 57.5 (3-OMe), 55.1 (d, C-2), 53.9 (C-1), 52.6 (C-12), 41.4 (C-10b), 39.2 (C-11), 25.2 (C-4)..

The NMR data was in agreement with literature data [126]

6.9.3 Chromatographic fingerprinting of *B. disticha* and *A. coranica*

Buphanisine is one of the marker compounds that are detected every time when the use of *B. disticha* is suspected. Epibuphanisine, the 3-epimer of buphanisine is according to the literature also found in the bulb scales of *A. coranica* [40] and may lead to false identification of the biological source of exposure. The SPE extracts of *B. disticha* and *A. coranica* were analysed on the ZMD system (Figure 6.10) and produced similar chromatographic profiles when monitoring the typical masses associated with *Boophone* alkaloids. By overlaying the chromatograms and zooming in on the most complex portion of the chromatogram, differences between the extracts could be detected.

These differences might merely be concentration differences and therefore might not be significant. However, a detailed analysis of the mass spectra of the individual peaks revealed that the two extracts might look similar at a first glance, but differed in compound profile quite substantially (Table 6.7). Of the 11 masses monitored in both bulbs, only two compounds with *m/z* 316 and 332 $(M+H)^+$ displayed identical mass spectra when analysed with the 1st scan function [**U** (CV 25 V to minimise fragmentation)] and the 2nd scan function [**F** (CV 45 V to inisiate ISCID)]. *B. disticha* had three unique compounds (m/z 286, 320 and 332) that displayed unique fragmentation with the 1st and 2nd scan functions.

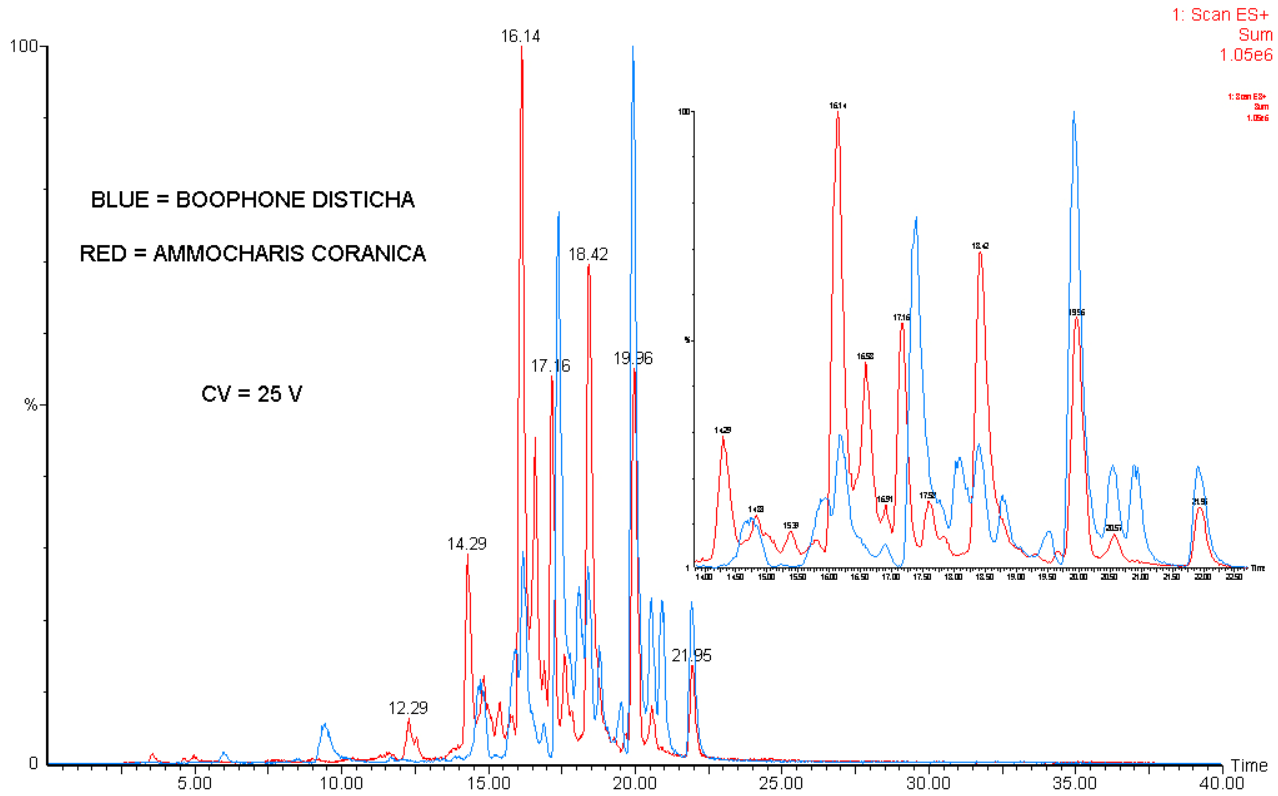


Figure 6.10

Overlaid ESI$^+$ chromatograms of the SPE extracts of the bulbs of *Ammocharis coranica* and *Boophone disticha* (ZMD system) by monitoring selected masses (286, 288, 302, 316, 320, 330 & 332 amu)

Eight of the masses monitored (*m/z* 288, 302, 318, 330, 332, 346 and 374), revealed compounds in both extracts that produced distinctively different fragmentation patterns and are therefore different compounds. *A. coranica* had two unique compounds (*m/z* 263 and 346) that were not detected in *B. disticha*. By monitoring these differences in the mass spectra of *B. disticha* and *A. coranica*, it would be possible to distinguish between these two plants.

6.9.4 Analysis of case exhibits and viscera samples

The presence of alkaloids from *B. disticha* has been confirmed in numerous cases – mainly in the plant exhibits or *muti* that were submitted to the laboratory. The detection of these alkaloids in viscera was only accomplished in cases where a massive overdose with these alkaloids occurred. Confirmation of these alkaloids was done by comparing the mass spectra of the suspected compounds with the NIST spectral library. The presence of low levels of these alkaloids could not be confirmed prior to the development of the ESI HPLC-MS method. Three cases were selected to emphasise the efficacy of the two methods used to screen for alkaloids (TMD and ZMD detectors), namely cases 1175, 121 and 1196.

Table 6.7 Comparison of the compounds detected in the bulb extracts of *Ammocharis coranica* and *Boophone disticha* (ESI^+ on ZMD system)

MASS (M+H)	*AMMOCHARIS*	*BOOPHONE*	R_t SIMILAR	UNIQUE	IDENTICAL
263 U F	✓ 263/209 263/209	×	N/A	Yes	N/A
286 U F	×	✓ 286 286/254/226/196	N/A	Yes 286(B)	No
288 U F	✓ 288/318 288/226/211/181	✓ 288 288/270/260	No	Yes Both	No
302 U F	✓ 302/332/272/252 302/270/252/226	✓ 302 302/284	No	Yes Both	No
316 U F	✓ 316 316/284/256/226	✓ 316/344 316/284/256/226	Yes	No	Yes
318 U F	✓ 318/227 318/268/227/199	✓ 318 318/300/282/241	No	Yes Both	No
320 U F	×	✓ 320/287 320/302/287/232	N/A	Yes 320/287(B)	N/A
330 (#1) U F	✓ 330/298/256/225 330/298/244/225	✓ 330/302/207 330/302/207/124	Yes	Yes Both	No
330 (#2) U F	✓ 330/270 330/270/226/211	✓ 330/271 330/320/270/255	No	Yes Both	No
332 (#1) U F	×	✓ 332/320 332/320/300/272	No	Yes 332/320(B)	No
332 (#2) U F	✓ 332/316/300/284 332/300/282/241	✓ 332 332/307/234/232	Yes	Yes Both	No
332 (#3) U F	✓ 332/252 332/300/282/271	✓ 332 332/300/282/271	Yes	No	Yes
346 (#1) U F	×	✓ 346/329 346/329/245/230	N/A	Yes 346/329(B)	No
346 (#2) U F	✓ 346(8)/330/318 346/330/318/270	×	N/A	Yes 346(8)/330(A)	No
346 (#3) U F	✓ 346/315/226 346/268/226/209	✓ 346 346/329/313/207	Yes	Yes Both	No
374 U F	✓ 374/362/282 374/362/282/165	✓ 374/314 374/314/297/267	No	Yes Both	No

CASE 1175

Two black males went to a traditional healer to obtain a *muti* to "clean their systems". They were given a plant bulb to boil in water. They administered the *muti* as an enema by using a rubber bulb, and drank the rest of the decoction. They both collapsed – one died and the other person was admitted to a hospital. The empty *muti* bottle and a small piece of the plant bulb was sent for analysis and sample preparation performed as described in §6.8.5. Five of the alkaloids common to the bulb of *B. disticha* (Table 6.8) were detected in the piece of plant bulb on the TMD detector (Figures 6.11 and 6.12), but the analysis of the empty bottle only indicated the possible presence of two compounds at trace levels (*m/z* 315 and 331). The analysis of the piece of bulb on the ZMD detector confirmed the results of the TMD detector, while the ESI positive analysis of the empty bottle, in full scan mode (100 – 400 amu), revealed the presence of all 6 alkaloids previously extracted and confirmed in the bulb of *B. disticha* (Figures 6.13 and 6.14).

CASE 121

A black female visited a traditional healer and was given a powder to mix with water and administer as an enema. This she did and died a few hours later. The last remains of the powdered *muti* sample and the rubber bulb used to administer the enema, was sent for analysis. The amount of powdered *muti* sample was insufficient to yield enough alkaloids for detection on the TMD system, but analysis on the ZMD system (Figure 6.15) produced a chromatogram that displayed the characteristic alkaloid fingerprint of the extract of the bulb of *B. disticha*. Identical results were obtained when the rubber bulb, used to administer the enema, was analysed.

CASE 1196

A black female consulted a traditional healer to obtain a *muti* to purge her system. She was handed a 750 ml bottle of *muti* and told to drink half of the bottle and to administer the other half as an enema. She administered the enema and drank the bottle of *muti,* but became violently sick, vomited repeatedly and died shortly thereafter. Stomach, liver and kidney samples as well as the empty *muti* bottle was sent for analysis. Analysis on the samples on the TMD system did not reveal the presence of any alkaloids, but on the ZMD system five alkaloids were detected in the empty *muti* bottle that were consistent with those detected in the *B. disticha* bulb extracts (Figures 6.16 and 6.17).

The analysis of the viscera also produced positive results; three alkaloids were detected in the stomach sample (*m/z* 302, 316 and 332) (Figure 6.18) and four alkaloids in the liver and kidney samples (*m/z* 286, 302, 316 and 332) (Figures 6.19 and 6.20 respectively)

Table 6.8 Identification of the compounds detected in the bulb extracts of Case 1175 by comparing to the NIST™ MS spectral library

PEAK R_t (min)	NIST™ FORWARD SEARCH	NIST™ REVERSE SEARCH
13.22	90% BUPHANAMINE	91% BUPHANAMINE
12.80	73% DIHYDROHEMANTHIDINE	74% DIHYDROHEMANTHIDINE
16.15	78% EPIPOWELLINE	83% POWELLINE
16.18	91% BUPHANISINE	91% BUPHANISINE
18.61	64% 3,4-DMETHOXY-5,N-DIMETHYL-MORPHAN-6-ONE	65% 3,4-DMETHOXY-5,N-DIMETHYL-MORPHAN-6-ONE
19.55	79% UNDULATINE	83% UNDULATINE
20.38	93% BUPHANIDRINE	95% BUPHANIDRINE

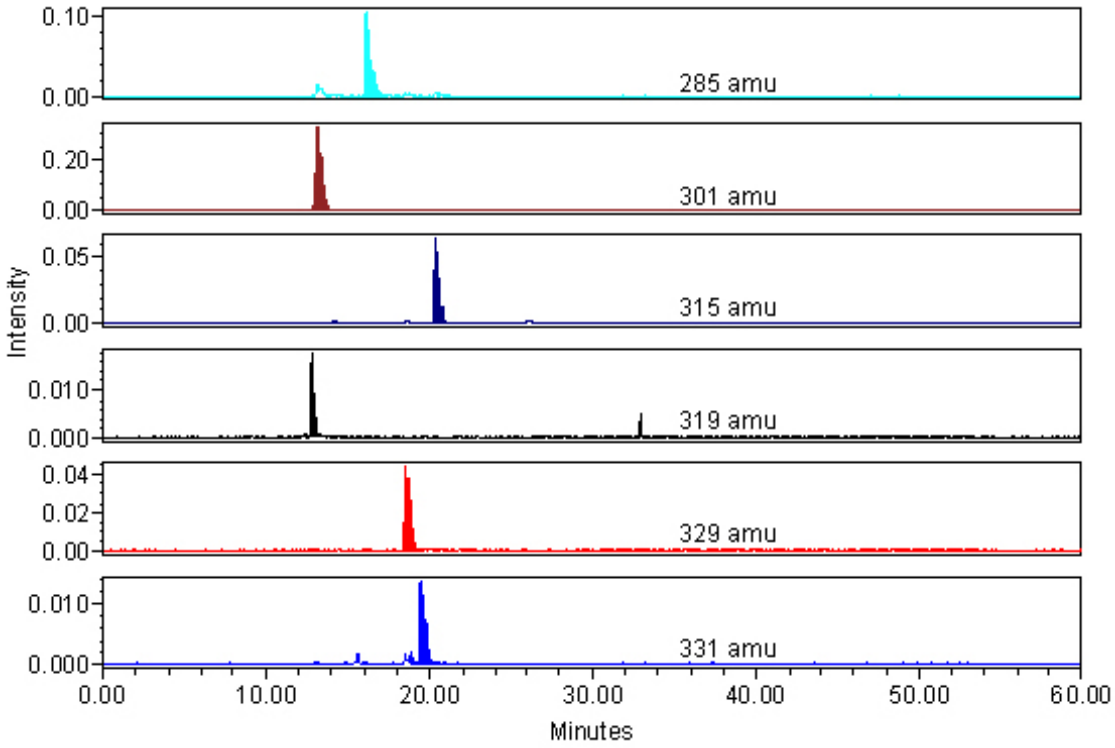


Figure 6.11

Stacked EI chromatograms of the bulb extract of the bulb submitted with Case 1175 (TMD system)

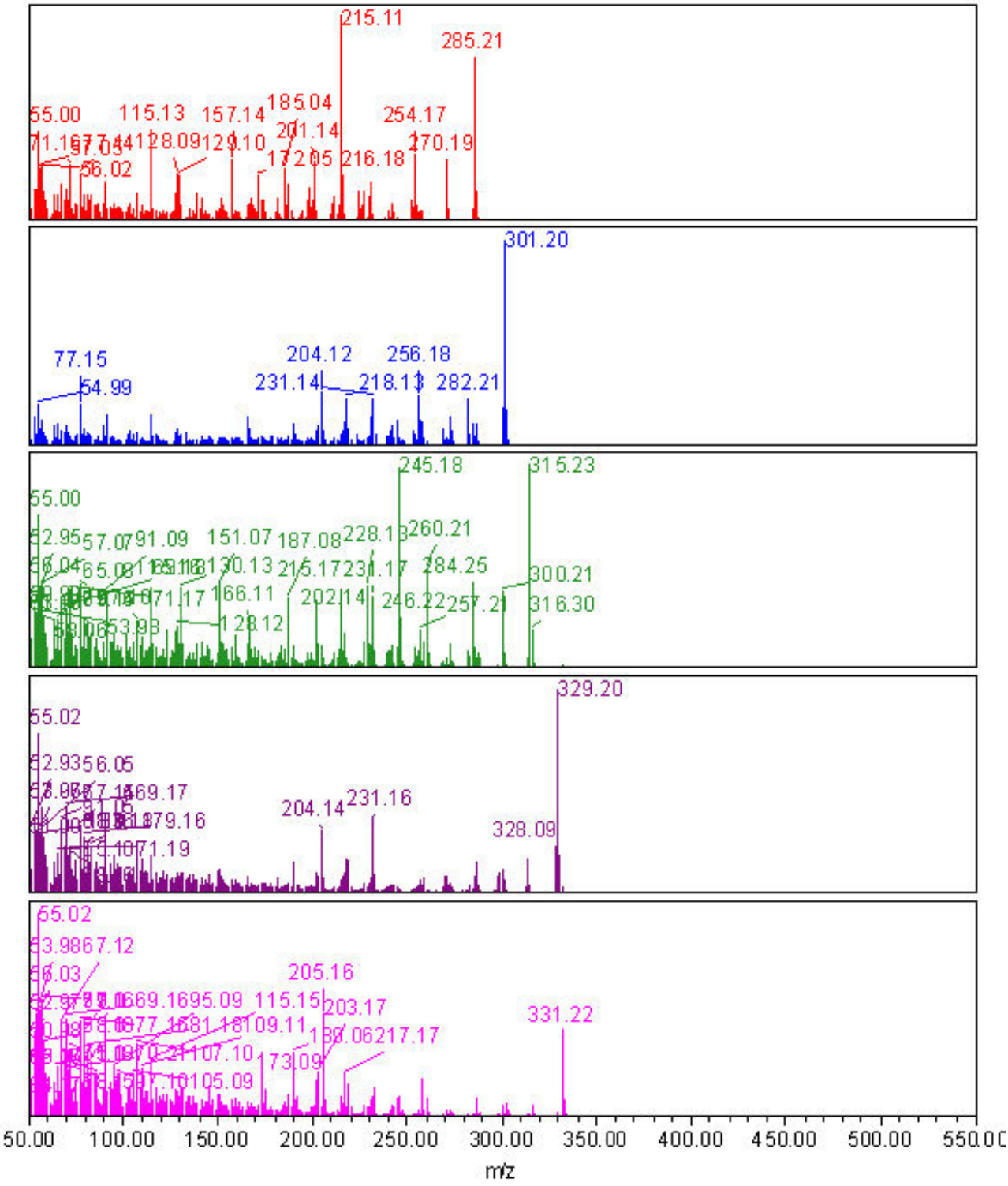


Figure 6.12

Stacked EI spectra of the bulb extract of the bulb submitted with Case 1175 (TMD system)

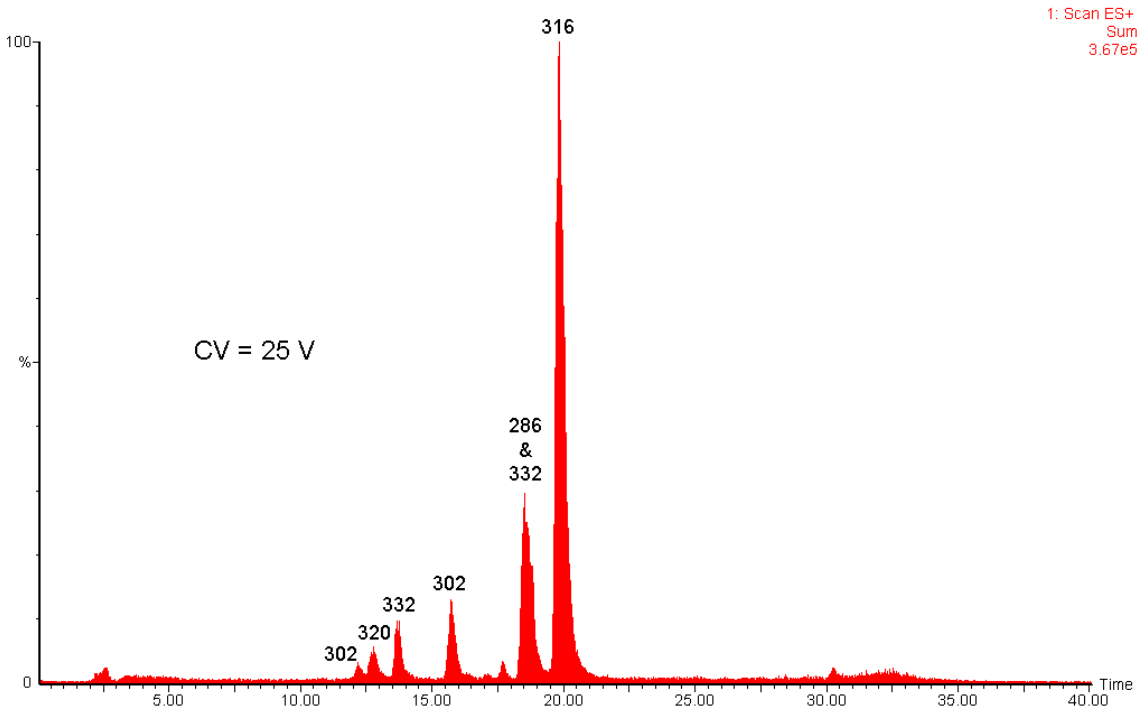


Figure 6.13

ESI$^+$ chromatogram of the SPE extract of the empty *muti* bottle of Case 1175. The nominal masses of the compounds are indicated (ZMD system)

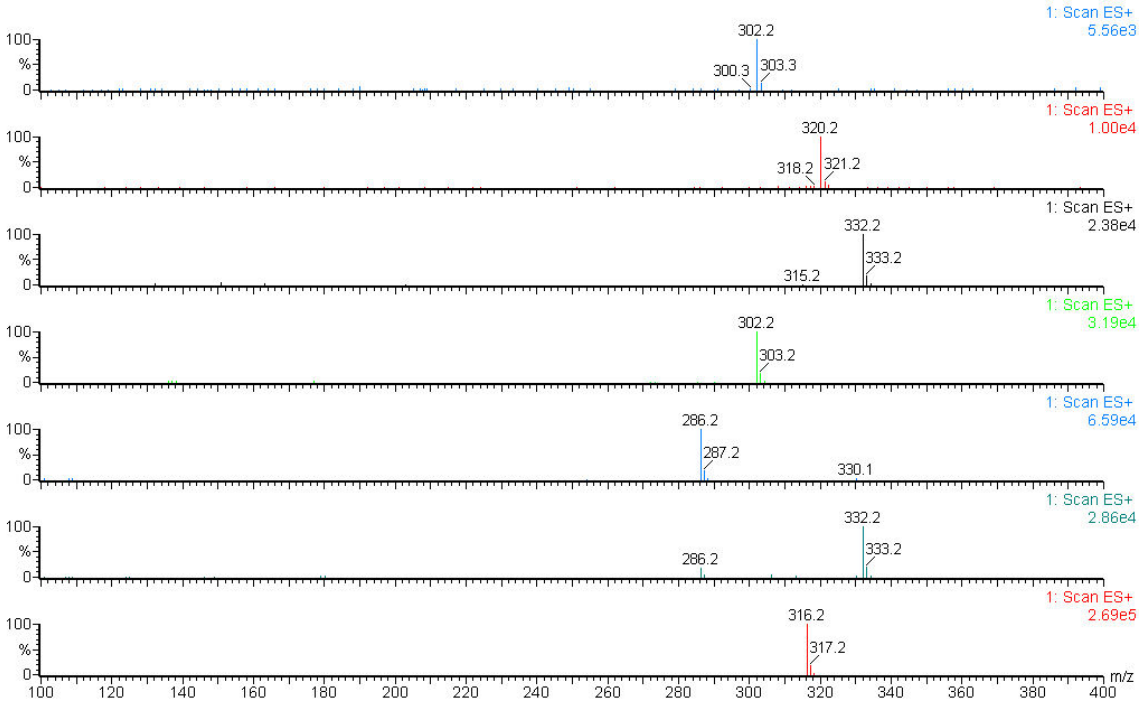


Figure 6.14

ESI$^+$ spectra of the SPE extract of the empty *muti* bottle of Case 1175 (ZMD system)

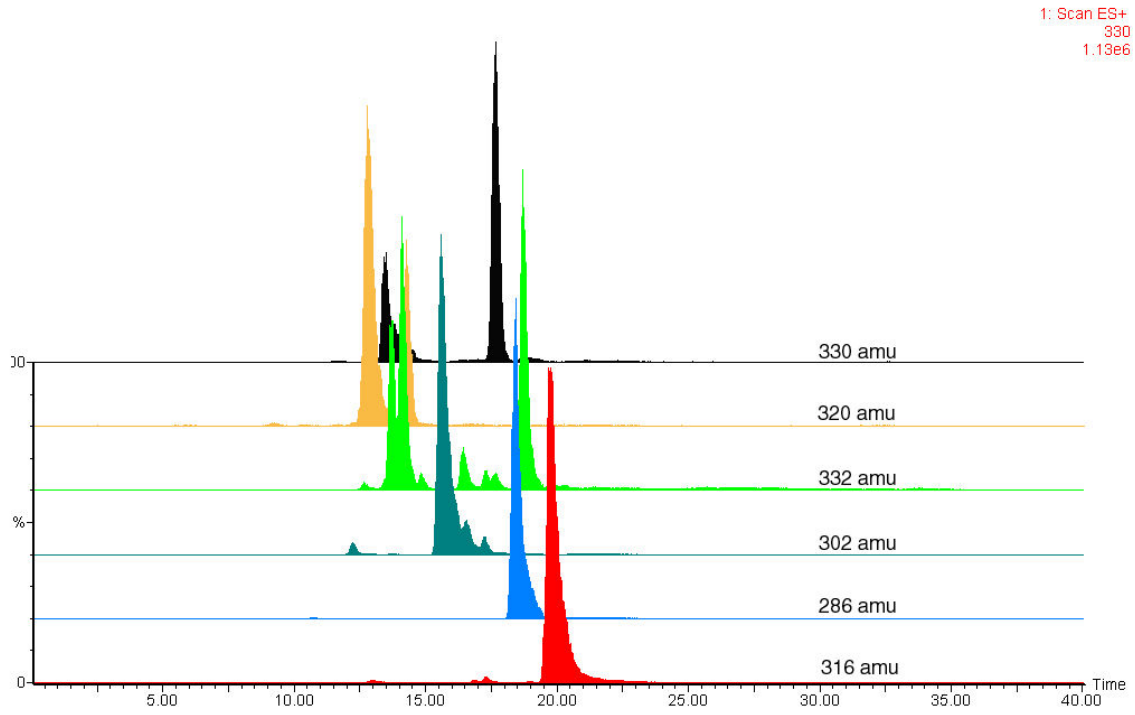


Figure 6.15

ESI$^+$ SIM chromatograms of the SPE extract of the powdered *muti* sample of Case 121. The SIM masses are indicated on the baseline of each chromatogram

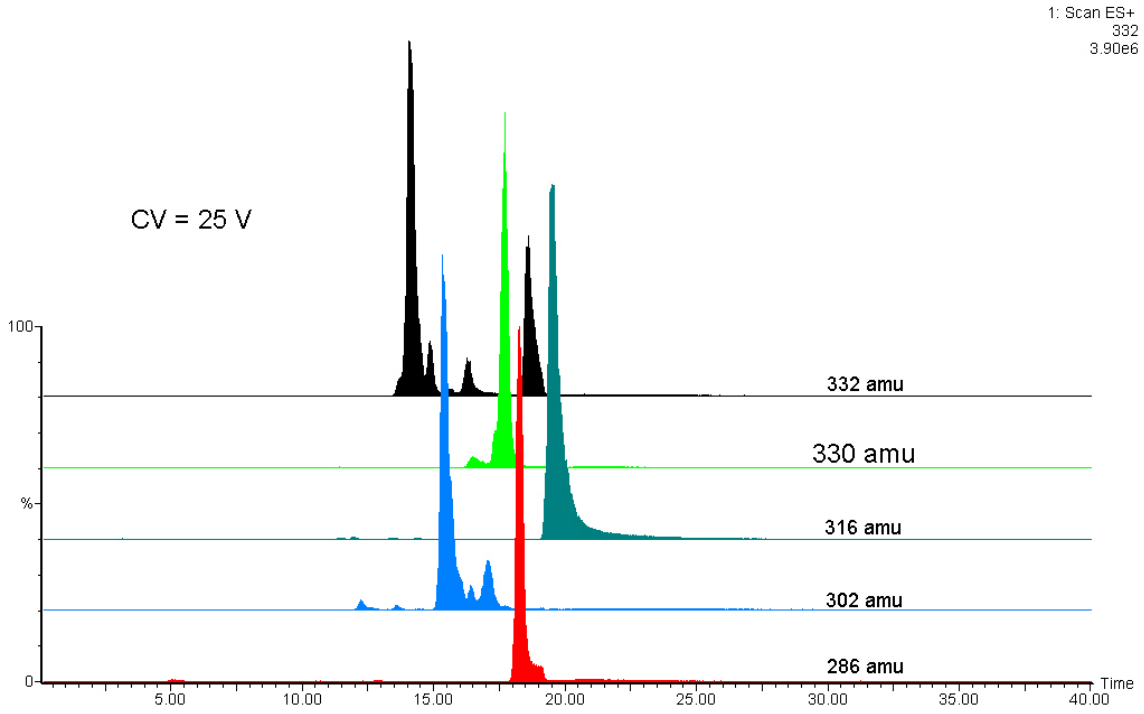


Figure 6.16

ESI$^+$ SIM chromatograms of the SPE extract of the empty *muti* bottle of Case 1196. Each SIM channel mass is displayed on the baseline of the chromatogram

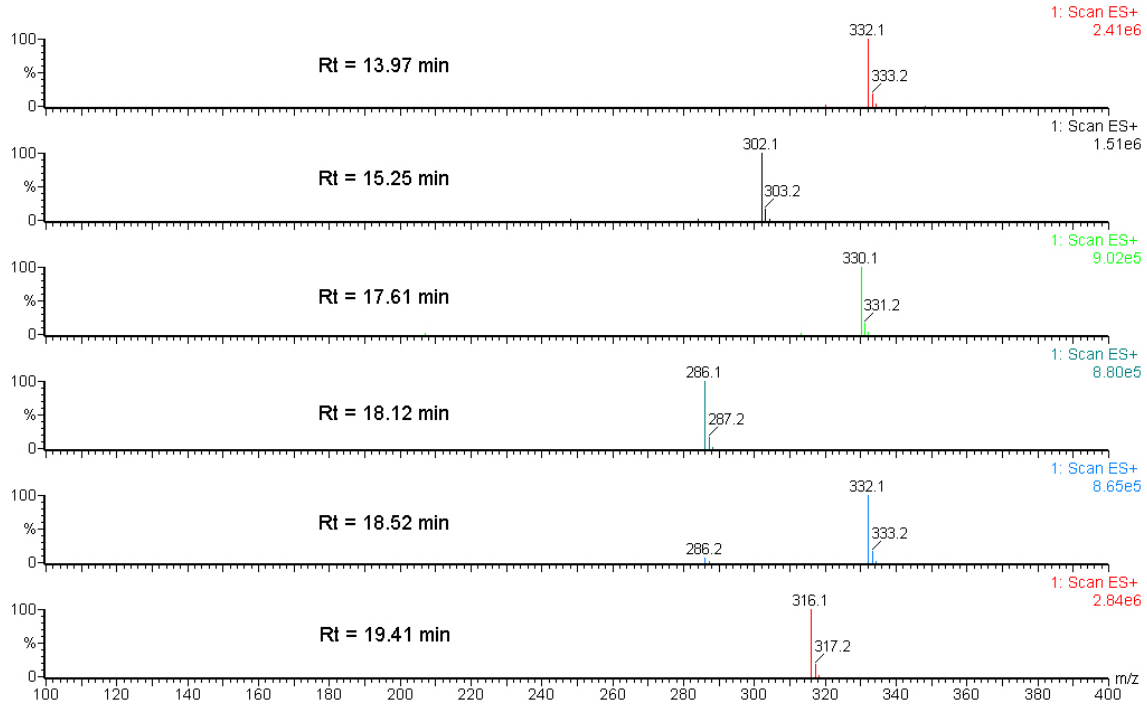


Figure 6.17

ESI$^+$ spectra of the main compounds detected Figure 6.17

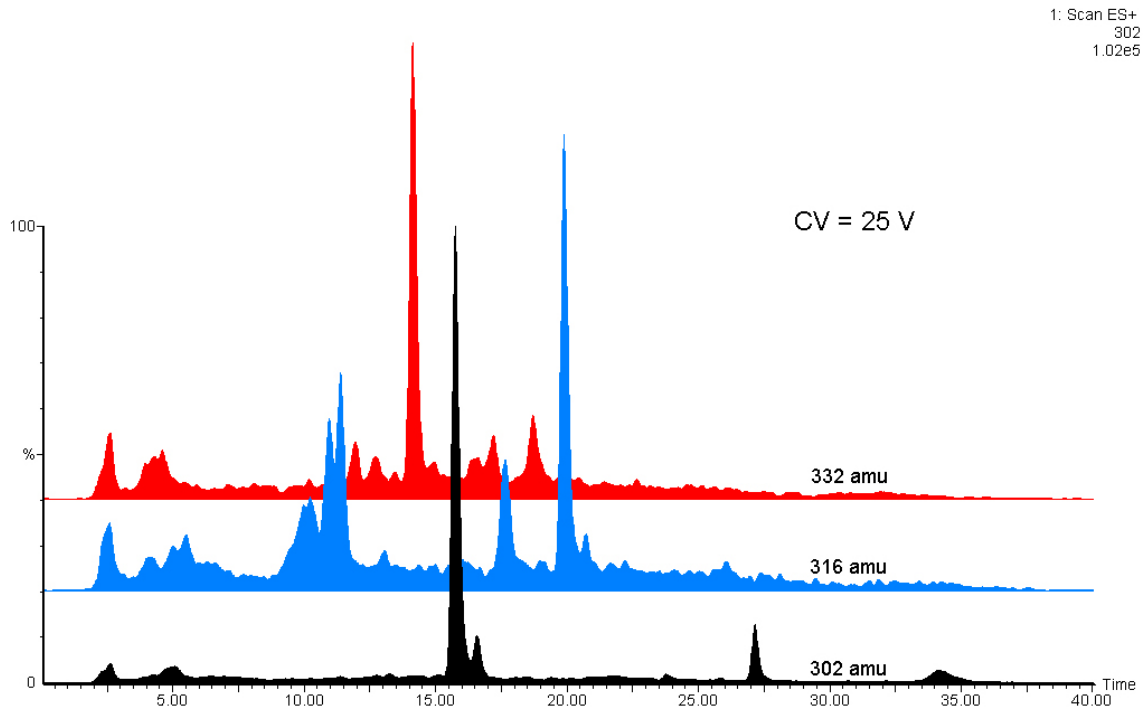


Figure 6.18

ESI$^+$ chromatograms at selected masses of the SPE extract derived from the stomach sample submitted for Case 1196

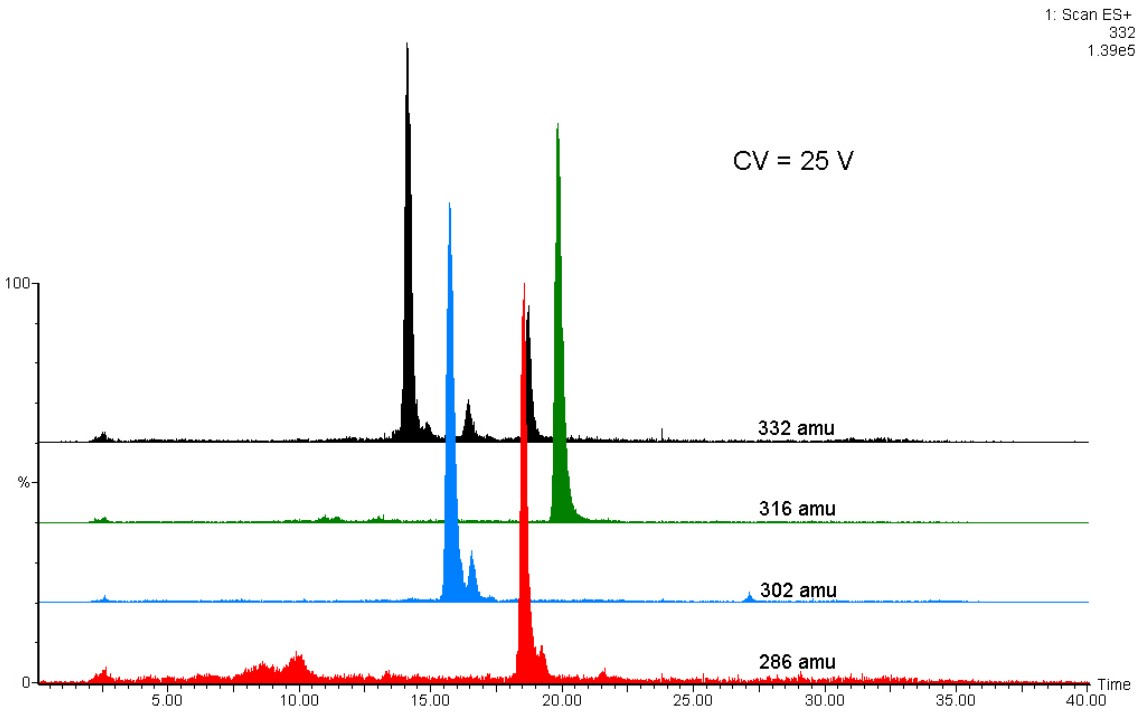


Figure 6.19

ESI$^+$ chromatograms at selected masses of the SPE extract derived from the liver sample submitted for Case 1196

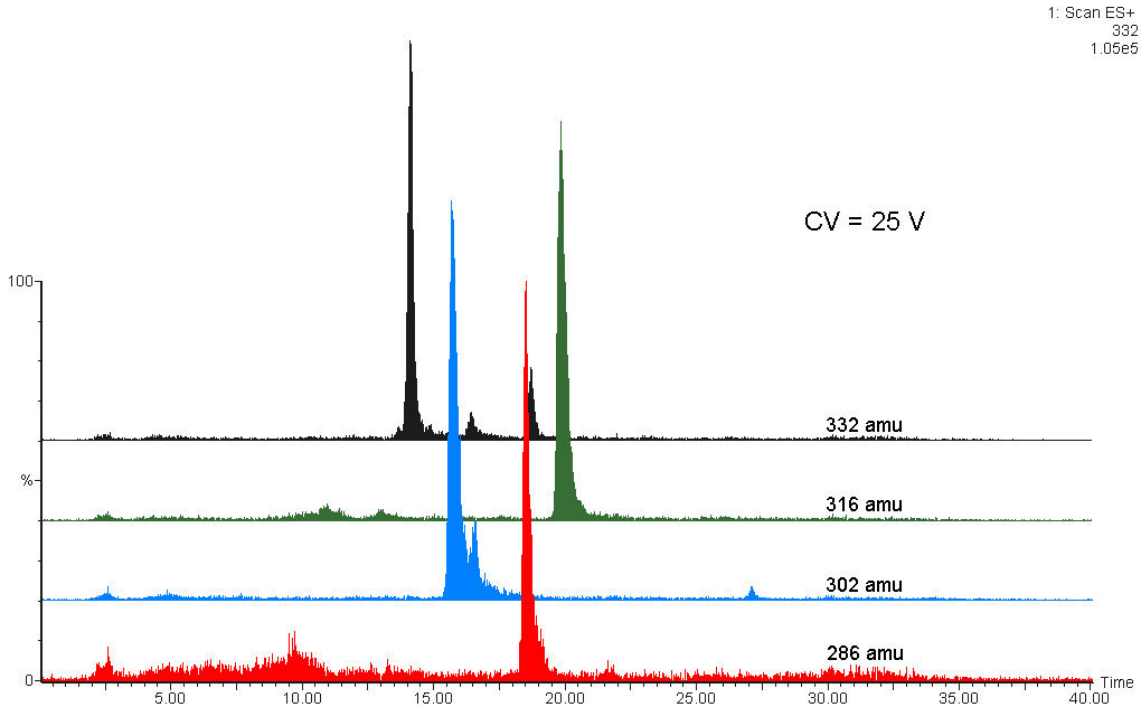


Figure 6.20

ESI$^+$ chromatograms at selected masses of the SPE extract derived from the kidney sample submitted for Case 1196

6.10 FORENSIC APPLICABILITY

The alkaloids present in the bulb of *B. disticha* were extracted successfully with organic solvents, and the major compounds were isolated. Four known alkaloids were identified [buphanamine, buphanisine, buphanidrine and *3-O*-methylcrinamidine (undulatine)] and the structure of distichamine was described for the first time. The use of organic solvents is not desirable as a general extraction procedure, and will also not be suitable for the extraction of viscera due to the presence of large quantities of fats and lipids in viscera samples. The extraction of the alkaloids of *B. disticha* has been done in the past by using the standards SPE methodology supplied with the Waters Oasis HLB cartridges. However, it was found that the extraction efficiency could be nearly doubled by decreasing the pH to 5.

The detection of high levels of these alkaloids can be accomplished with PDA detection as well as mass spectral detection (EI) on the TMD system. The general screening protocol in use on this system is not ideally suited for the separation of all the compounds, but the TMD detector was able to detect selectively the various alkaloids present. However, the analysis of low levels of these alkaloids posed a problem and would have gone undetected if the electrospray method was not developed. The ZMD detector is not the most sensitive detector currently available, and with a scan rate of 500 amu/s is unable to detect small transient peaks. The detector is however ideally suited for the routine screening of samples for the presence of alkaloids, and will easily detect *Boophone* alkaloids in samples where the TMD detector failed to detect them.

The combination of the adapted general SPE procedure and the ZMD detector improved the capability of the Forensic Toxicology Research Unit (FTRU) to detect the presence of Amaryllidacae alkaloids. This was displayed in a botanical matrix (bulb extract and unknown powdered *muti* samples) as well as in biological matrices (stomach, liver and kidney samples).

The presence of *Ammocharis coranica* can also be confirmed or excluded by monitoring the various masses common to *Boophone* (*m/z* 286, 320 and 332), or those common to *Ammocharis* (*m/z* 263 and 346). It was shown that the alkaloid profile of *Ammocharis coranica* appeared to be quite similar to that of *Boophone disticha*, but that a scrutiny of the mass spectra revealed that only two compounds were common to both plants.

CHAPTER 7

Abrus precatorius

7.1 BOTANICAL DESCRIPTION

Abrus precatorius L. (Fabaceae) is a climbing shrub with woody stems and pinnately compound leaves, each with 10 to 15 pairs of small oblong leaflets (Figure 7.1) [128, 129]. Pale purple, inconspicuous flowers are borne in dense groups, followed by clusters of flat pods (Figure 7.2). Each pod produces up to five seeds of about 5 mm in diameter. The pod valves curl back when they open to reveal pendulous seeds. The smooth bright red seeds have a black patch around the hilum and a hard and impermeable seed coat (Figure 7.3) [130]. Common names for this plant include Deadly crab's eye, Indian bead, Jequirity bead, Lucky bean, Minie-minie, Prayer beads, Rosary pea [131], Paternoster bean [132] and *umkoka*.(Zulu) [133]

7.2 ORIGIN AND DISTRIBUTION

Abrus precatorius has a wide distribution in the tropics, including Indonesia, India, Sri Lanka, Thailand, the Philippine Islands, South China, the West Indies and Florida [128, 129, 132] and is indigenous to Indonesia [132] and India [134]. It grows naturally in the eastern and northern parts of South Africa [130].

FIGURE 7.1

Leaves of *Abrus precatorius*

FIGURE 7.2

Leaves and flower of *Abrus precatorius*

FIGURE 7.3

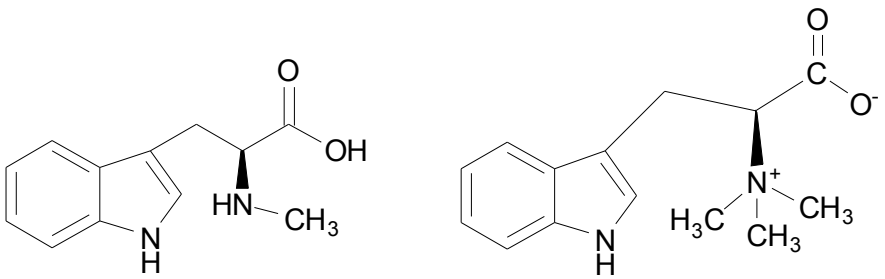
Unripe pods of *Abrus precatorius*

FIGURE 7.4

Seeds of *Abrus precatorius*

7.3 TYPE OF TOXIN

Abrus precatorius contains one of the most toxic compounds known to man, namely abrin. The term “abrin” refers collectively to five glycoproteins that have been purified from the seeds of *A. precatorius*: Abrus agglutinin and the toxic principles, abrins A - D. Abrus agglutinin is a tetramer of 134,900 Da while abrins A - D have molecular weights ranging from 63,000 to 67,000 Da [41]. The seeds also contain two indole alkaloids: abrine, with a molecular formula of $C_{12}H_{14}N_2O_2$ and molecular mass of 218.3, and hypaphorine, with a molecular formula of $C_{14}H_{18}N_2O_2$ and molecular mass of 246.3 [41, 135]. Although these alkaloids are not the major toxins present in *A. precatorius*, they can be used as chemical markers for *A. precatorius* seeds.



Abrine Hypaphorine

FIGURE 7.5

Chemical structures of abrine and hypaphorine

7.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

Abrus agglutinin (tetramer of 134,900 Da) is non-toxic to animal cells and a potent hemagglutinator. Abrins A - D are composed of two disulfide-linked polypeptide chains. The smaller A-chain inhibits protein synthesis and causes cell death; the larger B-chain binds to the cell's plasma membrane [41]. The intact seed is harmless due to the very resistant seed coat which even resists degradation by digestive juices. This situation changes drastically when the seed coat is breached by chewing or piercing, rendering the seed highly toxic. Abrin is extremely toxic with a LD_{50} of 0.02 mg/kg (mouse, IP) [134]. Abrin is antigenic and has chemical and pharmacological properties similar to those of ricin. The symptoms of intoxication are very severe gastroenteritis, dehydration, hypotension, weakness, confusion, convulsions, followed by coma and death [136].

7.5 ETHNOPHARMACOLOGICAL IMPORTANCE

Despite their toxicity, the seeds are used as aphrodisiacs, oral contraceptives or emetics [45]. Root or bark decoctions are taken for chest pain or pleurisy, leaf infusions are drunk if blood is expectorated and decoctions of the seed are taken for venereal disease [137]. The plant is also used for snakebite, sore throats, coughs, colds and fevers and malaria [138]. The seeds are fashioned into necklaces and braces and worn as a lucky charm. Seeds are also used for mythical purposes [139].

7.6 POISONING

7.6.1 Accidental

The bright colour of the seeds is one of the reasons why children are often poisoned by exposure to the seeds. Children have died from eating seeds and half a seed, chewed before swallowing, is reportedly enough to poison a person [45]. As mentioned before, the seeds are non-toxic if the seed coat is left intact, but chewing of the seeds or drilling into them to create a necklace or brace, will render seeds highly toxic. A case of non-fatal poisoning was reported where an herbal tea was contaminated after a rosary, made of *A. precatorius* seeds, was accidentally dipped into the tea [134].

7.6.2 Deliberate

No literature reference could be found to indicate deliberate poisoning with any part of this plant in South Africa. A single case report of deliberate seed ingestion was recorded by the poison control centre of Angers (France) in 1995 [134].

7.7 FCL JHB INVESTIGATION

Reference to *A. precatorius* was found in the FCL JHB database and in forensic case files submitted to the FCL JHB. The seeds are freely available on the traditional healer's market in Johannesburg, but have not been successfully detected or confirmed in forensic samples. This might be due to the unavailability of the necessary equipment and software to detect macromolecules like abrin and ricin, or equipment sensitive enough to detect the minor components like abrine and hypaphorine.

The latest instruments added to the analytical arsenal of FCL JHB, two API LC-MS systems that are capable of detecting small molecules at the ng/ml level, would be ideal for the analysis of the two indole alkaloids. However, no published method could be found for detection of abrine and hypaphorine by LC-MS. This necessitated the development of an extraction method and a HPLC-MS detection method for abrine and hypaphorine.

7.8 EXPERIMENTAL PROCEDURE

7.8.1 Standards and reagents

No standards are commercially available for abrine or hypaphorine. Potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (> 99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%) (NH_4OAc) and acetic acid (99%) were obtained from BDH Laboratory Supplies, England. Sulfuric acid (Suprapur 96%), hydrochloric acid (Suprapur 96%), phosphoric acid (Suprapur 96%), formic acid (98 - 100%) and ammonia (GR 25%) were obtained from Merck, Darmstadt, Germany. Acetonitrile (gradient quality, 200 nm UV cut-off) and methanol (gradient quality, 205 nm UV cut-off) were obtained from Romil, England. HPLC grade water (20 $M\Omega cm^{-1}$) was obtained from a Milli-Q / reversed osmosis system (Millipore, USA). The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [56]. The anhydrous salts of KCl (0.20 g), NaCl (8.00 g), KH_2PO_4 (0.20 g) and Na_2HPO_4 (1.15 g) were dissolved in 1 L deionised water and the pH adjusted to 5.0 with 10% hydrochloric acid.

Oasis HLB solid phase extraction (SPE) cartridges (3 ml with 60 mg of sorbent) were obtained from Waters Corporation, Milford, USA. A 12-port vacuum manifold was used for all extractions.

7.8.2 Instruments and conditions

A Waters 2690 HPLC system equipped with both a 996 photodiode array (PDA) detector and a Termabeam mass selective detector (TMD) [electron impact (EI) mode; maximum mass 1000 *m/z*] from Waters was used for initial screening and high concentration studies. A Waters 2690 HPLC system equipped with a 996 PDA detector and a Z spray mass selective detector (ZMD)

(Micromass, UK) (electrospray mode; maximum mass 2000 *m/z*) was used for low concentration studies. ^{1}H and ^{13}C NMR spectra were obtainded on either a Varian Inova 2000 300 MHz or a Varian 500 MHz spectrometer. A Phenomenex Aqua C18 HPLC column (250 x 2.1mm, 5 µm) was used on the TMD system while a Waters Xterra MS C18 HPLC column (250 mm x 2 mm, 5 µm) was used on the ZMD system. A Harvard syringe pump (Model 11) was obtained from Harvard Apparatus, Holliston, USA and was used for all direct injection experiments on the ZMD detector. Preparative HPLC was done on the ZMD system but a Waters Xterra MS C18 column (300 x 7.8, 10 µm) was used to separate the 100 µl injections of the seed extract.

To ensure the maximum retention of alkaloids on the Xterra MS C18 column, the initial chromatographic conditions were 90% water containing 10 mM ammonium acetate, the pH adjusted to 10 with ammonia (25%) and acetonitrile. After injection, the chromatographic conditions were gradually changed through a linear gradient profile to 90% acetonitrile and 10% of the original aqueous mobile phase in 20 minutes. These conditions were kept stable for 5 minutes where after the HPLC system re-equilibrated the analytical column to the initial conditions. The flow rate was kept constant at 0.2 ml/min and the column temperature was stabilised at 40 ^{o}C. The Phenomenex Aqua column was used with an acidic mobile phase of 0.1% formic acid in the water (pH 2.3), starting at 95% water: 5% acetonitrile and slowly ramping the organic component to 10% methanol: 90% acetonitrile in 35 minutes. These conditions were kept for 10 minutes where after the HPLC column was re-equilibrated with the initial conditions. The gradient profile is summarised in Table 7.1. The HPLC column temperature was maintained at 40 ^{o}C.

The nebuliser of the TMD detector was optimised with a caffeine test solution (100 µg/ml) to ensure efficient nebulisation and droplet formation. The electrospray ionization (ESI) probe of the ZMD detector was optimised by direct injection via a syringe pump using a tryptophan test solution (10 µg/ml). The optimised conditions for the TMD detector are listed in Table 7.2 and those for the ZMD detector in Table 7.3. The optimised chromatographic conditions for the Xterra MS C18 column are listed in Table 7.4.

7.8.3 Chemical extraction of *A. precatorius* seeds

Seeds were purchased from the Traditional healer's market in Johannesburg and dried at 40 ^{o}C to constant weight. The seeds were finely ground using an Ika Werk A10 water-cooled mill (Janke & Kunkel, Straufen, Germany) and 5 g of powdered seeds was placed in two seperate 50 ml polypropylene screw-top centrifuge sample tubes. PBS-HCl buffer (pH 5) (30 ml) was added to one tube while PBS-NH_3 buffer (pH 9) (30 ml) was added to the second tube. The tubes were capped and shaken on a Heidolph rotating sample mixer for thirty minutes. Oasis HLB SPE cartridges were prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used.

Table 7.1 HPLC gradient profile of the general screening method on the TMD system

	TIME min	FLOW ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		0.20	0	0	95	5	6
2	1.00	0.20	0	0	95	5	4
3	10.00	0.20	0	0	80	20	6
4	12.00	0.20	0	0	80	20	4
5	20.00	0.20	0	30	50	20	5
6	35.00	0.20	0	10	0	90	6
7	37.00	0.20	0	10	0	90	6
8	40.00	0.25	0	10	0	90	6
9	45.00	0.25	0	10	0	90	6
10	50.00	0.20	0	0	95	5	5

TABLE 7.2 Optimised MS conditions for the detection of abrine and hypaphorine on the TMD detector

TMD CONDITIONS	ABRINE HYPAPHORINE
Mode	EI positive
Ionisation energy (eV)	70
Gain	10
Nebuliser temperature (°C)	80
Expansion temperature (°C)	90
Source temperature (°C)	225
Capillary tip temperature (^{o}C)	160
Desolvation gas flow (L/h)	30
Sampling rate	1.0
Scan range (amu)	50 - 550
Ion Energy	1.0
Multiplier gain (V)	1250

TABLE 7.3 Optimised MS conditions for the detection of abrine and hypaphorine on the ZMD detector

ZMD CONDITIONS	ABRINE HYPAPHORINE
Mode and polarity	ESI positive
Capillary voltage (KV)	2.50
Cone voltage (V)	15
Desolvation temperature (°C)	450
Extractor voltage (V)	5.0
RF lens (V)	0.5
Source temperature (°C)	120
Cone gas flow (L/h)	40
Desolvation gas flow (L/h)	400
Ion Energy	1.0
Multiplier (V)	-650
Resolution (LMR & HMR)	15.1

Table 7.4 HPLC gradient profile of the alkaloid screening method on the ZMD system

	TIME min	FLOW ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		0.20	0	0	90	10	1
2	2.00	0.20	0	0	90	10	1
3	25.00	0.20	0	0	10	90	6
4	25.00	0.20	0	0	10	90	6
5	34.00	0.20	0	0	90	10	4

After centrifugation on a Hermle Z 300 centrifuge at 4000 rpm for 15 minutes, the aqueous phases were passed through the conditioned Oasis HLB cartridges and the absorbed compounds stripped from the solid phase material with 5 ml methanol. The cartridges were washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. The cartridges were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates combined into two separate collection tubes. The collection tubes were placed into a Zymark

Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of a 50:50 methanol: water mixture.

7.8.4 Isolation of abrine and hypaphorine from *A. precatorius* seeds

A portion of the ground seeds (see 7.8.3) (20 g) was extracted with the acidic PBS buffer as described above but the aqueous phase was passed through a preparative Oasis HLB cartridge (20 ml/ 1g). The SPE cartridge was prepared by washing consecutively with 20 ml of methanol, water and the extracting buffer used. The cartridge was washed with 20 ml extracting buffer and dried under vacuum for 10 minutes. The cartridge was then eluted with 20 ml methanol, 20 ml acidic methanol (10 ml formic acid per liter methanol), 20 ml basic methanol (10 ml ammonia per liter methanol) and 20 ml acetonitrile and the eluates combined into one collection flask and evaporated to dryness at 40 °C under reduced pressure. The dry residue was reconstituted using the minimum amount of a 50:50 methanol: water mixture.

The chromatographic conditions used on the Waters Xterra MS C18 analytical column was fed into the Prep Calculator software of the Waters Corporation to recalculate the conditions that must be used for a Waters Xterra MS C18 column (300 mm x 7.8 mm, 10 µm). The same gradient profile as described for the analytical column was used but the flow rate was increased to 2.76 ml/min. These conditions were used to fractionate abrine and hypaphorine from 100 µl injections of the re-dissolved residue by means of a LKB 2211 Superrac fraction collector and monitoring the UV spectrum of the PDA detector between 200 and 600 nm. The structures of abrine and hypaphorine were confirmed by NMR spectroscopy.

Abrine:

^{1}H NMR: δ_H (MeOH-d_4, 500 MHz) 7.68 (1H, d, *J* 7.8 Hz, H-4 or 7), 7.36 (1H, d, *J* 8.1 Hz, H-4 or 7), 7.21 (1H, s, H-2), 7.12 (1H, t, *J* 7.5 Hz, H-5 or 6), 7.05 (1H, t, *J* 7.5 Hz, H-5 or 6), 3.76 (1H, dd, *J* 8.0, 4.6 Hz, H-α), 3.49 (1H, dd, *J* 15.4, 4.6 Hz, H-β), 3.27 (1H, dd, *J* 15.4, 8.0 Hz, H-β).
^{13}C NMR: δ_C (MeOH-d_4, 125 MHz) 138.1 (C-9), 128.3 (C-8), 125.1 (C-7), 122.7 (C-6), 120.1 (C-5), 119.2 (C-4), 112.3 (C-2), 108.7 (C-3), 65.2 (C-α), 33.1 (NCH_3), 27.5 (C-β) (Signal of carboxylic acid was not observed due to low signal-to-noise ratio).

Hypaphorine:

^{1}H NMR: δ_H (D_2O, 300 MHz) 7.65 (1H, d, *J* 7.5, H-4 or 7), 7.51 (1H, d, *J* 8.0, H-4 or 7), 7.23 (2H, m, H-5,6), 3.90 (1H, dd, *J* 11.2, 3.8 Hz, H-α), 3.30 (2H, m, H-β), 3.25 [9H, s, N-$(CH_3)_3$].
^{13}C NMR: δ_C (D_2O, 75 MHz) 174.4 (CO_2H), 139.0(C-9), 129.2 (C-8), 127.5 (C-7), 124.9 (C-6), 122.3 (C-5), 121.0 (C-4), 114.9 (C-2), 109.9 (C-3), 81.7 (C-α), 54.8 [N-$(CH_3)_3$], 25.6 (C-β).

7.9 RESULTS AND DISCUSSION

7.9.1 Chromatography and detection of abrine and hypaphorine

Abrine and hypaphorine ionised easily under acidic conditions which rendered them polar and difficult to retain on reversed-phase HPLC columns. The Phenomenex aqua column was stable with highly aqueous mobile phases, well suited to retain intermediate polarity compounds but unstable under basic chromatographic conditions. A 5 µl injection of the acidic extract on the TMD system (Figure 7.6) produced seven major UV-active compounds, while a similar volume injection of the basic extract (Figure 7.7) produced five major UV-active compounds. Both extracts contained abrine (R_t 11.9 min), hypaphorine (R_t 13.8 min) and two apolar but unknown compounds (R_t 28.7 min and R_t 30.8 min).

The fractionated abrine and hypaphorine samples were injected on the TMD system. The UV and EI chromatograms and mass spectra of abrine and hypaphorine are depicted in Figures 7.8 and 7.9 respectively. Both compounds displayed unspecific UV spectra that are very similar to commonly detected indole putrifacts. The EI mass spectra of both compounds were also quite insignificant and no molecular ion could be observed. Although the EI spectra of the compounds are significantly different, the lack of a strong molecular ion peak will make low level detection of both compounds by means of EI ionisation difficult.

During the optimisation of the ESI interface of the ZMD system, it was found that tryptophan ionised easily by using a gentle cone voltage of 15 V. The test compound was also readily fragmented by ISCID if the cone voltage was raised to 25 V or higher. Abrine and hypaphorine are both polar compounds and not well retained on the Xterra MS C18 column. To retain these compounds, the initial conditions were maintained for two minutes before the gradient was introduced.

The fractionated abrine and hypaphorine samples were injected on the ZMD system and a single compound was observed in each fraction. Fraction 1 (Figure 7.10) contained only abrine (R_t 6.17 min) and the mass spectrum produced by a cone voltage of 15 V was dominated by the protonated species of abrine ($[M+H]^+$ = 219) and some minor fragmentation ions.

By raising the cone voltage to 25 V abrine was extensively fragmented ([M+H-$NHCH_3$ = 188], [M+H-$C_2H_3NO_2$ = 146] and [M+H-$C_3H_5NO_2$ = 132]; Figures 7.10 and 7.12). Fraction 2 (Figure 7.11) contained only hypaphorine (R_t 8.25 min) and the mass spectrum produced by a cone voltage of 15 V was dominated by the protonated species of hypaphorine ($[M+H]^+$ = 247) and a minor fragmentation ion. By raising the cone voltage to 25 V hypaphorine was extensively fragmented ([M+H-$C_2H_3O_2$ = 188] and [M+H-$C_4H_7NO_2$ = 146]; Figures 7.11 and 7.12).

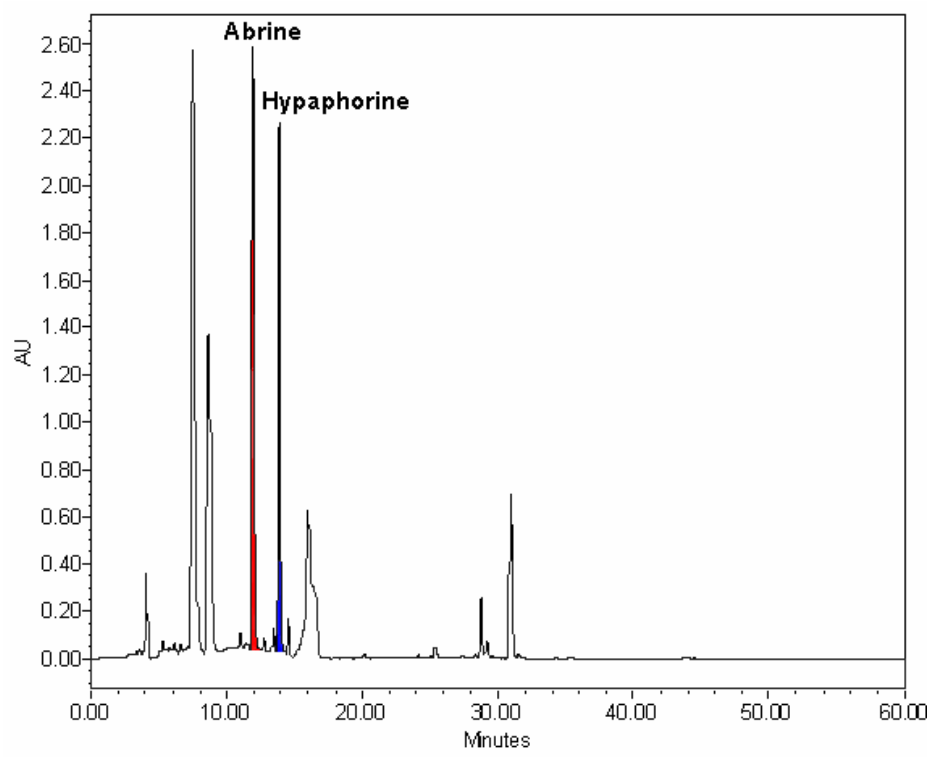


Figure 7.6

HPLC PDA chromatogram of the acidic extract of *Abrus precatorius* seeds on the TMD system

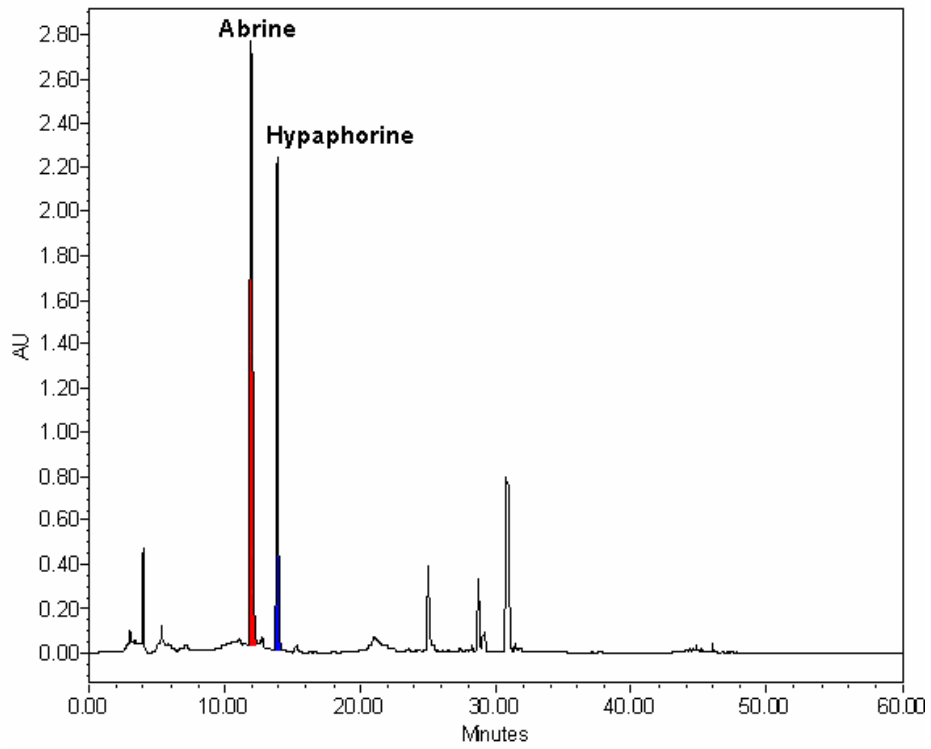


Figure 7.7

HPLC PDA chromatogram of the basic extract of *Abrus precatorius* seeds on the TMD system

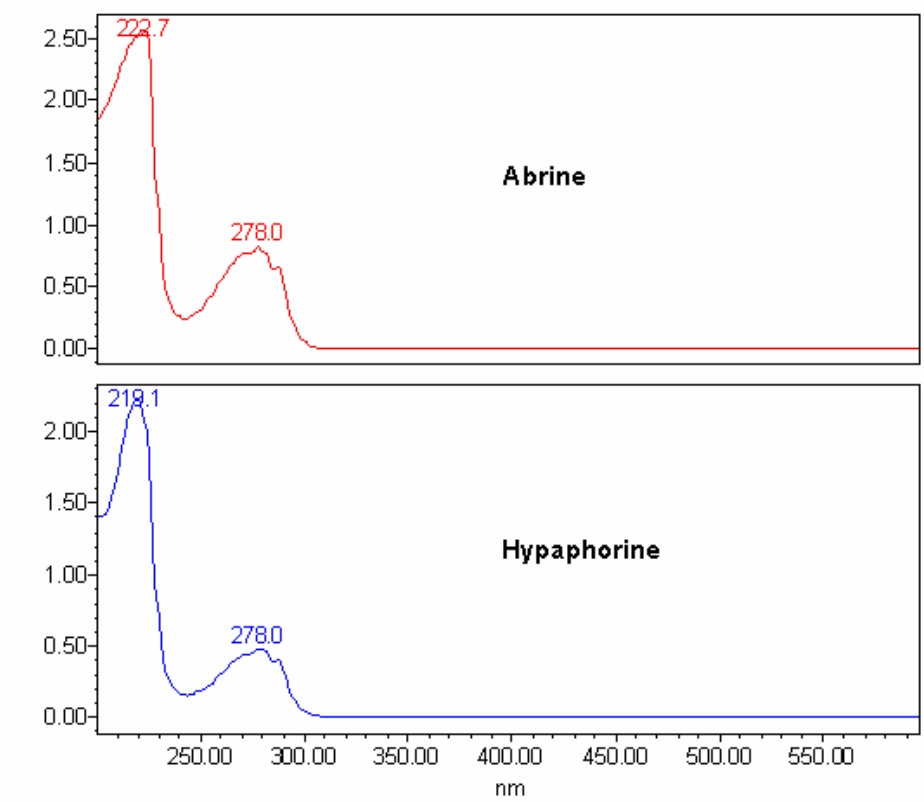


Figure 7.8

UV spectra of abrine and hypaphorine (TMD system)

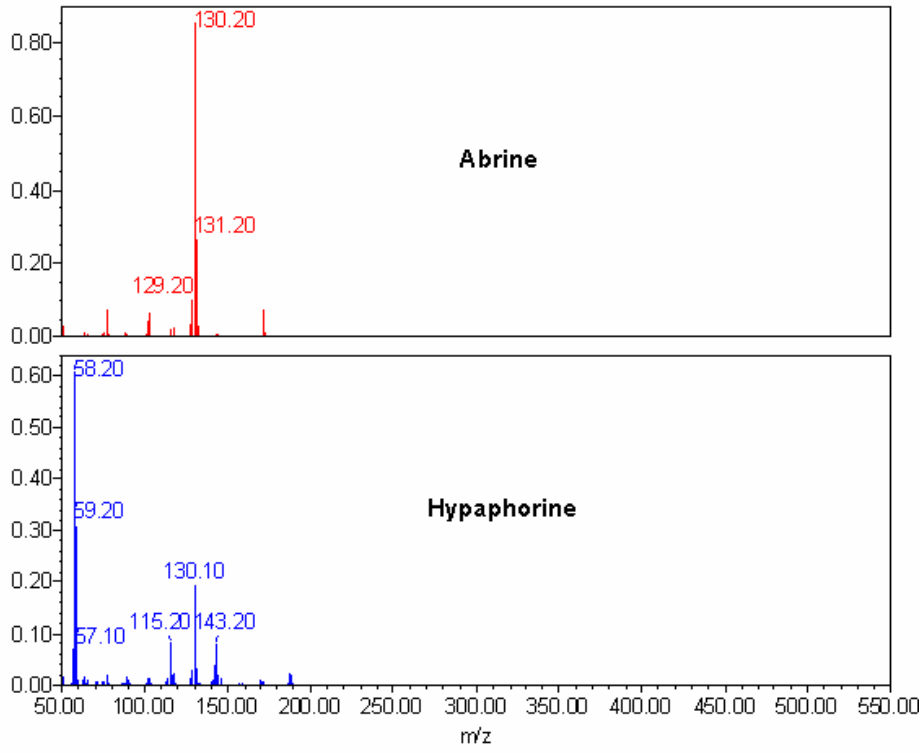


Figure 7.9

EI spectra of abrine and hypaphorine (TMD system)

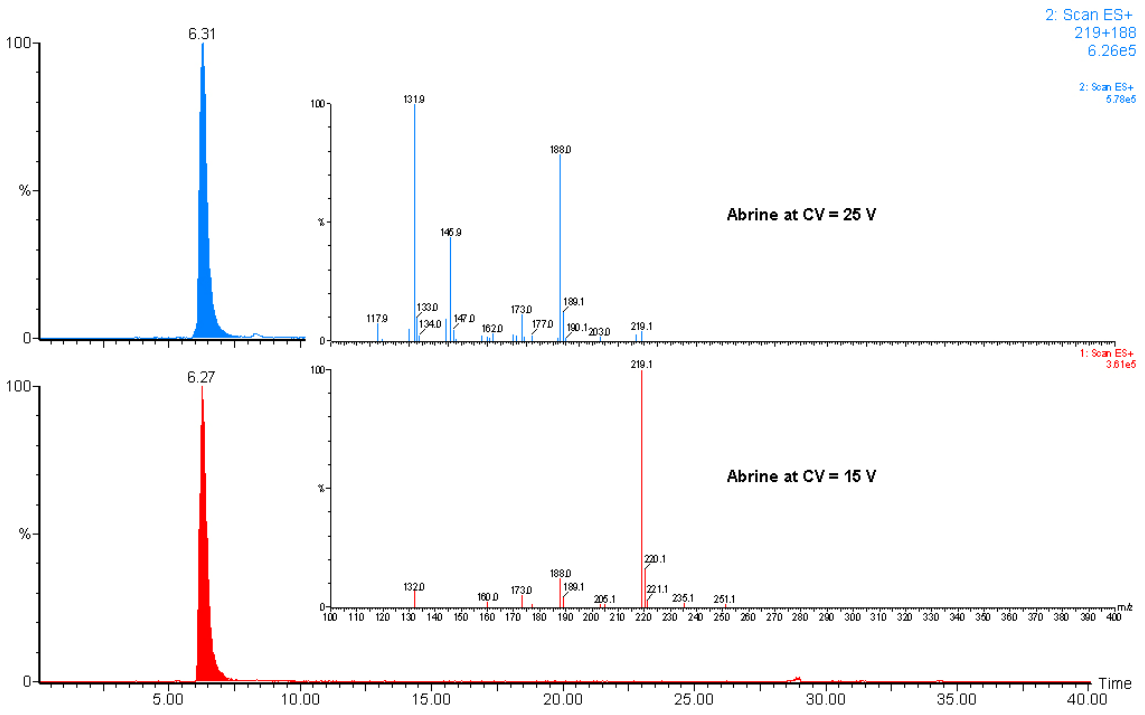


Figure 7.10

Chromatogram and ESI$^+$ spectra of the 1st fraction containing abrine (ZMD system)

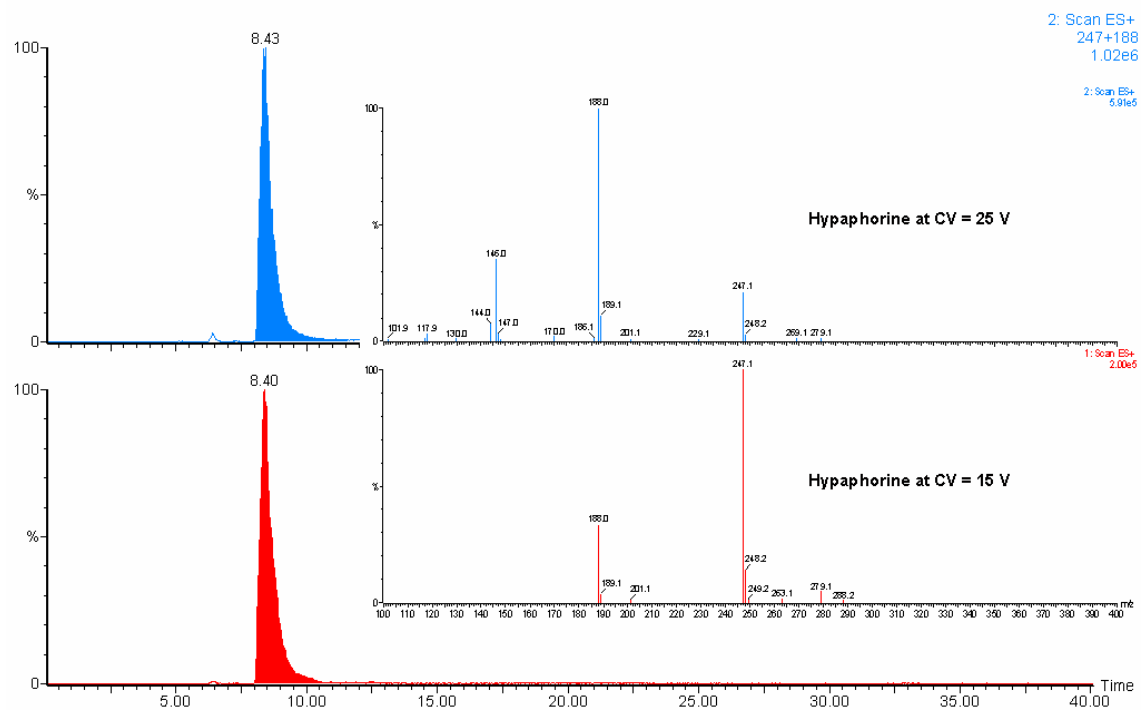


Figure 7.11

Chromatogram and ESI$^+$ spectra of the 2nd fraction containing hypaphorine (ZMD system)

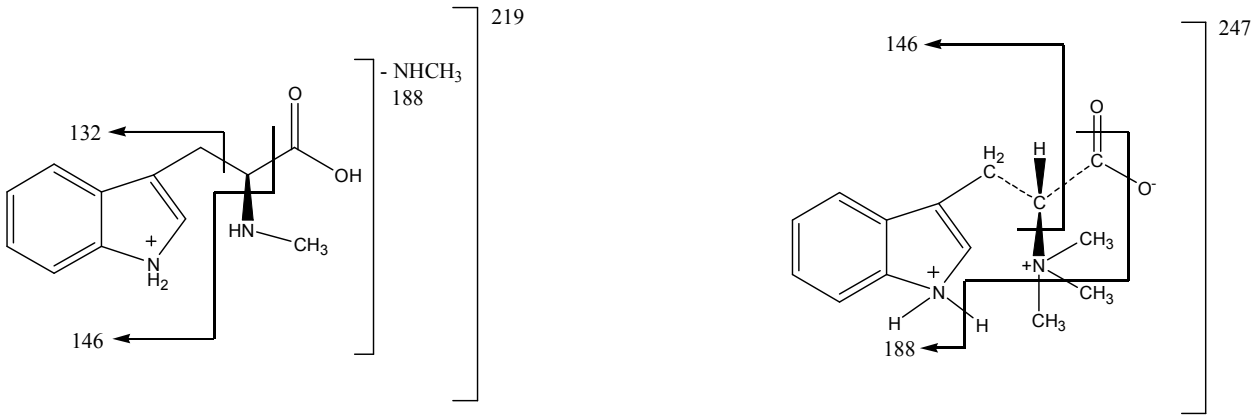


FIGURE 7.12

ESI$^+$ ISCID fragmentation of abrine and hypaphorine (ZMD system)

Both the acidic and basic extracts of the ground seeds contained abrine and hypaphorine, but the acidic extract (Figure 7.12) contained the most product and displayed the two well separated peaks of main components with R_t 6.2 min ($[M+H]^+$ = 219; abrine) and R_t 8.3 min ($[M+H]^+$ = 247; hypaphorine). The identity of both compounds could easily be confirmed by the characteristic ISCID fragmentation of the compounds at a cone voltage of 25 V (Figures 7.13 and 7.14).

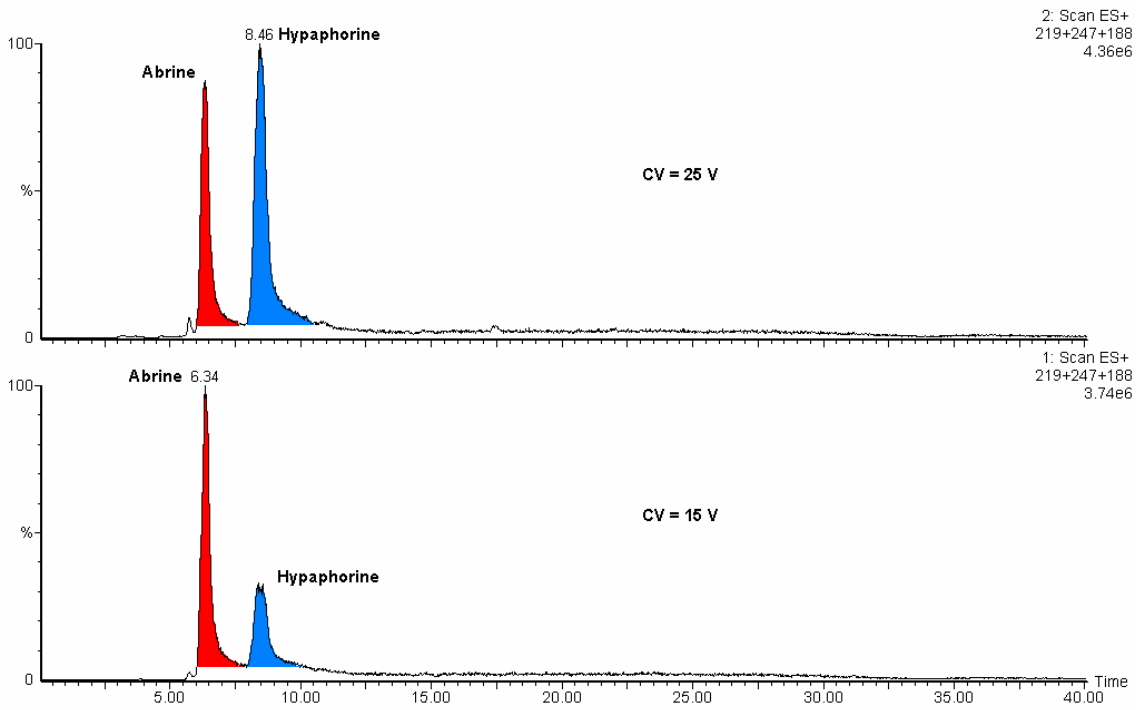


Figure 7.13

ESI$^+$ chromatograms of the acidic extract of *Abrus precatorius* on the ZMD system at cone voltages of 15 V and 25 V

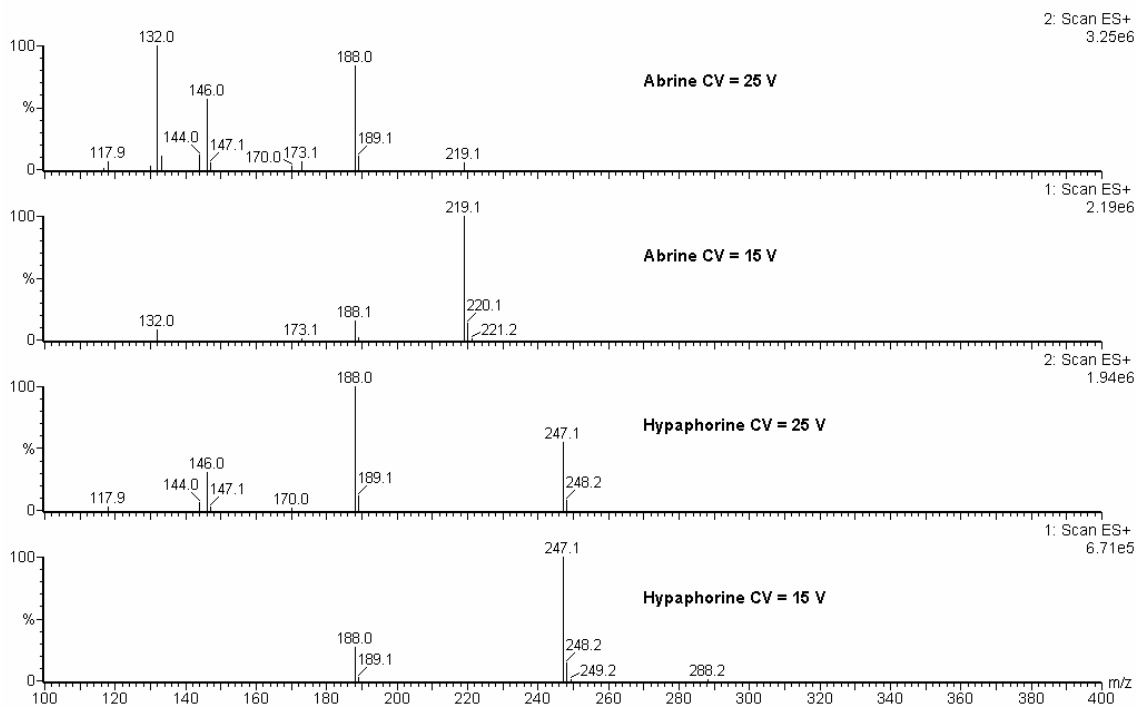


Figure 7.14

ESI$^+$ spectra of abrine and hypaphorine as detected in the acidic extract at cone voltages of 15 V and 25 V

7.9.2 Chemical extraction of *A. precatorius* seeds

Due to the polar nature of abrine and hypaphorine, Oasis HLB solid phase cartridges were used to extract the compounds of interest. Both compounds were successfully retained but rapidly eluted with the addition of methanol or acetonitrile. It was interesting to note that the bed of the cartridge turned wine red under acidic conditions but turned dark green under basic conditions. This might be useful when extracting unknown powdered samples with this method. Abrine and hypaphorine were retained in both acidic (pH 4.5) and basic (pH 9) extracts although the acidic extract rendered the highest yields of the two compounds and was the method of choice for all further experiments.

7.9.3 Purification of abrine and hypaphorine

The preparative Oasis HLB cartridges (20 ml/ 1 g) displayed similar retention and chromatographic characteristics as the small analytical scale (3 ml/ 60 mg) cartridges. The retention behaviour was strongly dependent on the amount of organic modifier present and the smallest amount of methanol or acetonitrile resulted in loss of product due to bleeding. The same chromatographic conditions as used for the analytical column (Table 7.3) were used for the preparative column and only the flow rate was increased to 2.76 ml/min. This was done to ensure similar chromatographic behaviour of the compounds of interest. The 10 µl syringe of the

injector was replaced with a 250 µl syringe to allow injection volumes of 100 µl per preparative run. Although the preparative column could handle larger injection volumes, the injection system was limited to 100 µl injections due to the internal sample loop volume of 100 µl on the 2690 Separation module.

The UV spectrum (200 – 600 nm) was monitored and the two main components were fractionated on the fraction collector by using a retention time trigger from the 2690 Separation module and fixed collection windows on the fraction collector. Due to the concentrated nature of the crude extracts, the samples started precipitating. To alleviate this problem, the temperatures of the sample compartment and column compartment were raised to 40 ^{o}C. This eliminated the precipitation problem and predictably improved peak separation and peak shape.

Analyses of the purified fractions on the TMD and ZMD systems have been discussed in a previous section. The results indicated that abrine and hypaphorine could be successfully extracted on preparative Oasis HLB cartridges and that the fractionation procedure used were able to produce abrine and hypaphorine of >95% purity.

7.10 FORENSIC APPLICABILITY

Abrus precatorius is one of the plants that have not been successfully detected at the FCL JHB. The only report of the presence of *A. precatorius* in a forensic exhibit was based on the apolar UV components (Figure 7.15) of the seeds that were detected in a spiked chocolate cake.

A recent forensic case (Case 114) was received at the FCL JHB of a high-profile person who went to a traditional healer for a tonic. After taking his tonic, the person became ill and died a few days later. It was alleged that he was poisoned by the traditional healer and the stomach and its contents as well as a blood sample was submitted for chemical analysis. From previous experience it was found that the HCl-PBS buffer constantly produces the best extraction yields, and this method was used to extract the samples.

The samples were analysed on the TMD system (UV and EI), and two peaks were observed on the PDA detector, in all the samples, that closely resembled abrine and to a lesser extent hypaphorine (UV spectra and retention time; Figure 7.16). The EI mass spectra of the two compounds were matched with the NIST MS library and indicated possible matches with tryptophan and tryptamine derivatives. The matches were good enough to warrant a second look but were not conclusive. The first spectrum was particularly interesting as a strong 130 amu base peak was observed that strongly resembled abrine (Figure 7.17).

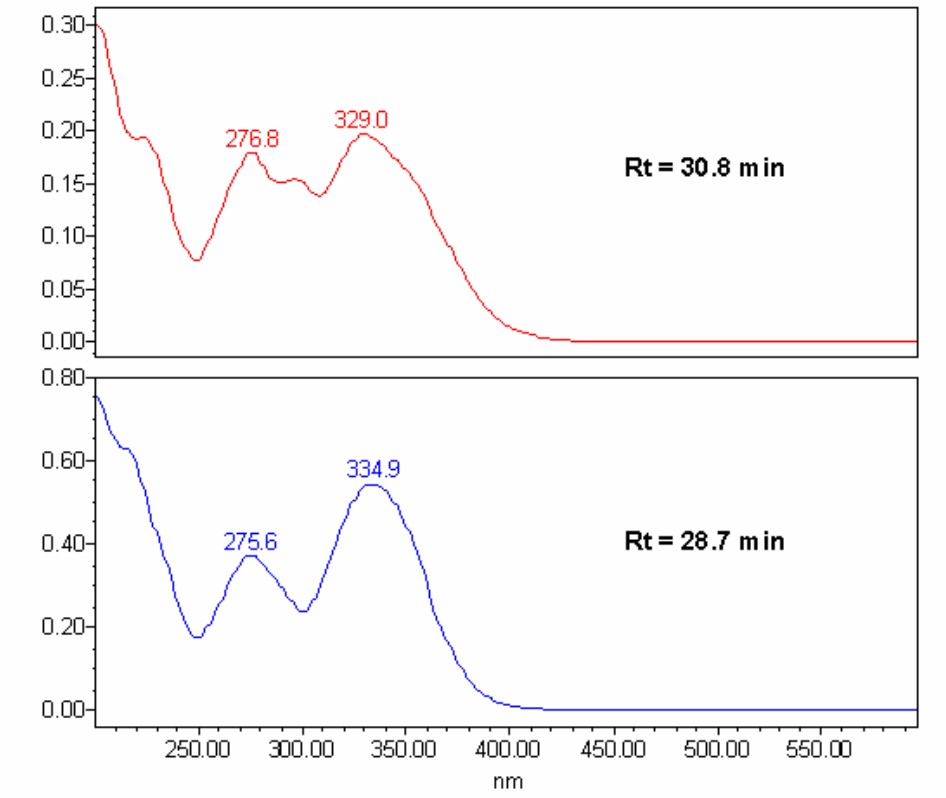


Figure 7.15

UV Spectra of the apolar compounds extracted form *Abrus precatorius* seeds

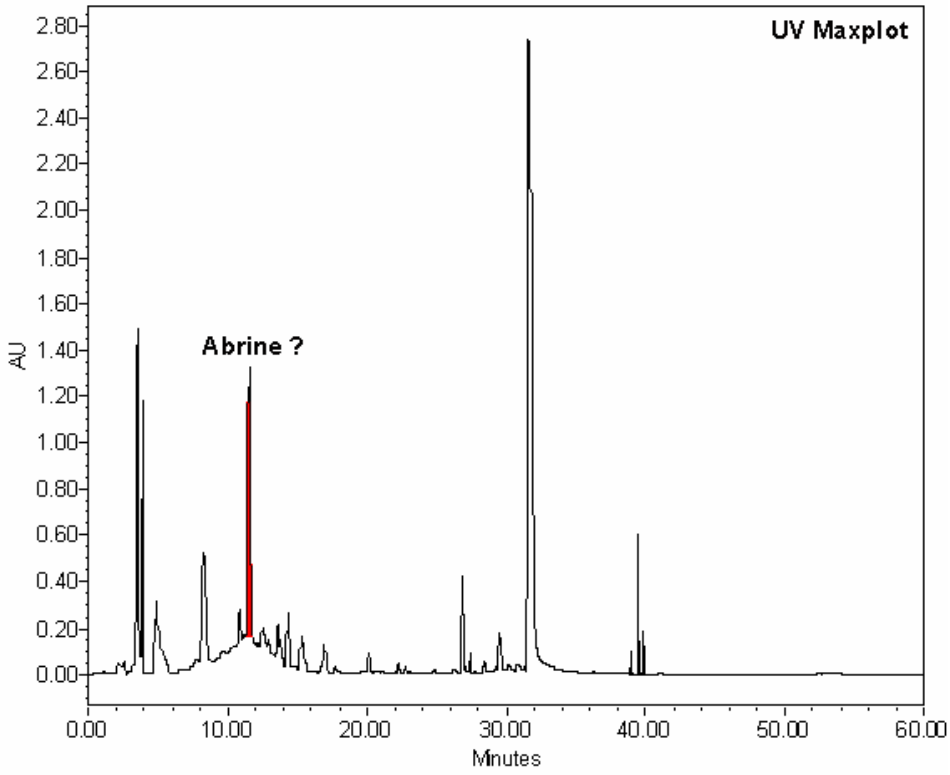


Figure 7.16

Maxplot UV chromatogram of the analysis of the blood sample of Case 114 (TMD system)

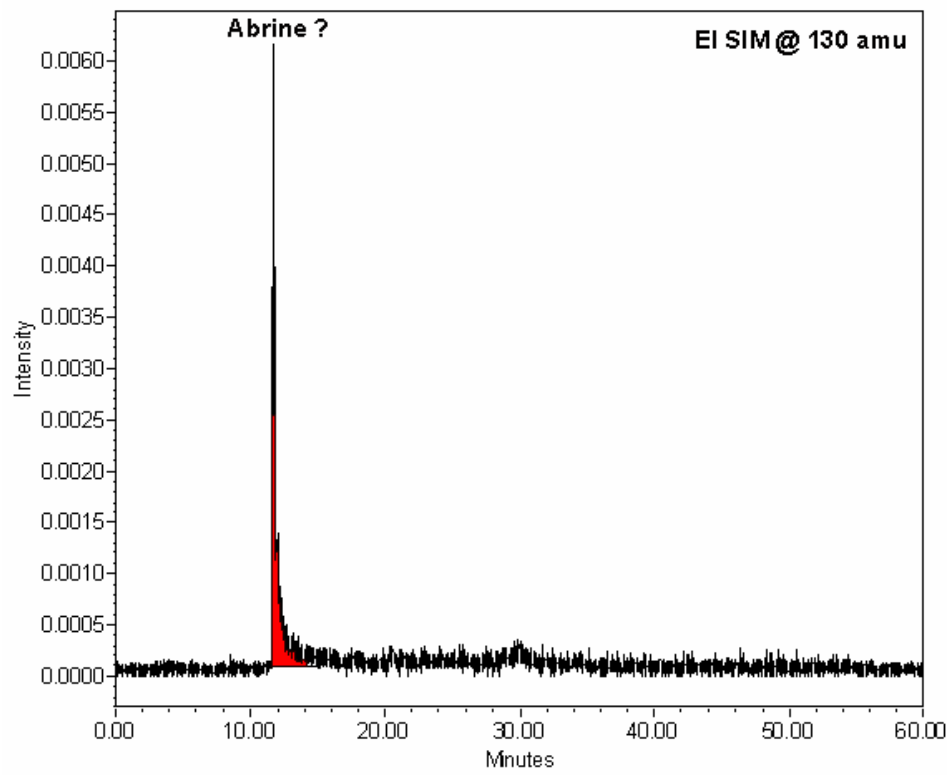


Figure 7.17

EI SIM chromatogram of the analysis of the blood sample of Case 114 (TMD system)

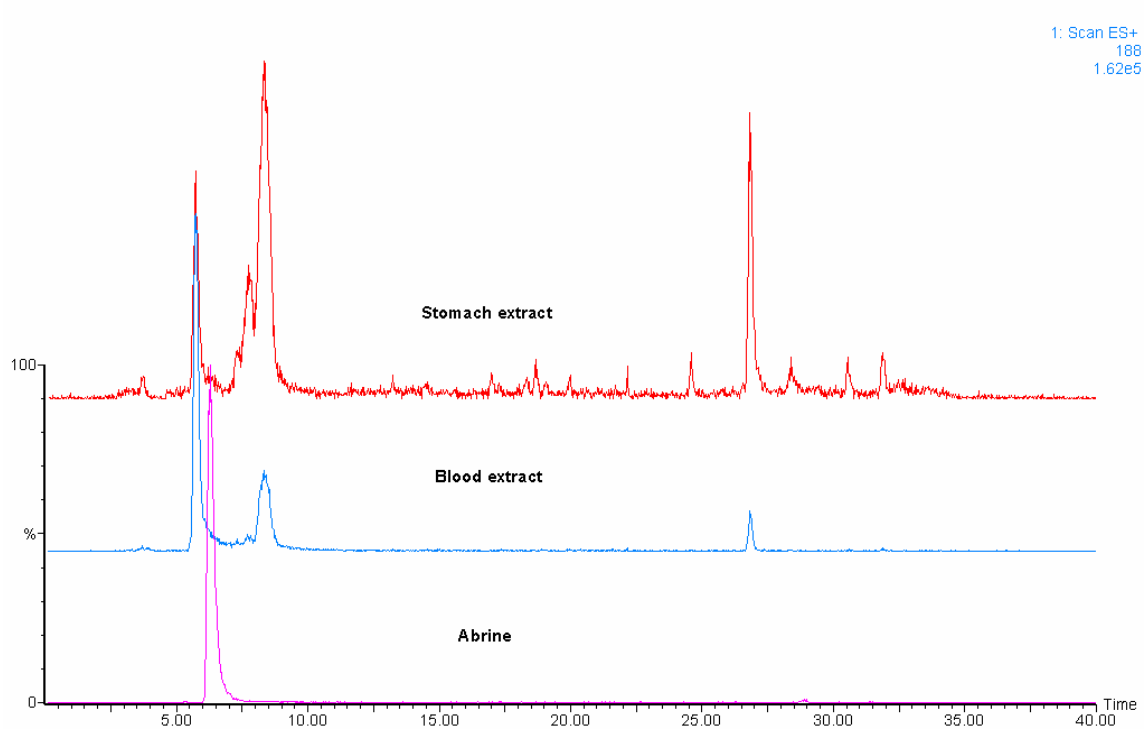


Figure 7.18

ESI$^+$ chromatograms of the analysis of the blood sample of Case 114. Overlaid the chromatogram of the purified abrine fraction (both ZMD system)

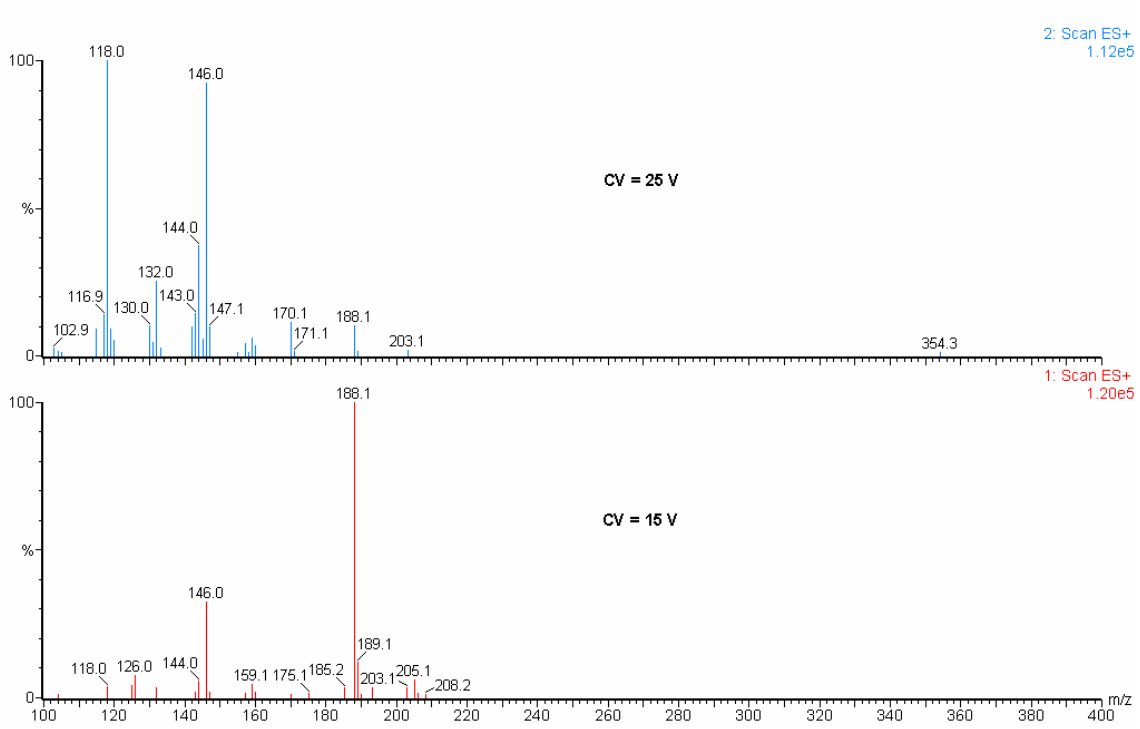


Figure 7.19

ESI$^+$ spectra of the suspected abrine peak detected in the analysis of the blood sample of Case 114 (ZMD system)

The samples were re-analysed on the ZMD system to obtain the characteristic ESI$^+$ spectra of the two compounds. Although peaks were observed at the correct retention time, the ESI$^+$ spectra did not match those of abrine or hypaphorine.

If this type of analysis was attempted on a UV-based chromatographic system, a false positive might have been reported. The addition of EI and ESI$^+$ mass spectral data conclusively ruled out the use of a *muti* containing the compounds of *Abrus precatorius*.

The combination of the UV-EI and UV-ESI$^+$ data obtained from the TMD and ZMD systems respectively, will assist in eliminating false-positive results when indole putrifacts are observed in human viscera.

CHAPTER 8

Ricinus communis

8.1 BOTANICAL DESCRIPTION

*Ricinus com*munis L. (Euphorbiaceae) is a large shrub or small tree of up to 4 m in height with red-purplish stems, which could be annual or perennial depending on the climatic conditions. The leaves are large, hand-shaped on long, stout leaf stalks. The flowers are gathered in clusters – the female flowers above the male flowers on the tips of the inflorescences. The fruits are three-lobed, three-seeded capsules armed with soft spikes. The seeds, 10 – 12 mm long, have a smooth and shiny seed coat and are irregularly mottled with reddish-brown, brown, silver and black [140, 141, 142]. Although listed as a problem plant, it has been used as an ornamental plant in gardens [143, 144]. It is commonly known as the castor oil plant or *umhlakuva* (Xhosa and Zulu).

FIGURE 8.1

Purple form of *Ricinus communis*

FIGURE 8.2

Seeds of *Ricinus communis*

8.2 ORIGIN AND DISTRIBUTION

The true origin of *R. communis* is unclear but it is believed to be indigenous to north-eastern Africa and India. The plant has become a weed in many parts of the world and usually grows in

tropical, subtropical and temperate climates in disturbed soil or uncultivated lands. It is widely distributed throughout South Africa and commercially cultivated for the seed oil in many countries including South Africa, Brazil, Ecuador, Paraguay, India, Tanzania, USA and USSR [144].

8.3 TYPE OF TOXIN

Ricinus communis is similar to *Abrus precatorius* in that it contains a highly toxic lectin, ricin and a piperidine alkaloid ricinine [145]. The term "ricin" refers collectively to the four glycoproteins that have been purified from the seeds of *R. communis*. Two ricin agglutinins and two toxins have been identified: RCL I, RCL II (agglutinins), RCL III or Ricin D and RCL IV (toxins). All four lectins consist of two different polypeptide chains joined by a disulfide bond; the toxins are dimers of an A-chain (30,000 Da) and a B-chain (33,000 Da) and the agglutinins occur as tetramers composed of two 30,000 and two 33,000 molecular weight subunits [41]. Ricinine has a molecular formula of $C_8H_8N_2O_2$ and a molecular weight of 164.2. The oil from the seeds of *R. communis*, ricinoleic acid (castor oil), has a molecular formula of $C_{18}H_{34}O_3$, a molecular mass of 298.5 and is cold-pressed from the seeds.

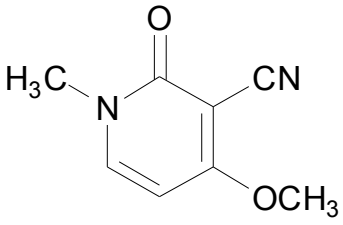


FIGURE 8.3
Chemical structure of ricinine

8.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

All the compounds isolated from *R. communis* have pharmacological effects. The B-chain (sub-unit) of ricin is a lectin and allows the toxin to bind to cell membranes containing galactosyl sites. This first step is required before the A-chain (sub-unit), an enzyme, can inhibit protein synthesis [141]. The four glycoproteins that make up ricin have been shown to have anti-tumor properties [41], but are destroyed by heat and light and may be unstable in the human digestive tract. Ricin agglutin is reportedly nontoxic if taken orally [144]. Ricinine is highly toxic and produces similar symptoms as abrin and ricin [41]. The drastic cathartic properties (purgative) of castor oil is well known and are due to ricinoleic acid which alters the intestinal cell membrane and causes the loss of water and electrolytes [141].

8.5 ETHNOPHARMACOLOGICAL IMPORTANCE

Castor oil is a well-known purgative medicine and very effective, but has a very disagreeable taste. The seeds are used as a purgative by many of the ethnic groups in South Africa [45, 146, 147] but not by the Sotho and Zulu. One to three intact seeds are taken as a strong purgative with a much more drastic action than castor oil. Leaf infusions are administered orally or rectally (enema) for stomach ache, while root and leaf poultices are widely applied to wounds, sores and boils [45, 115, 147]. The roots are used as an abortifacient or pounded into a paste and used against toothache. A hot water extract of the seeds are taken to improve fertility and leaves are inserted directly into the anus to cure haemorrhoids [147]. Spiritual / magical uses of the plant have also been documented [148].

8.6 POISONING

8.6.1 Accidental

The seeds are considered as non-toxic as long as the seed coat remains intact, but maceration of the seeds renders them highly toxic. Chewing of the seeds result in a burning sensation of the mouth and throat. After a latent period of three to six hours (or sometimes several days) nausea, vomiting, severe stomach pains, diarrhoea and excessive thirst develop leading to acute dehydration, hypotension and a circulatory failure. These symptoms are followed by blurred vision, loss of consciousness, convulsions, liver necrosis, haemolysis and renal failure [144].

Fatal poisoning by *R. communis* as a result of the ingestion of seeds as a purgative, has been reported [45, 144]. A dose of one to three seeds (taken whole) are considered as “safe”. Three macerated seeds are considered a fatal dose [45] but one macerated seed may kill a child. Non-fatal poisonings have also been reported after the ingestion of seeds. It is interesting that people have fully recovered after the ingestion of 31 seeds but that others have died after the ingestion of three to eight seeds. Damaging of the seed coat in any way will drastically increase the chance of a fatal poisoning, but seasonal variations in toxicity or the dimensions of the seeds ingested may also affect the outcome of ingestion of *R. communis* seeds. Individual susceptibility or resistance to the toxic agents may also play a role as will the age of the patient [141].

8.6.2 Deliberate

In the past, deliberate poisoning with *R. communis* was not common and no reference to any deliberate poisoning in South Africa could be found. In 1978, Georgi Markov, an exiled Bulgarian broadcaster was jabbed with an umbrella in the leg. He developed the typical symptoms associated with ricin poisoning and died three days later. At autopsy a small 1.5 mm metal sphere was recovered from the wound site. The sphere had two tiny holes and could hold a volume of 0.28 mm^3. A pig injected with a similar dose of ricin died after 26 hours. In the USA

(1991 – 1997) three ricin-related cases were reported where the intent was to kill using ricin. In December 2002 a "ricin laboratory" was discovered in Manchester, England which led to the arrest of six suspected terrorists. On the 3rd of February 2004 three US Senate buildings were closed after ricin was discovered in the mailroom that serves these buildings [149].

8.7 FCL JHB INVESTIGATION

Reference to *R. communis* was found in the FCL JHB database and it has quite frequently been mentioned in forensic case files submitted to the FCL JHB. The seeds are freely available due to the widespread distribution of the plant but have not been successfully detected or confirmed in forensic samples. As for *A. precatorius*, this might be due to the unavailability of the necessary equipment and software to detect macromolecules like abrin and ricin, or the availability of equipment sensitive enough to detect the minor components like abrine and ricinine. The lack of a suitable analytical standard also hampered the development of an analytical method in the past, and therefore ruled out the detection of a minor compound like ricinine in viscera samples and exhibits.

Ricinine has been analysed by GC-MS in high concentrations [150] but HPLC methods are sadly lacking. With the addition of the API LC-MS systems to the analytical arsenal of the FCL JHB, the capability was introduced to detect small, stable molecules at the ng/ml level, but no published method could be found for ricinine. This necessitated the development of an extraction method and a HPLC-MS detection method for ricinine.

8.8 EXPERIMENTAL PROCEDURE

8.8.1 Standards and reagents

Ricinine is not commercially available. Ricinine, ring-$^{13}C_5$, cyano-^{13}C (99% by GC) (ricinine $^{13}C_6$) was purchased from Cerilliant Corporation, Texas, USA. Potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (> 99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%) and acetic acid (99%) were obtained from BDH Laboratory Supplies, England. Sulfuric acid (Suprapur 96%), hydrochloric acid (Suprapur 96%), phosphoric acid (Suprapur 96%), formic acid (98 - 100%) and ammonia (GR 25%) were obtained from Merck, Darmstadt, Germany. Acetonitrile (gradient quality, 200 nm UV cut-off) and methanol (gradient quality, 205 nm UV cut-off) were obtained from Romil, England. HPLC grade water (20 $M\Omega cm^{-1}$) was obtained from a Milli-Q / reversed osmosis system (Millipore, USA). The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [56]. The anhydrous salts

of KCl (0.20 g), NaCl (8.00 g), KH_2PO_4 (0.20 g) and Na_2HPO_4 (1.15 g) were dissolved in 1 L deionised water and the pH adjusted to 5.0 with 10% hydrochloric acid.

Oasis HLB solid phase extraction (SPE) cartridges (3 ml with 60 mg of sorbent) were obtained from Waters Corporation, Milford, USA. A 12-port vacuum manifold was used for all extractions.

8.8.2 Instruments and conditions

A Waters 2690 HPLC system equipped with both a 996 photodiode array (PDA) detector and a Termabeam mass selective detector (TMD) [electron impact (EI) mode; maximum mass 1000 *m/z*) from Waters was used for initial screening and high concentration studies. A Waters 2690 HPLC system equipped with a 996 PDA detector and a Z spray mass selective detector (ZMD) (Micromass, UK) (electrospray mode; maximum mass 2000 *m/z*) was used for low concentration studies. A Phenomenex Aqua C18 HPLC column (250 x 2.1mm, 5 µm) was used on the TMD system while a Waters Xterra MS C18 HPLC column (250 mm x 2 mm, 5 µm) was used on the ZMD system. A Harvard syringe pump (Model 11) was obtained from Harvard Apparatus, Holliston, USA and was used for all direct injection experiments on the ZMD detector. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 MHz spectrometer.

The Phenomenex Aqua column was used with an acidic mobile phase of 0.1% formic acid in the water, starting at 95% water: 5% acetonitrile and slowly ramping the organic component to 10% methanol: 90% acetonitrile in 40 minutes. These conditions were kept for 10 minutes where after the HPLC column was re-equilibrated with the initial conditions. The complete gradient profile of the 2695 SM is summarised in Table 8.1.

To ensure maximum retention of the alkaloid on the Xterra MS C18 column, the initial chromatographic conditions were 90% water containing 10 mM ammonium acetate, the pH adjusted to 9.5 with ammonia (25%) and acetonitrile. After injection, the chromatographic conditions were gradually changed through a linear gradient profile to 90% acetonitrile and 10% of the original aqueous mobile phase in 20 minutes. These conditions were kept stable for 5 minutes where after the HPLC system re-equilibrated the analytical column to the initial conditions. The flow rate was kept constant at 0.2 ml/min.

The nebuliser of the TMD detector was optimised with a caffeine test solution (100 µg/ml) to ensure efficient nebulisation and droplet formation. The electrospray ionization (ESI) probe of the ZMD detector was optimised by direct injection via a syringe pump using a very dilute solution of the $^{13}C_6$ ricinine standard (100 ng/ml).

Table 8.1 HPLC gradient profile of the general screening method (TMD system)

	TIME min	FLOW ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		0.20	0	0	95	5	6
2	1.00	0.20	0	0	95	5	4
3	10.00	0.20	0	0	80	20	6
4	12.00	0.20	0	0	80	20	4
5	20.00	0.20	0	30	50	20	5
6	35.00	0.20	0	10	0	90	6
7	37.00	0.20	0	10	0	90	6
8	40.00	0.25	0	10	0	90	6
9	45.00	0.25	0	10	0	90	6
10	50.00	0.20	0	0	95	5	5

8.8.3 Chemical extraction of *R. communis* seeds

Seeds were purchased from the Traditional healer's market in Johannesburg and dried at 40 ^{o}C to constant weight. The seeds were finely ground using an Ika Werk A10 water-cooled mill (Janke & Kunkel, Straufen, Germany) and 5 g of powdered seed was placed in a 50 ml polypropylene screw-top centrifuge sample tubes. Thirty milliliters (30 ml) of PBS-HCl buffer was added, the tube capped and shaken on a Heidolph rotating sample mixer for thirty minutes. After centrifugation at 4000 rpm using a Hermle Z 300 centrifuge for 15 minutes the aqueous phase was passed through an Oasis HLB cartridge and the absorbed compounds stripped from the solid phase material with 5 ml methanol. The SPE cartridge was prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used. The cartridge was washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. The cartridge was then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates combined into one collection tube. The collection tube was placed into a Zymark Turbovap and evaporated at 40 $^{\circ}$C under a regulated stream of nitrogen gas. The dry residue was reconstituted with 1 ml of a 50:50 methanol: water mixture.

8.8.4 Isolation of ricinine from *R. communis* seeds

A portion of the ground seeds (see §7.8.3) (15 g) was extracted as described above but the aqueous phase was passed through a preparative Oasis HLB cartridge (20 ml/ 1g). The SPE cartridge was prepared by consecutively washing with 20 ml of methanol, water and the extracting buffer used. The cartridge was washed with 20 ml extracting buffer and dried under

vacuum for 10 minutes. The cartridge was then eluted with 20 ml methanol, 20 ml acidic methanol (10 ml formic acid per liter methanol), 20 ml basic methanol (10 ml ammonia per liter methanol) and 20 ml acetonitrile and the eluates combined into one collection flask and evaporated to dryness at 50 ^{o}C under reduced pressure. The dry residue was reconstituted using the minimum amount of a 50:50 methanol: water mixture.

The chromatographic conditions used on the Waters Xterra MS C18 analytical column was fed into the Prep Calculator software of the Waters Corporation to recalculate the conditions that must be used if a Waters Xterra MS C18 column (7.8 mm x 300 mm, 10 µm) was used. The modified conditions were used to fractionate ricinine from 100 µl injections of the re-dissolved residue by means of a LKB 2211 Superrac fraction collector and monitoring the UV spectrum of the PDA detector.

The structure of ricinine was confirmed by NMR spectroscopy:

Ricinine:

^{1}H NMR: δ_H (MeOH-d_4, 500 MHz) 7.95 (1H, d, *J* 7.8 Hz, H-6), 6.44 (1H, d, *J* 7.8 Hz, H-5), 4.04 (3H, s, OCH_3), 3.53 (3H, s, NCH_3).

^{13}C NMR**:** δ_C (MeOH-d_4, 125 MHz) 174.7 (C-2), 163.8 (C-4), 146.8 (C-6), 114.7 (CN), 95.7 (C-5), 88.2 (C-3), 58.2 (OCH_3), 37.8 (NCH_3).

8.9 RESULTS AND DISCUSSION

8.9.1 Chemical extraction of *R. communis* seeds

Ricinine is less polar than the alkaloids from *A. precatorius* and can be retained on most silica-based C18 SPE cartridges. The choice of a trifunctional SPE C18 cartridge is advisable to ensure that any residual charge on the molecule will not disrupt the binding process onto the active sites of the SPE cartridge. The fact that the silica-based cartridges are prone to dewetting and poor recoveries if allowed to run dry makes the choice of a polymeric trifunctional C18 cartridge essential. No problems were encountered during the SPE extraction of the ground seeds but the evaporation of the combined column eluates on the Turbovap were severely hampered if the SPE cartridges were not completely dried – either by applying vacuum to the cartridges or by passing instrument quality nitrogen gas through the cartridges. It was found that both drying techniques required a minimum of 10 minutes to effectively dry the cartridges. The slow evaporation of the samples in the Turbovap due to the presence of water could be alleviated by the frequent addition of methanol to azeotrope the remaining water.

8.9.2 Chromatography and detection of ricinine

Ricinine ionised as easily as abrine and hypaphorine under acidic conditions and displayed similar chromatographic behaviour as these two compounds. Analysis of the $^{13}C_6$ ricinine standard on the TMD system produced a single peak on the PDA detector (Figure 8.4) with a very characteristic UV spectrum (Figure 8.5). The TMD detector produced similar results and a single peak was observed (Figure 8.6) with an EI spectrum that is consistent with the molecular formula of $^{13}C_6{}^{12}C_2H_8N_2O_2$ and a molecular weight of 170.2 (Figure 8.7). The conditions for the TMD detector were similar to those used for the analysis of abrine and hypaphorine (Table 7.2).

The acidic extract of the seeds were analysed on the TMD system and six UV-active compounds were detected on the PDA detector (Figure 8.8). The UV spectrum of the main component (R_t 13.64 min) was consistent with that of the $^{13}C_6$ ricinine standard. Only one component (R_t 13.76 min) was detected on the TMD detector (Figure 8.9) and the EI spectrum (Figure 8.10) displayed a molecular ion of 164 amu (which was also the base peak) that is consistent with the molecular formula and molecular weight of ricinine ($C_8H_8N_2O_2$ and 164.2 amu).

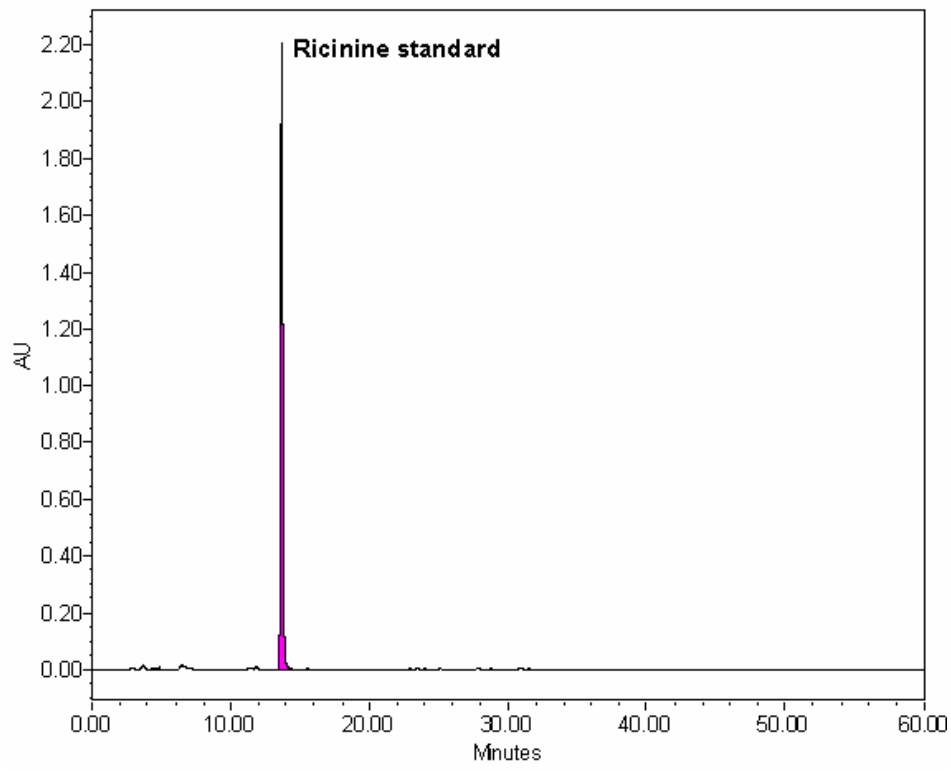


Figure 8.4

PDA Maxplot chromatogram of the $^{13}C_6$ ricinine standard (TMD system)

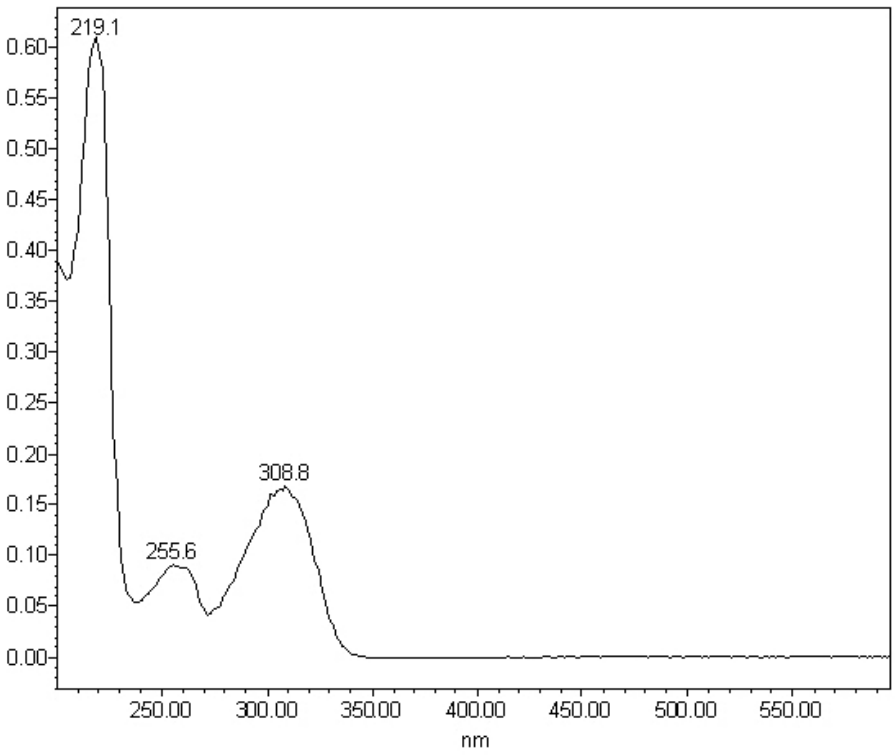


Figure 8.5

PDA spectrum of the $^{13}C_6$ ricinine standard (TMD system)

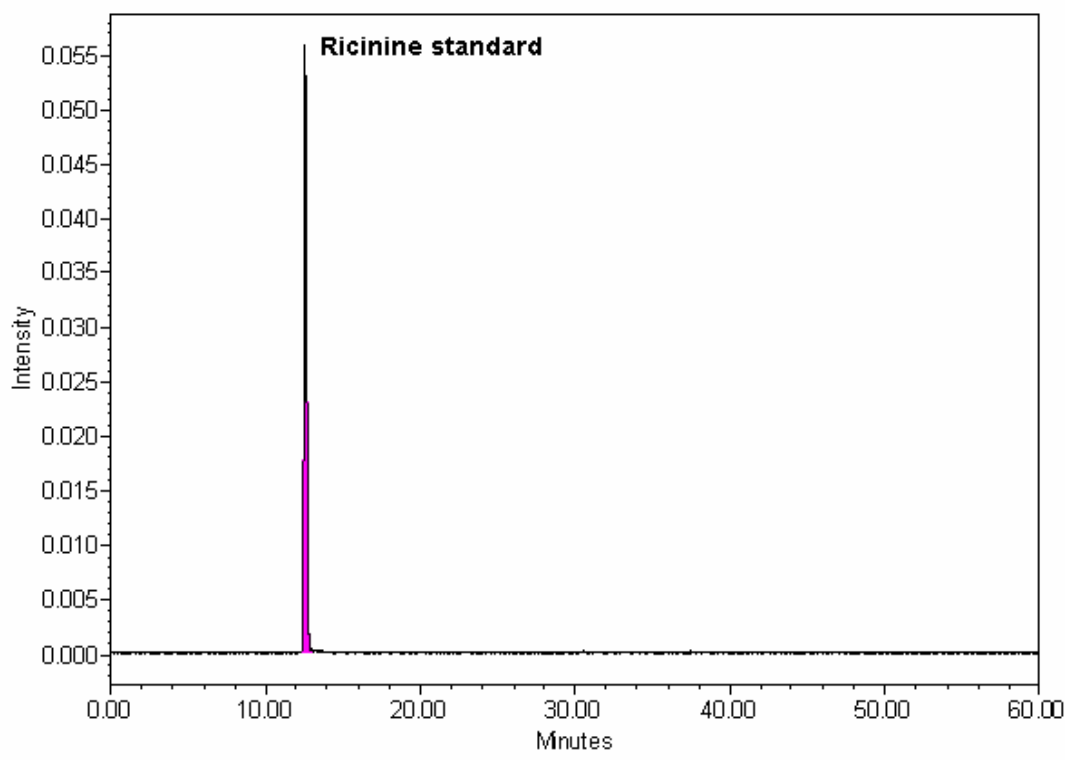


Figure 8.6

EI chromatogram of the $^{13}C_6$ ricinine standard (TMD system)

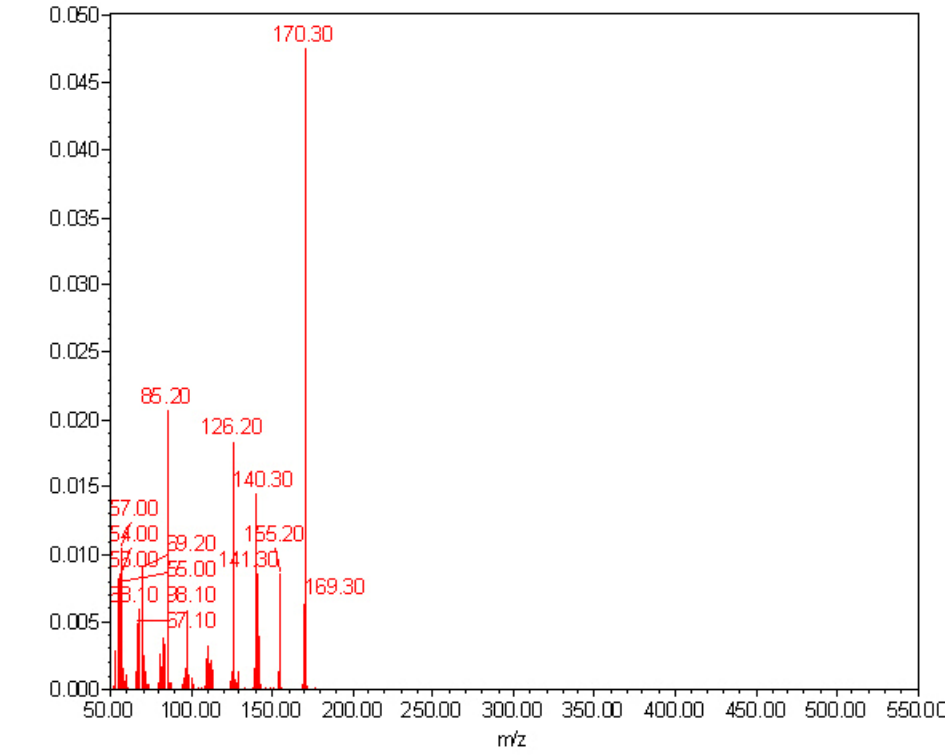


Figure 8.7

EI spectrum of the $^{13}C_6$ ricinine standard (TMD system)

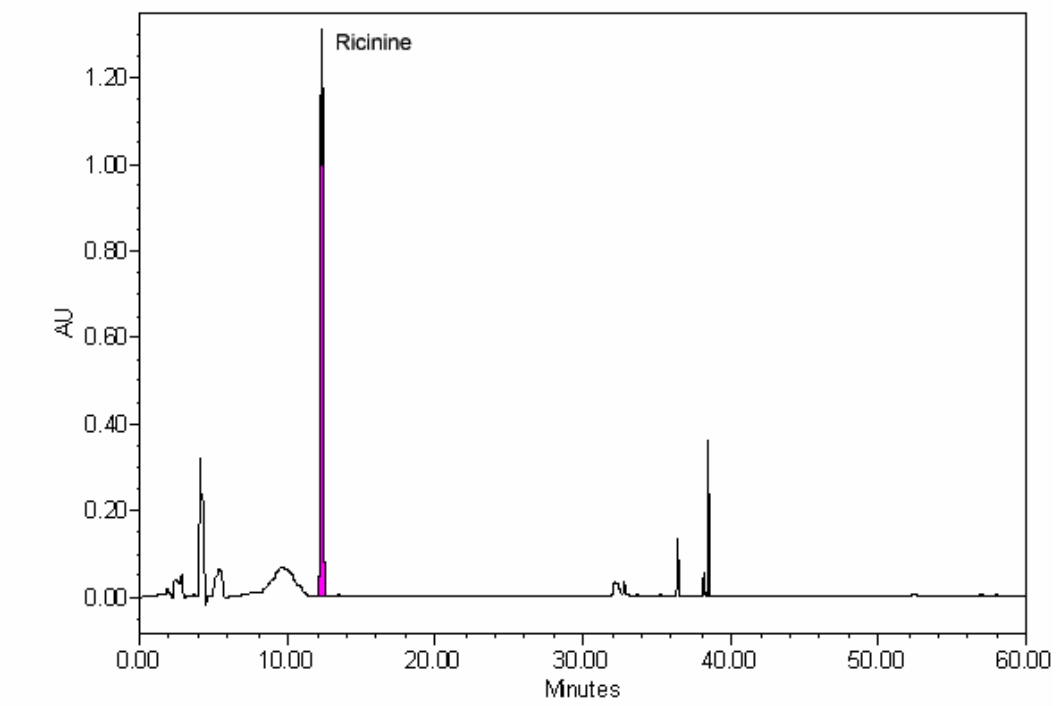


Figure 8.8

PDA Maxplot chromatogram of the acidic extract of the seeds of *Ricinus communis* containing ricinine

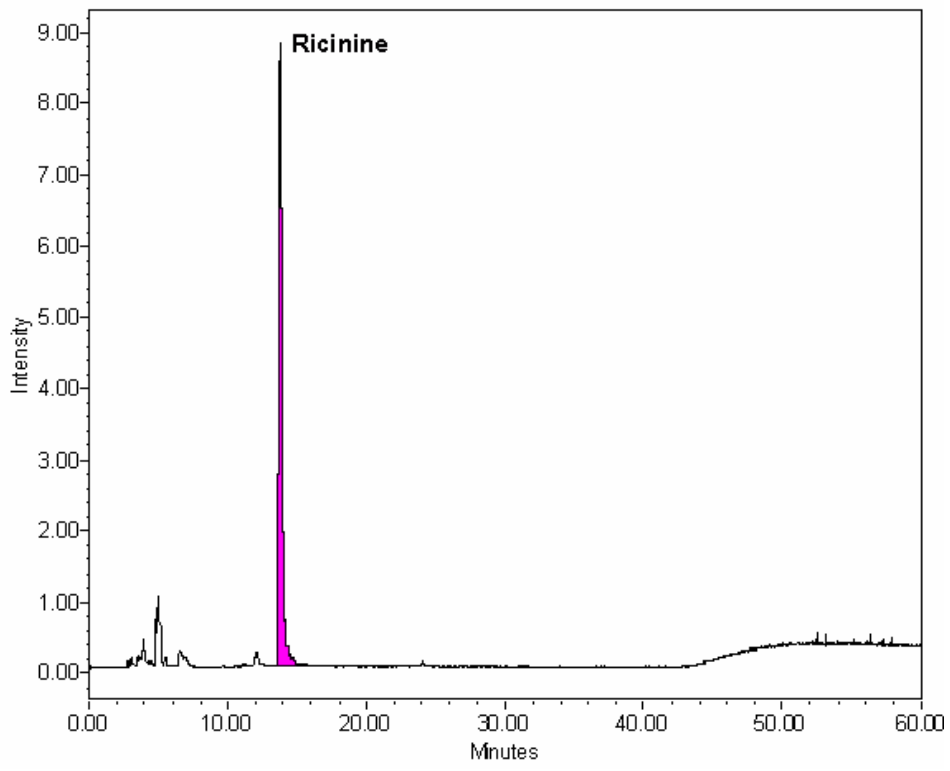


Figure 8.9

TMD EI chromatogram of the acidic extract of the seeds of *Ricinus communis* containing ricinine

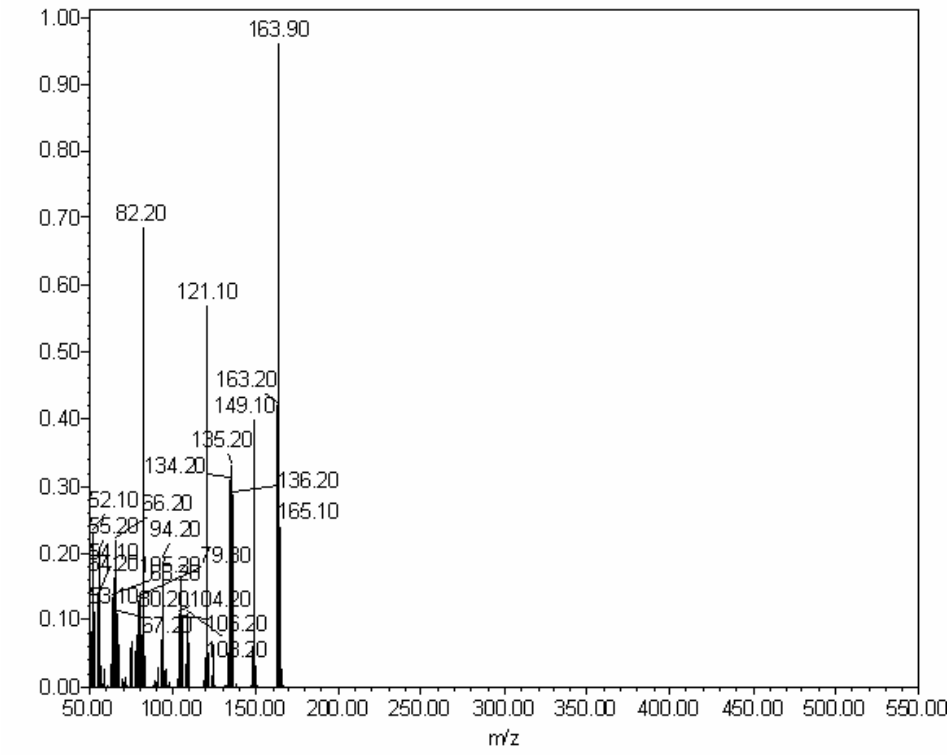


Figure 8.10

EI spectrum of the extracted ricinine (TMD system)

8.9.3 Purification of ricinine

The purification of ricinine on preparative Oasis HLB cartridges (20 ml/ 1 g) displayed similar retention and chromatographic characteristics as the small analytical scale (3 ml/ 60 mg) cartridges. The retention behaviour of ricinine was less dependent on the amount of organic modifier present than abrine and hypaphorine, and the loss of product due to bleeding was negligible. The same chromatographic conditions as used for the analytical column (Table 8.3) were used for the preparative column and only the flow rate was increased to 2.76 ml/min. This was done to ensure similar chromatographic behaviour of the compounds of interest. The 10 µl syringe of the injector was replaced with a 250 µl syringe to allow injection volumes of 100 µl per preparative run. Although the preparative column could handle larger injection volumes, the injection system was limited to 100 µl injections due to the internal sample loop volume of 100 µl on the 2690 Separation module.

The UV spectrum (200 – 600 nm) was monitored at 309 nm and the main component, ricinine, was fractionated on the fraction collector by using a retention time trigger from the 2690 Separation module and fixed collection window on the fraction collector. The UV spectrum of the fractionated compound was continually monitored to ensure that the characteristic UV spectrum of ricinine was observed. Due to the concentrated nature of the crude extract, the sample started precipitating. To alleviate this problem, the temperature of the sample compartment and column compartment were raised to 40 ^{o}C. This partially eliminated the precipitation problem and dilution of the sample with methanol became necessary to ensure that all the sample components remained in solution. The fractionated component from the seeds of *R. communis* was analysed on the TMD system and only one compound was observed on the UV and TMD detectors. The UV spectrum was identical to that of the $^{13}C_6$ ricinine standard while the EI spectrum was consistent with literature (151). The API interface of the ZMD system was optimised to enable low-level detection of ricinine in ESI^+ mode. The optimised conditions are summarised in Table 8.2.

Ricinine displayed greater stability to ionisation than the alkaloids of *A. precatorius* and required higher cone voltages to ionise and fragment. A typical chromatogram of the fractionated ricinine (Figure 8.11) displayed a single peak (R_t 7.64 min) and the ESI^+ spectra (Figure 8.12) confirmed the nominal mass of the compound as 164 amu. The ESI^+ spectrum of ricinine at CV = 25 V was dominated by the protonated species $[M+H]^+$ = 165 amu and displayed no ISCID fragmentation.

The compound started fragmenting at a CV = 35 V and at a CV = 45 V displayed significant ISCID fragmentation. The main fragmentation product (Figure 8.13) was formed by the loss of CN ($[M+H-CN]^+$ *m/z* 138).

TABLE 8.2 Optimised MS conditions for detecting ricinine on the ZMD detector

ZMD CONDITIONS	RICININE
Mode and polarity	ESI positive
Capillary voltage (KV)	2.50
Cone voltage (V)	25
Desolvation temperature (°C)	450
Extractor voltage (V)	5.0
RF lens (V)	0.5
Source temperature (°C)	120
Cone gas flow (L/h)	40
Desolvation gas flow (L/h)	400
Ion Energy	1.0
Multiplier (V)	-650
Resolution (LMR & HMR)	15.5

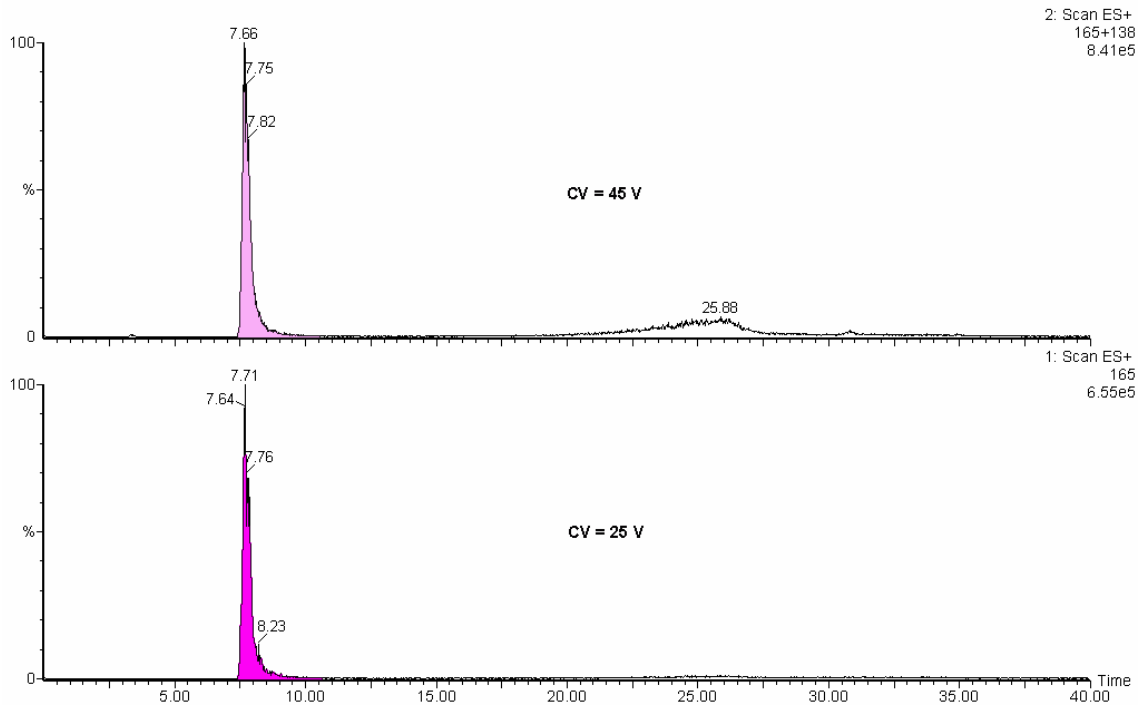


Figure 8.11

ESI$^+$ chromatograms of the extracted ricinine at two cone voltages (25 V and 45 V) on the ZMD system

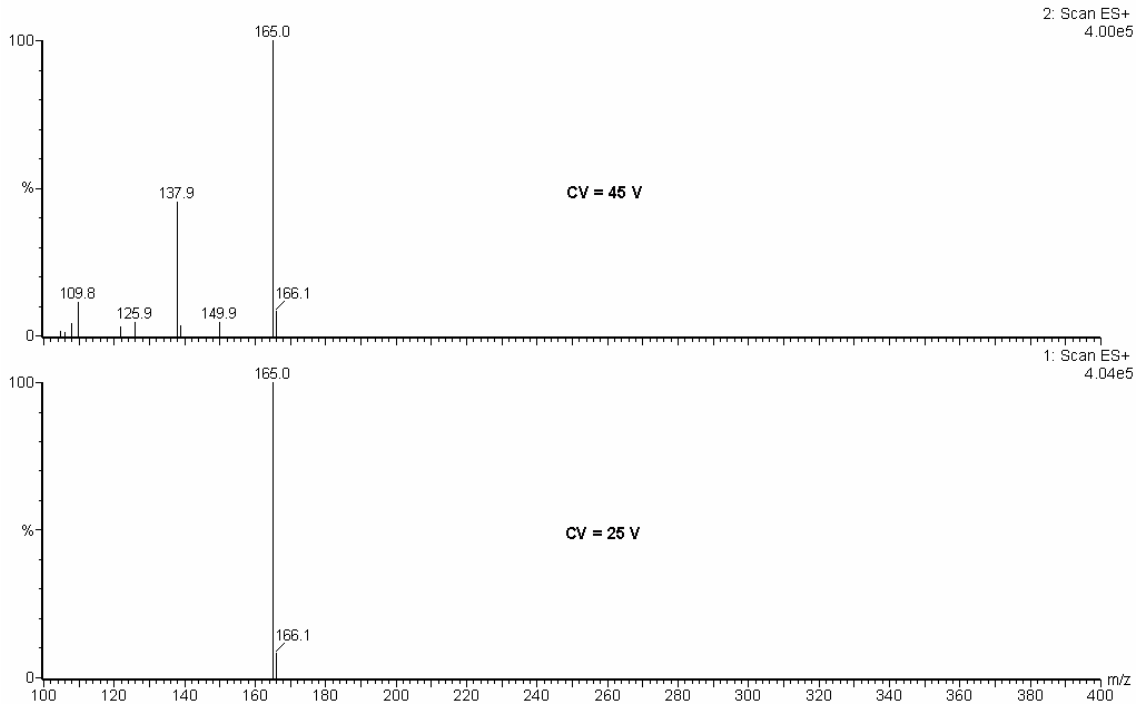


Figure 8.12

ESI$^+$ MS spectra of the extracted ricinine at cone voltages of 25 V and 45 V on the ZMD system

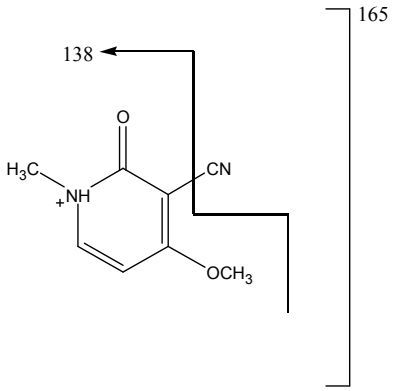


Figure 8.13

ESI$^+$ ISCID of the extracted ricinine (ZMD system)

8.10 FORENSIC APPLICABILITY

This method is a valuable addition to forensic chemistry and will assist in the detection of ricinine at low concentration levels. The detection of ricinoleic acid in any forensic exhibits might be indicative of the presence of ricinine, and will necessitate the re-extraction of the forensic exhibits with this extraction method. The detection and confirmation of low molecular weight compounds is usually problematic with MS as detection technique, but the strong ionisation and reproducible ISCID fragmentation of ricinine under ESI conditions will assist in the detection and confirmation of ricinine at very low concentration levels.

CHAPTER 9

Nerium oleander / Thevetia peruviana

9.1 BOTANICAL DESCRIPTION

Nerium oleander L. (Apocynaceae) is a robust, woody, evergreen shrub of up to 6 m high (Figure 9.1). The leaves are oblong, with a prominent, white midrib and numerous parallel lateral veins [152]. The flowers can be single or double in a variety of colours – white, pink, purple (dark pink) or red [153] (Figure 9.2). When the long and narrow seed capsules burst open, they release numerous small, brown seeds with long, reddish-brown hairs [152]. *N. oleander* is also called the common oleander.

FIGURE 9.1
Typical *Nerium oleander* shrub

FIGURE 9.2
Some colour forms of *Nerium oleander*

Thevetia peruviana (Pers.) Schumann (Apocynaceae) is a leafy shrub of up to 10 m high, with glossy, spirally arranged leaves (Figure 9.3) and attractive, trumpet-shaped, yellow flowers (Figure 9.4). The triangular fruits are slightly fleshy and green at first, but turn black when they

ripen, and contain 2 – 4 flat seeds (Figures 9.4 and 9.5). The plant is commonly known as the yellow oleander.

FIGURE 9.3
Typical *Thevetia peruviana* shrub

9.2 ORIGIN AND DISTRIBUTION

The origin of *N. oleander* is uncertain but is thought to be native to the Near East [153]. It occurs naturally in a large area that stretches from the Mediterranean region to Western China and is particularly well suited to the Mediterranean climates. It adapts well to drought and is a hardy and popular garden shrub. In South Africa it has become an invader plant and is widely distributed through the more temperate climate regions [154].

FIGURE 9.4
Flower and fruit of *Thevetia peruviana*

FIGURE 9.5
Opened fruit and seed of *Thevetia peruviana*

Thevetia peruviana occurs naturally in Mexico and the northern parts of South America and is widespread in Australia. It is a popular garden shrub favouring the warmer climates of the north-eastern parts of South Africa [155].

9.3 TYPE OF TOXIN

Nerium oleander and *T. peruviana* both contain cardiac glycosides of the cardenolide type (Figure 9.6). The main cardiac glycoside of *N. oleander* is oleandrin with a molecular formula of $C_{32}H_{48}O_9$ and a molecular mass of 576.7. The main cardiac glycosides of *T. peruviana* are thevetin A and thevetin B with molecular formulae of $C_{42}H_{64}O_{19}$ for thevetin A and $C_{42}H_{66}O_{18}$ for thevetin B. The molecular mass of thevetin A is 872.0 and 858.0 for thevetin B.

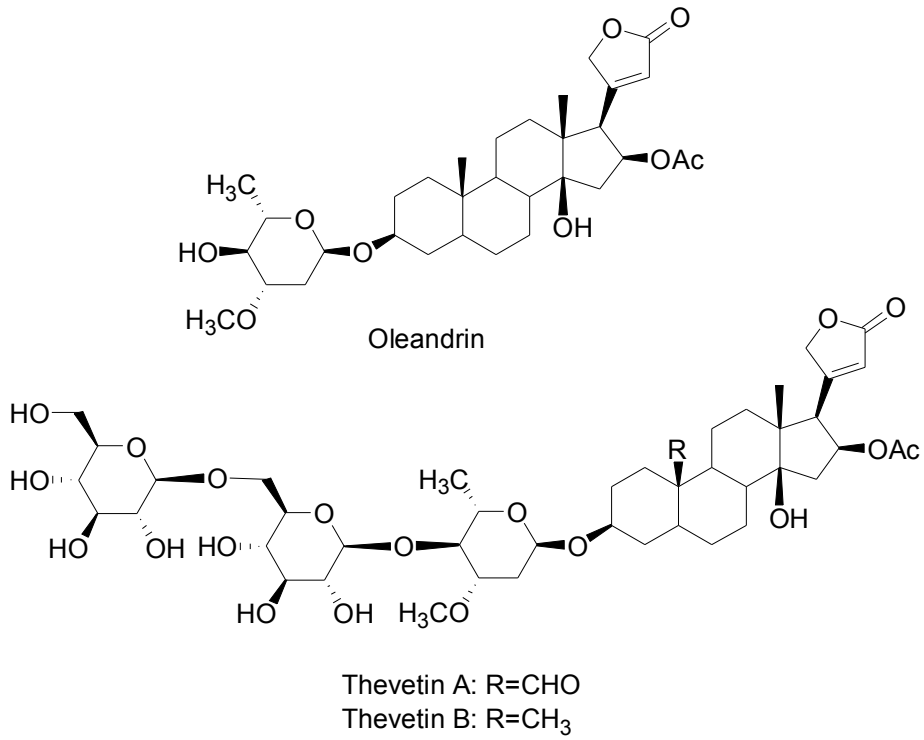


FIGURE 9.6

Chemical structures of oleandrin, thevetin A and thevetin B

9.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

Oleander leaves and seeds contain more than 30 different cardiac glycosides with oleandrin as the main cardiac glycoside. Oleandrin was formerly used as a cardiac tonic and diuretic, and extracts are still used in homeopathy [152]. Numerous cardiac glycosides are known from *T. peruviana*, of which thevetin A and B are the main components [152]. A mixture of thevetin A and B was previously available as a commercial cardiac stimulant under the name thevetin [153].

The cardiac glycosides of *N. oleander* and *T. peruviana* produce typical clinical signs of cardiac glycoside poisoning. The first signs are gastrointestinal discomfort, nausea and vomiting. These are followed by neurological symptoms that usually include weakness, mental confusion and visual disturbances. Next to appear are cardiac symptoms, usually bradycardia due to conduction problems and the onset of an A-V block resulting in fibrillation [153]. The physiological action of cardenolides are attributed to their binding to the Na^+/K^+-sensitive membrane-bound enzymes, thereby disturbing the Na^+/K^+ transport and leading to increased intracellular Ca^{2+} levels [156]. Thevetin has a direct stimulant action on the smooth muscles of the intestine, bladder, uterus and blood vessel walls [45].

9.5 ETHNOPHARMACOLOGICAL IMPORTANCE

Even though *N. oleander* and *T. peruviana* are known to be toxic, both are used medicinally.

***Nerium oleander*:**

Finely powdered leaves are sniffed for colds and headache. Syphilis is treated by eating small quantities of bark. Leaf extract is rubbed in to clear up rashes and venereal diseases are treated by drinking leaf infusions [157]. A patented hot-water extract of *N. oleander* have been used in the development of a treatment for cancer, viral infections and skin disorders [158].

Thevetia peruviana:

The leaf sap is dripped into the nostrils for colds and severe headache and migraine. Bark decoction is drunk to reduce fever while amenorrhea is treated by drinking a maceration of leaves and bark [159]. The seeds are used as an abortifacient in Bengal and neighbouring provinces [45]. Another interesting use of *T. peruviana* in India is the utilisation of aqueous extracts to assist local fishermen in their fish catching practices [160].

9.6 POISONING

9.6.1. Accidental

Accidental poisoning can be direct or indirect. Direct accidental poisoning would be if the leaves, seeds or flowers are accidentally consumed. This is mostly the case with children who experiment out of curiosity. Misidentification of leaves may lead to the preparation of an infusion thought to be from eucalyptus but turning out to be oleander. Indirect accidental poisoning would occur by the consumption of stagnant water contaminated with leaves or flowers, or ingestion of meat skewered on *N. oleander* stems [153]. All parts of *N. oleander* are poisonous to man, animals and certain insects. Inhalation of smoke or the ingestion of sap or honey produced from this plant has resulted in poisoning of humans and animals [156]. Accidental exposure to *N.*

oleander in children is quite common in Australia, but the mortality associated with these events is negligible [161]. In the USA oleander poisoning occurs regularly with quite an equal distribution between the ages 1 – 90 years. The highest number of exposures occurred in the one year-old age group. These results indicate that oleander exposure in the USA is more accidental than intentional [162].

Young children are enticed by the attractive fruit of *T. peruviana*, resulting in numerous poisonings. Their natural curiosity and tendency to "mouth" objects in their surroundings, place young children at increased risk of exposure to *N. oleander* and *T. peruviana*. Another example of accidental exposure is the preparation of an herbal tea containing *T. peruviana* leaves [163]. The kernels of about 10 fruits may be fatal in adults but the kernel of one fruit may prove fatal to a child [165]. In Namibia two children were poisoned after eating the kernel of a seed, resulting in the death of one child six hours later [45].

9.6.2. Deliberate

An analysis of the well-documented cases shows that the leading cause of severe intoxication with *N. oleander* in humans is attempted suicide which is often successful [153]. A case was reported of a 55-year-old male who prepared a cocktail comprising of 25 *N. oleander* leaves and five flowers which he blended with a soft drink and consumed. He survived this suicide attempt due to specialised medical treatment and the fact that he vomited severely after ingestion of the blend and the remaining pulp [164].

Numerous suicide attempts have been recorded by the ingestion of *T. peruviana* seeds, especially the kernel [165]. One or two seeds will only cause gastrointestinal distress; three or more seeds will lead to severe cardiac distress and may lead to death [153]. An evaluation of the reported *T. peruviana* exposures in Sri Lanka indicated that very few cases (2%) involved young children and that the majority of cases (65%) were suicide attempts involving people in the 16 - 25 year age group [161, 166]. The deliberate ingestion of *T. peruviana* seeds in Sri Lanka has become a popular method of self harm with a fatality rate of at least 10% and 40% of the cases requiring specialised medical treatment [167].

9.7 FCL JHB INVESTIGATION

Oleandrin and oleandrigenin were previously detected at the FCL JHB and were identified using commercial spectral libraries like NIST on the GC-MS (EI) and HPLC-MS (EI) systems. This was only accomplished in case exhibits and *muti,* but no cardiac glycosides could be detected or confirmed in viscera samples. Compounds have been detected in case exhibits and *muti* that gave reasonable (but not absolute) matches to known cadiac glycosides in NIST, but the

unsatisfactory match qualities are indicative that these compounds might be other cardiac glycosides from indigenous plants and therefore not included in commercial mass spectral libraries. Reference to *N. oleander* was found in the FCL JHB database and in Forensic case files submitted to the FCL JHB. Plant material is freely available due to the widespread distribution of the plant. However it could only be detected by the FCL JHB if present in the high µg/ml concentration range and necessitated the development of extraction and LC-MS detection methods for this plant.

The first aim was to develop a chromatographic and mass spectral detection protocol for the analysis of heart glycosides and oleandrin, in particular. The second aim was to develop a method to extract and detect the presence of *N. oleander* in forensic samples by matching the mass spectral profiles of unknown samples to that of extracts made of *N. oleander* leaves. The third aim was to be able to develop a protocol to allow the unambiguous differentiation between *N. oleander* and *T. peruviana* in forensic samples by pattern recognition (comparing the mass spectral BPI or TIC patterns of unknown samples with the mass spectral BPI or TIC pattern of *T. peruviana* leaves). The fourth aim was to adapt the mass spectral detection technique to detect oleandrin at a low concentration in viscera samples.

9.8 EXPERIMENTAL PROCEDURE

9.8.1. Standards and reagents

No standards are commercially available for thevetin A or B. Oleandrin was previously available from Sigma but was discontinued. Oleandrin (1 mg; >90% purity) was obtained from Dr. G. Verdoorn (previously from RAU). Digitoxin, neriifolin, strophanthin G, strophanthin K, bufalin, helveticoside, gitoxin, convallatoxin, digoxin and lanatoside C (>95% purity by TLC) were purchased from Sigma-Aldrich. Potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (>99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%) and acetic acid (99%) were obtained from BDH Laboratory Supplies, England. Sulfuric acid (Suprapur 96%), hydrochloric acid (Suprapur 96%), phosphoric acid (Suprapur 96%), formic acid (98 - 100%) and ammonia (GR 25%) were obtained from Merck, Darmstadt, Germany. Acetonitrile (gradient quality, 200 nm UV cut-off) and methanol (gradient quality, 205 nm UV cut-off) were obtained from Romil, England. HPLC grade water (20 $M\Omega cm^{-1}$) was obtained from a Milli-Q / reversed osmosis system (Millipore, USA). The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [56]. The anhydrous salts of KCl (0.20 g), NaCl (8.00 g), KH_2PO_4 (0.20 g) and Na_2HPO_4 (1.15 g) were dissolved in 1 L deionised water and the pH adjusted to 6.0 with 10% hydrochloric acid.

Oasis HLB solid phase extraction (SPE) cartridges (3 ml with 60 mg of sorbent) were obtained from Waters Corporation, Milford, USA. A 12-port vacuum manifold was used for all extractions.

9.8.2. Instruments and conditions

A Waters 2690 HPLC system equipped with both a 996 photodiode array (PDA) detector and a Termabeam mass selective detector (TMD) [electron impact (EI) mode; maximum mass 1000 *m/z*] from Waters was used for initial screening and high concentration studies. A Waters 2695 HPLC system equipped with a 2996 PDA detector and a Z spray mass selective detector (ZQ) (Micromass, UK) [Multi-mode ESCi, maximum mass 4000 *m/z*, ESCi (+/-) mode] was used for low concentration studies. A Phenomenex Aqua C18 HPLC column (250 x 2.1mm, 5 µm) was used on the TMD system while a Waters Xterra MS C18 HPLC column (250 mm x 2 mm, 5 µm), a Waters Xterra RP C18 HPLC column (250 mm x 2 mm, 5 µm) and a Phenomenex Synergi Fusion C18 HPLC column (250 x 2.1mm, 4 µm) was evaluated on the ZQ system. The built-in syringe pump was used for all direct injection experiments on the ZQ detector.

The Phenomenex Aqua column was used with an acidic mobile phase of 0.1% formic acid in the water, starting at 95% water: 5% acetonitrile and slowly ramping the organic component to 10% methanol: 90% acetonitrile in 40 minutes. These conditions were kept for 10 minutes where after the HPLC column was re-equilibrated with the initial conditions. The complete gradient profile of the 2695 SM is summarised in Table 9.1.

The nebuliser of the TMD detector was optimised with a caffeine test solution (10 µg/ml) to ensure efficient nebulisation and droplet formation. Flow injection experiments were done on the multi-mode ionization probe (ESCi) of the ZQ detector to determine the optimal solvent composition and mass spectral conditions to ionise cardiac glycosides. Initial experiments were done with a 100 µg/ml digitoxin solution in acetonitrile but was replaced in later experiments with a 10 µg/ml solution in methanol. After initial optimisation on digitoxin as test compound, a 10 µg/ml oleandrin test solution was used to do the final optimisation. The optimised mass spectral conditions for oleandrin are summarised in Table 9.2.

A test solution of the 11 cardiac glycosides was prepared in methanol. The chromatographic conditions were varied to obtain separation between the components. The separation was initially monitored with the UV detector but later changed to the ZQ detector. To ensure maximum retention of the more polar cardiac glycosides on the Synergi Fusion C18 column, the initial chromatographic conditions were highly aqueous in nature, but a solvent composition higher than 75% water seriously affected the ionisation of the early-eluting cardiac glycosides. The optimised chromatographic conditions are listed in Table 9.3. The flow rate was kept constant at 0.2 ml/min and the column temperature was kept constant at 40 ^{O}C.

Table 9.1 HPLC gradient profile of the general screening method (TMD system)

	TIME min	FLOW ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		0.20	0	0	95	5	6
2	1.00	0.20	0	0	95	5	4
3	10.00	0.20	0	0	80	20	6
4	12.00	0.20	0	0	80	20	4
5	20.00	0.20	0	30	50	20	5
6	35.00	0.20	0	10	0	90	6
7	37.00	0.20	0	10	0	90	6
8	40.00	0.25	0	10	0	90	6
9	45.00	0.25	0	10	0	90	6
10	50.00	0.20	0	0	95	5	5

TABLE 9.2 Optimised ESI^+-MS conditions for the detection of oleandrin (ZQ system)

CONDITION	OLEANDRIN
Mode and polarity	ESI positive
Capillary voltage (KV)	2.50
Cone voltage (V)	50
Desolvation temperature (°C)	500
Extractor voltage (V)	1.0
RF lens (V)	0.1
Source temperature (°C)	120
Cone gas flow (L/h)	0
Desolvation gas flow (L/h)	450
Ion Energy	1.1
Multiplier (V)	-650
Resolution (LMR & HMR)	15.2

TABLE 9.3 Optimised HPLC conditions for the detection of oleandrin (ZQ system)

VARIABLE	OPTIMISED
Column	Phenomenex Synergi Fusion C18 250 x 2.1 mm (4 µm)
Column temperature (°C)	40 ± 1
Mobile phase components	Methanol H_2O (0.1% Formic acid pH 2.3)
Gradient profile	Initial conditions of 70:30 H_2O:MeOH Gradient to 40:60 H_2O:MeOH in 20 minutes (G3) Gradient to 10:90 H_2O:MeOH at 40 minutes (G9) Isocratic conditions of 10:90 H_2O:MeOH for 5 minutes Return to initial conditions within 5 minutes (G3) Equilibrate for 10 minutes
Mobile phase flow rate	0.2 ml/min
Degassing	In-line (normal mode)
Sample temperature (°C)	5 ± 1

9.8.3. Chemical extraction of *N. oleander* and *T. peruviana* leaves

The leaves of *N. oleander* (white, white hybrid, light pink, dark pink and red forms) and *T. peruviana* (yellow) were collected and separately dried to constant weight at 40 °C. The leaves were ground to a powder using an Ika Werk A10 water-cooled mill (Janke & Kunkel, Straufen, Germany). Initial experiments were done on the light pink form of *N. oleander*. Ground leaf portions (1g) were extracted with chloroform, methanol, PBS buffer (pH 7) and HCl-PBS buffer (pH 5) by placing the ground leaf portions in separate 50 ml polypropylene screw-top centrifuge sample tubes. Chloroform, methanol PBS buffer and HCl-PBS buffer (30 milliliters of each) were added to the tubes where after the tubes were capped and shaken on a Heidolph rotating sample mixer for thirty minutes. After centrifugation in a Hermle Z 300 centrifuge at 4500 rpm for 15 minutes, the organic phases were placed into a Zymark Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml methanol. The aqueous phases were passed through conditioned 3 ml (60 mg) Oasis HLB cartridges and the cartridges washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. The cartridges were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates of each tube combined into a single collection tube for each experiment. The collection tubes were placed into a Zymark Turbovap and evaporated at 40 °C

under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of methanol.

The effect of pH was evaluated by placing seven 1g ground leaf samples (*N. oleander* light pink form) into separate 50 ml polypropylene screw-top centrifuge sample tubes. HCl-PBS buffer (pH 3, 4, 5, 6, 7, 8, and 9 with HCl or NH_4OH) was added to the tubes, the tubes capped and shaken on a Heidolph rotating sample mixer for thirty minutes. The rest of the sample preparation was the same as for the aqueous samples described above.

9.8.4. Chemical extraction and analysis of viscera and case exhibits

An aliquot of each liquefied viscera sample [stomach (including contents), liver, kidney (3 g each), case exhibit (1 g) and blood (3 ml)] were placed in separate 50 ml polypropylene screw-top centrifuge sample tubes. Thirty milliliters (30 ml) of PBS-HCl buffer (pH 5) was added to each sample and the tubes capped and shaken on a Heidolph rotating sample mixer for thirty minutes. Oasis HLB SPE cartridges (3 ml – 60 mg) were prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used. After centrifugation of the sample tubes at 4500 rpm (Hermle Z 300 centrifuge) for 15 minutes, the aqueous phase of each sample was passed through a conditioned Oasis HLB cartridge and the cartridges were washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. They were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates of each cartridge combined into one collection tube. The collection tubes were placed into a Zymark Turbovap and evaporated to dryness at 40 ^{o}C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of a 50:50 methanol: water mixture.

9.9 RESULTS AND DISCUSSION

9.9.1. Chromatography and detection of cardiac glycosides

Cardiac glycosides are a specific group of steroidal glycosides comprising of two basic types, the cardenolides and the bufadienolides. The cardenolides have a five-membered lactone ring attached to C-17, for example oleandrin (present in *N. oleander*), while the bufadienolides have a six-membered lactone ring at C-17, for example bovoside A, the active principle of *Bowiea volubilis* (Chapter 10) [154, 168, 171].

Cardenolides have a very non-specific UV spectrum with a single UV-maximum between 210 – 225 nm (Oleandrin λ_{max} 216 nm). The bufadienolides have a stronger UV absorption at λ 280 – 320 nm (Bufalin: λ_{max} 298 nm) (Figure 9.7) which makes them quite distinctive and detectable compared to the cardenolides. In the past the FCL JHB relied solely on UV detection of cardiac

glycosides, but this detection method was not reliable or conclusive. A HPLC method was developed for the detection of cardiac glycosides on the TMD system, but only the UV detector provided useful data as most cardiac glycosides displayed poor stability during EI ionisation. Another problem was that few of the cardiac glycosides were present in the NIST spectral database which ruled out positive identification or confirmation by library matching.

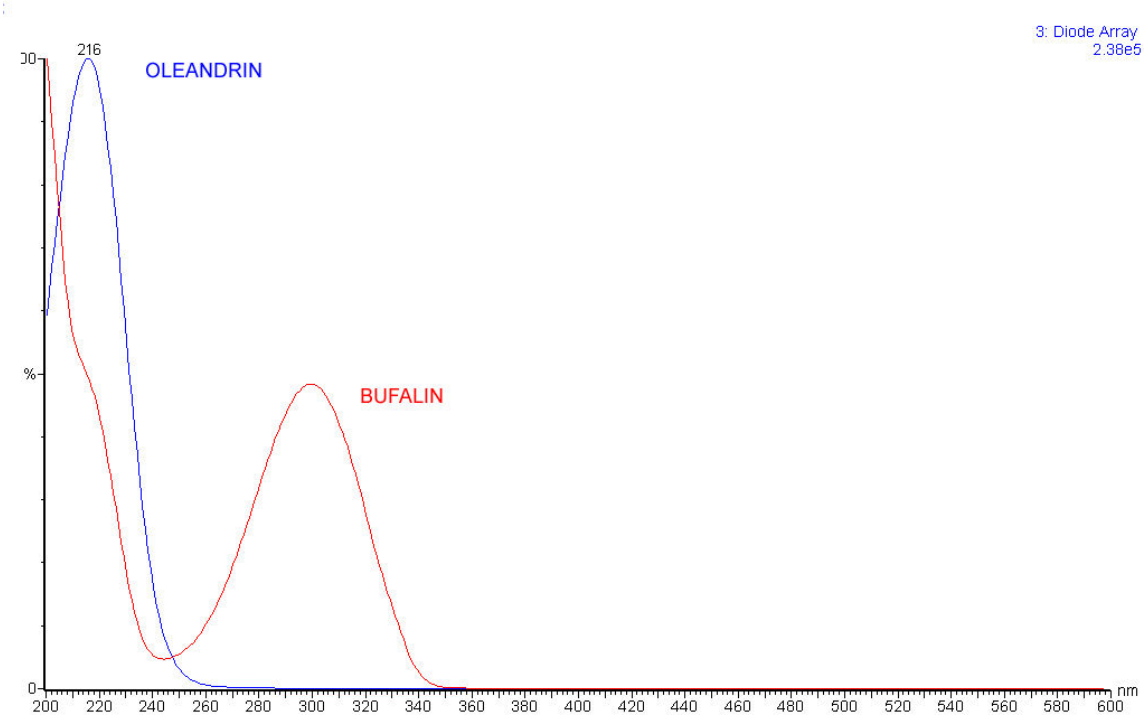


Figure 9.7

Comparison of the UV spectra of oleandrin and bufalin on the PDA detector (TMD system)

The solubility of cardiac glycosides in solvents varies quite significantly with the only common characteristic being the very low solubility in water. Quite a few compounds display solubility in chloroform, while others do not dissolve in chloroform alone but in a mixture of chloroform and methanol. Acetone is also one of the more common solvents but not all the cardiac glycosides display sufficient solubility to make it the common solvent. The best compromise is methanol as most cardiac glycosides dissolve in “alcohol” and all standards were prepared in HPLC grade methanol.

An evaluation of the structures of the selected cardiac glycosides (Figure 9.7) suggested that ESI negative would be the most probable ionisation mode due to the presence of hydroxyl groups, but these compounds displayed very poor ionisation in this mode irrespective of the mobile phase composition and these results were consistent with published methods [169, 170]

The only compound that displayed noteworthy ESI negative ionisation was bufalin, but it was not comparable to the strong ESI positive ionisation signal observed. Digitoxin was used as the initial test compound for optimisation of the ESI interface and solvent composition. Although acetonitrile is the solvent of choice in ESI analysis (strong solvent characteristics and high volatility) it resulted in ionisation suppression and was replaced with methanol in all analytical experiments. Strong ionisation was observed in highly organic mobile phases but the addition of water reduced the ionisation signal strength. The addition of more than 75% water severely affected ionisation and limited the starting mobile phase composition to less than 75% water.

Various buffers were evaluated to ensure ionisation of the cardiac glycosides, namely acetic acid, formic acid, ammonium acetate, ammonium formate and ammonium hydroxide. For ESI detection, the compound must be charged in solution which may occur naturally or is induced by the addition of a buffer (mobile phase modifier) and the adjustment of the pH. Formic acid and ammonium acetate were the only two buffers that allowed reproducible ESI positive ionisation, with dilute formic acid (0.05 – 0.5% v/v) being the better of the two buffers. A buffer concentration of 0.5% produced the best chromatography but suppressed ionisation, while a buffer concentration of 0.05% produced the strongest ionisation signal for digitoxin but was unable to stabilise the chromatography. The optimal buffer concentration of 0.1% v/v produced stable chromatography without any noteworthy suppression of ionisation.

Initial experiments on the TMD system (EI ionisation) suggested that all the cardiac glycosides were fragile compounds that fragmented easily and could not be detected with the glycoside moiety still attached to the steroidal aglycone. ESI experiments were adapted to compensate for this fragile nature of the compounds by using low cone and capillary voltages as well as low nebulisation temperatures. However, these mild settings resulted in poor ionisation of all the test compounds and settings were increased to enhance ionisation efficiency. The optimal mass spectral conditions for the detection of the selected 11 cardiac glycosides are listed in Table 9.2.

Under these MS conditions and by using the optimised HPLC conditions listed in Table 9.3, all the cardiac glycosides could be separated and detected (Figures 9.9 and 9.10). The only two components that overlapped partially were neriifolin (R_t 31.83 min) and bufalin (R_t 31.97 min). However, these compounds could easily be distinguished by their unique ESI positive spectra (Figure 9.9). A summary of the mass spectral interpretations for each compound is summarised in Table 9.4. Most of the compounds were subject to cationisation with sodium while a few compounds also formed adducts with methanol (bufalin, neriifolin, oleandrin and to a lesser extent strophanthin K).

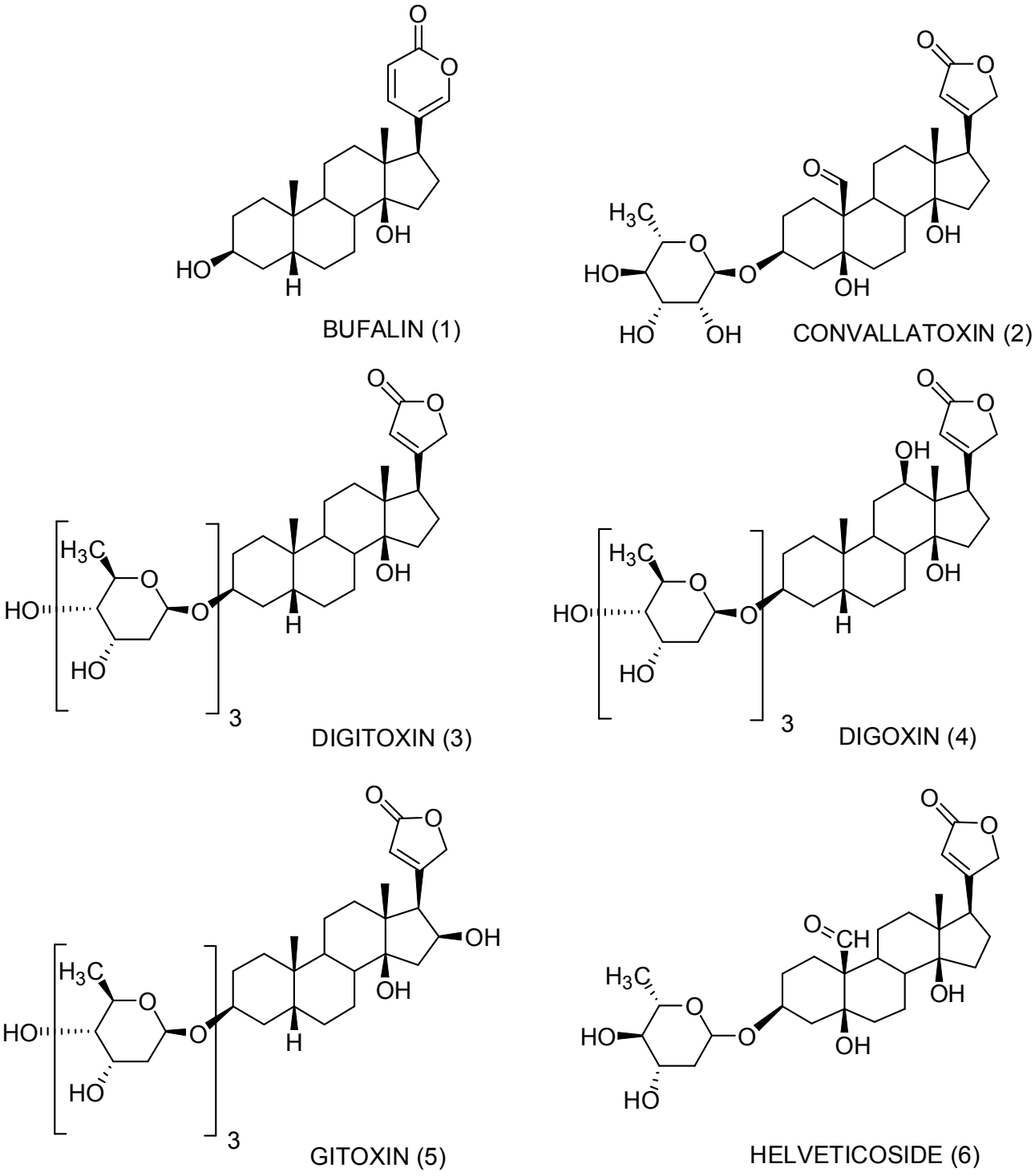
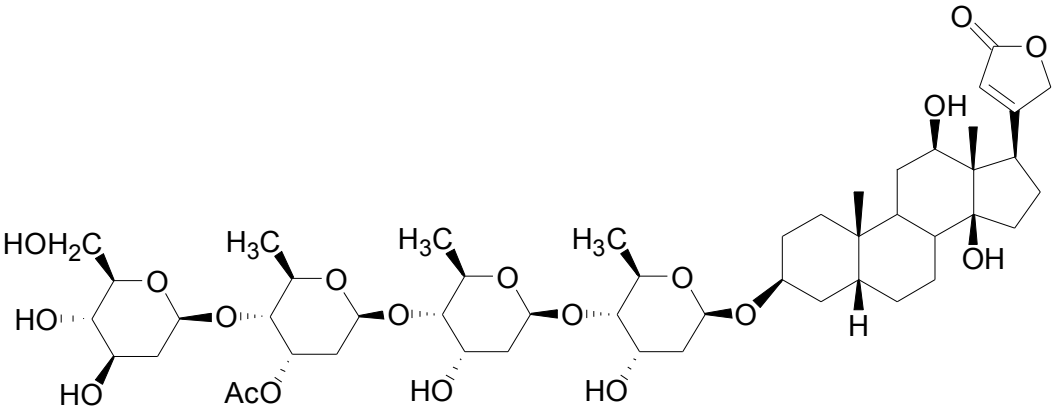
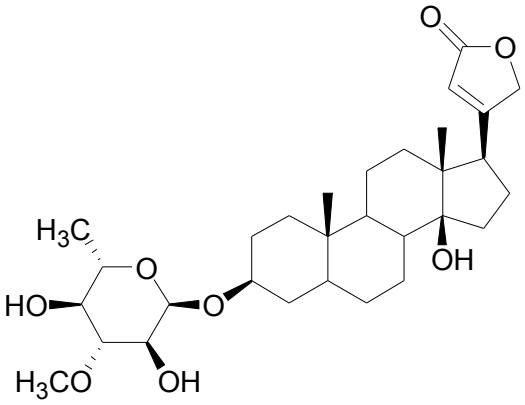


Figure 9.8 (a)

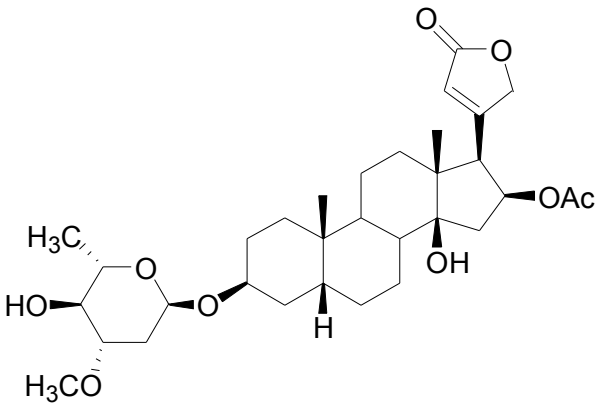
Chemical structures of the selected cardiac glycosides



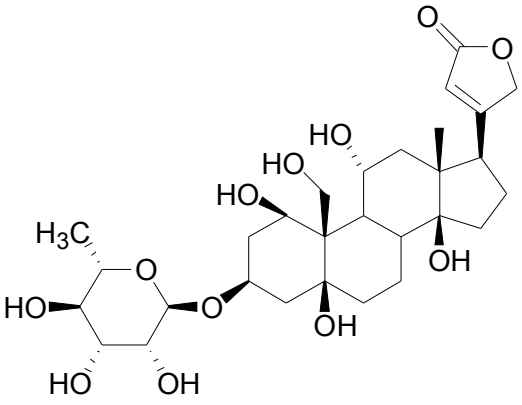
LANATOSIDE C (7)



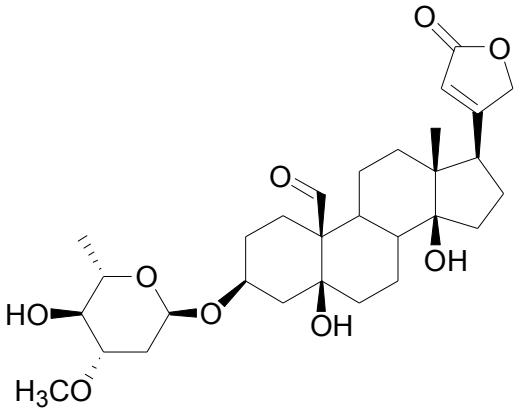
NERIIFOLIN (8)



OLEANDRIN (9)



STROPHANTHIN G (10)

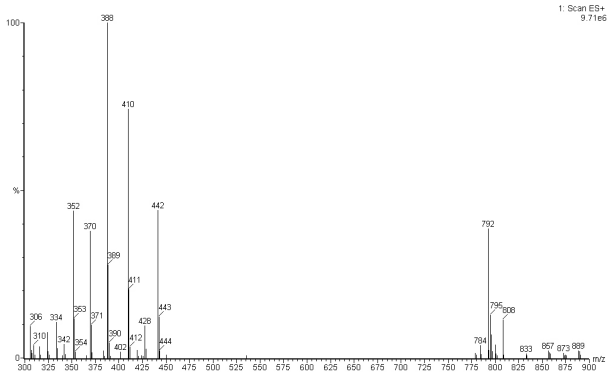


STROPHANTHIN K (11)

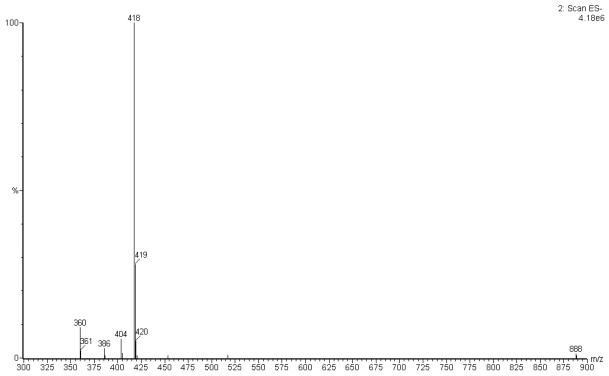
Figure 9.8 (b)

Chemical structures of the selected cardiac glycosides

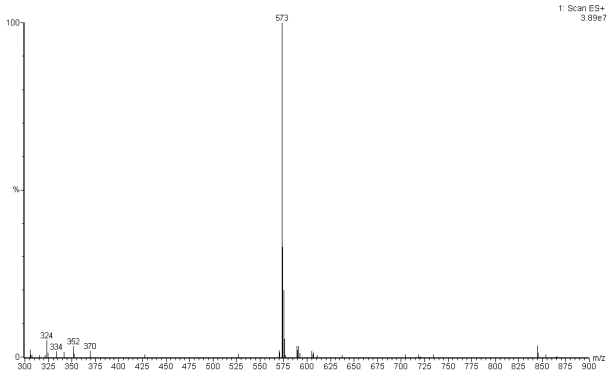
BUFALIN (ESI^+)



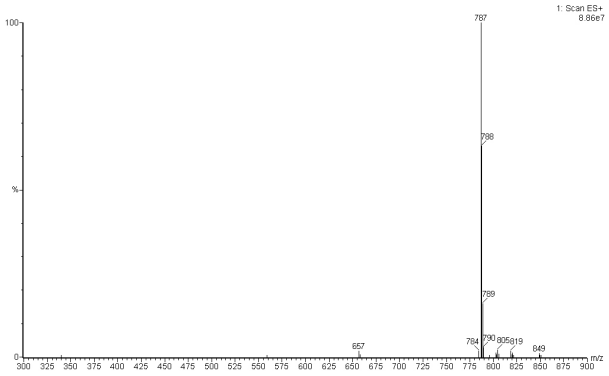
BUFALIN (ESI^-)



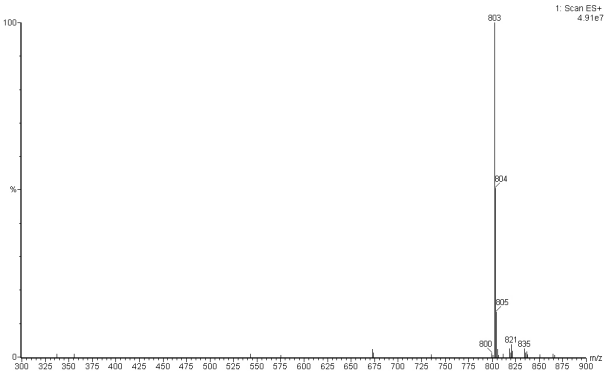
CONVALLATOXIN (ESI^+)



DIGITOXIN (ESI^+)



DIGOXIN (ESI^+)



GITOXIN (ESI^+)

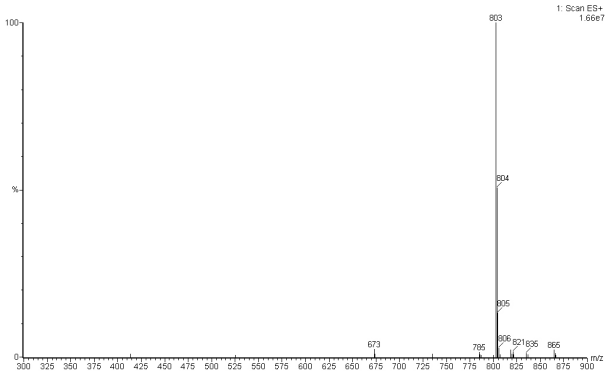
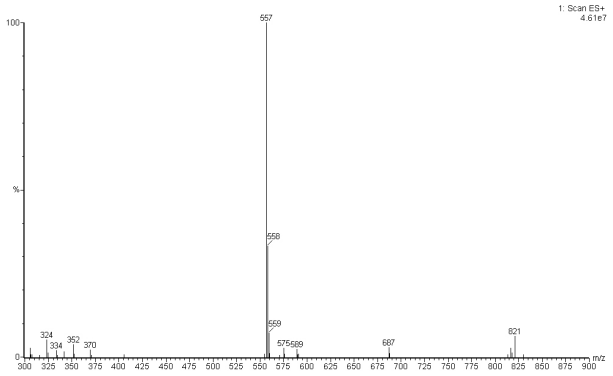


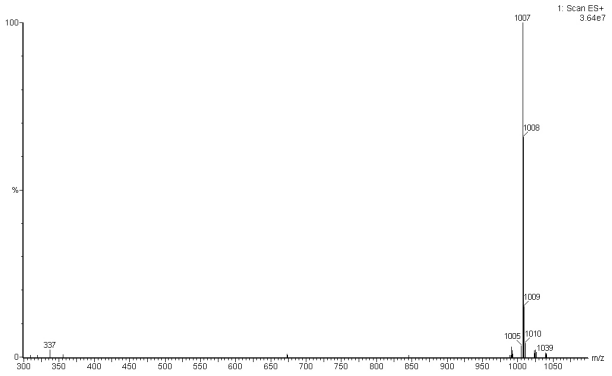
Figure 9.9 (a)

ESI spectra of the selected cardiac glycosides (ZQ system)

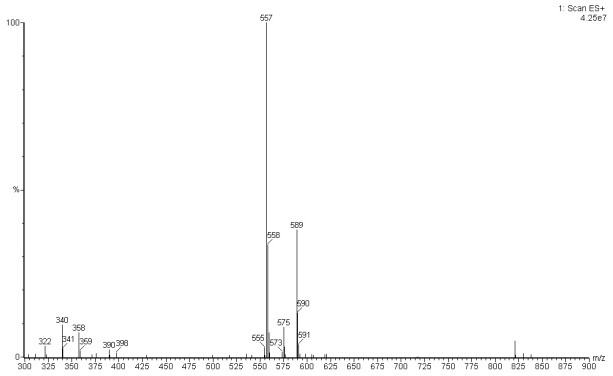
HELVETICOSIDE (ESI^+)



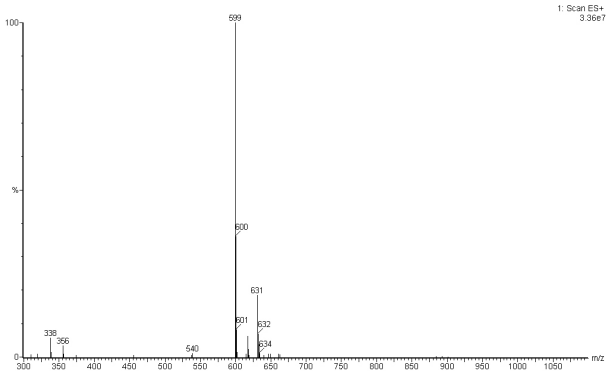
LANATOSIDE C (ESI^+)



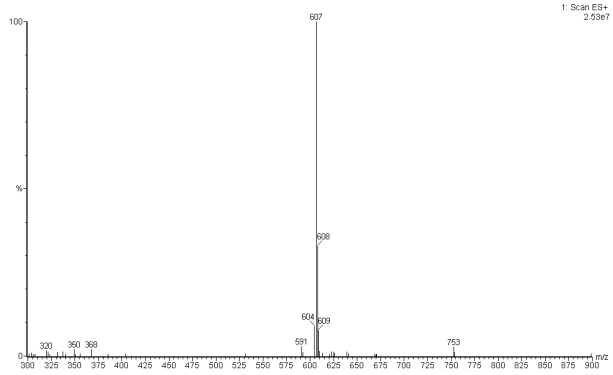
NERIIFOLIN (ESI^+)



OLEANDRIN (ESI^+)



STROPHANTHIN G (ESI^+)



STROPHANTHIN K (ESI^+)

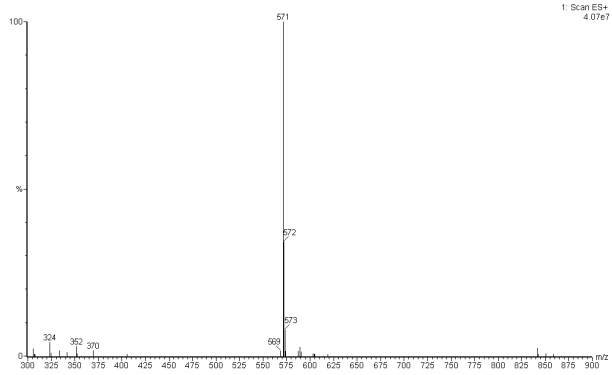


Figure 9.9 (b)

ESI spectra of the selected cardiac glycosides (ZQ system)

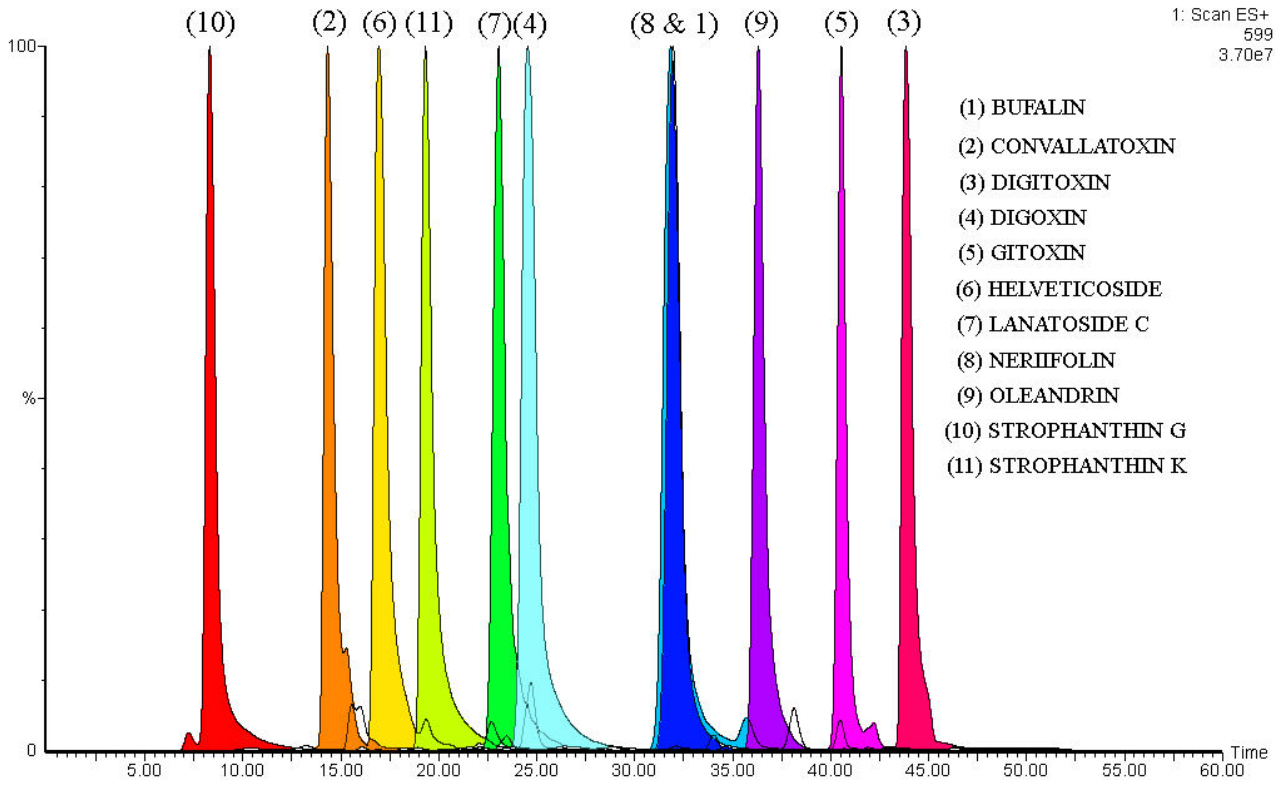


Figure 9.10

Overlaid ESI chromatograms of the selected cardiac glycosides (ZQ system)

TABLE 9.4 Mass spectral interpretation of the ESI spectra obtained for the selected cardiac glycosides (ZQ system)

COMPOUND	BASE PEAK	PEAK #2	PEAK #3
BUFALIN (ESI^+)	388 ($M+OH-CH_3$)	410 (M+H+Na)	442 ($M+H+Na+CH_3OH$)
BUFALIN (ESI^-)	418 ($M+CH_3OH$)		
CONVALLATOXIN	573 (M+Na)		
DIGITOXIN	787 (M+Na)		
DIGOXIN	803 (M+Na)		
GITOXIN	803 (M+Na)		
HELVETICOSIDE	557 (M+Na)		
LANATOSIDE C	1007 (M+Na)		
NERIIFOLIN	557 (M+Na)	589 ($M+Na+CH_3OH$)	
OLEANDRIN	599 (M+Na)	631 ($M+Na+CH_3OH$)	
STROPHANTHIN G	607 (M+Na)		
STROPHANTHIN K	571 (M+Na)	603 ($M+Na+CH_3OH$)	

It is interesting to note that those compounds that displayed the formation of a methanol adduct all had a methoxy group on the glycoside portion of the structure and it would seem as if the presence of this functional group is a prerequisite for the formation of a methanol adduct.

Various HPLC columns were evaluated during the development of this method. All the evaluated columns were able to separate most of the 11 cardiac glycosides, but it was only the Phenomenex Synergi Fusion column that could partially separate bufalin and neriifolin and fully separate digoxin and lanatoside C. These columns, as well as the Xterra RP C18 column, were the only columns that gave adequate retention of strophanthin G (R_t 8.29 min). This compound was extremely sensitive to the percentage of organic solvent present in the eluent and adequate equilibration time must be allowed at the end of each analysis run to obtain stable and reproducible chromatography.

Flow injection analysis (FIA) of the compounds was subject to various problems. Direct infusion of the methanolic standards at flow rates of 10 – 50 µl/min gave very prominent protonated base peaks, but the observed signals were unstable and fluctuated unacceptably. Infusion of the methanolic standards at similar flow rates into a mobile phase of 50:50 methanol:water mixed at 0.2 ml/min gave excellent results and also allowed the formation of adducts.

An attempt was made to induce ISCID of the selected compounds by varying the cone and capillary voltages. Each compound was tested by stepwise adjusting the cone voltage from 20 V to 150 V and the capillary voltage from 0.5 to 3.5 KV while infusing the standard into a 50:50 methanol:water eluent. It was found that none of the compounds could be gradually fragmented by ISCID and that they were destroyed by cone voltages above 70 V. Capillary voltages above 2.8 KV caused consistent arching between the capillary tip and the outer sample cone and could not be considered for future experiments.

9.9.2 Chemical extraction of *N. oleander* and *T. peruviana* leaves

The extraction of *N. oleander* and *T. peruviana* leaves with organic solvents produced mixed results. The extracts were extremely “dirty” and precipitated easily in the sample cooler or during injection. Although various cardiac glycosides were observed in these extracts, the frequent system maintenance required when analysing these samples ruled out the use of organic solvents. The PBS buffer extracts (phosphate and HCl buffers) were much cleaner and did not precipitate as severely as the organic solvent extracts when cooled to 5 ^{o}C. The UV/ ESI positive analysis of these extracts indicated the presence of several cardiac glycosides and the PBS-HCl buffer produced better results than the PBS-PO_4 buffer. The optimal pH of the PBS-HCl buffer was determined and a pH of 5 was found to extract the most oleandrin as well as the highest number of compounds.

The analysis of an oleandrin standard and the leaf extract of *N. oleander* under optimised conditions are depicted in Figure 9.11. A peak similar to that of oleandrin was detected in the leaf extract and was resolved from the other cardiac glycosides and matrix compounds. The mass spectrum of the oleandrin standard was added to the MassLynx user library and used as reference spectrum to confirm the identity of the suspected oleandrin peak in the leaf extract. The mass spectral matches were excellent (92% Forward match; 99% Reverse match). An evaluation of the detected compounds in the leaf extract indicated the presence of three other cardiac glycosides. The chromatograms of the three compounds are depicted in Figure 9.12 and their mass spectra in Figure 9.13. Figure 9.12 was generated by extracting selective masses (SIM) at 541, 557, 589 and 615 amu from the full scan data. The mass spectra were compared with those of the 11 cardiac glycosides and one compound was identified, namely nerriifolin (Forward match 81%; Reverse match 89%).

According to literature [170] odoroside ($C_{30}H_{46}O_7$; MW 518.32) and neritaloside ($C_{32}H_{48}O_{10}$; MW 592.32) are common constituents of *N. oleander*. Under the mass spectral and chromatographic conditions of this method, odoroside would cationise and form a protonated ion $[M+Na]^+$ with *m/z* 541 and would also display the formation of a methanol adduct $[M+MeOH]^+$ with *m/z* 573. Neritaloside would behave similarly and from a protonated ion $[M+Na]^+$ with *m/z* 615 and would also display the formation of a methanol adduct $[M+MeOH]^+$ with *m/z* 647. The observed mass spectra of the other two compounds matched very well with these theoretical predictions, and indicated the presence of neritaloside at R_t 30.14 min and odoroside at R_t 36.70 min.

The leaves of *T. peruviana* were extracted with the PBS-HCl method and the chromatographic profile was compared to that of the *N. oleander* leaf extract that was obtained under similar extraction conditions (Figure 9.14).

Differentiation between the two plants was possible by a visual comparison of the two chromatograms: the *N. oleander* extract contained more apolar compounds whereas the *T. peruviana* extracts were more polar in nature. By comparing these two extracts in SIM mode and using the common masses of *N. oleander*, the two plants could be clearly distinguished (Figure 9.15).

The same was true if the extracts were compared by using the common masses of *T. peruviana* (Figure 9.16). Although the mass spectral and chromatographic conditions were not optimised for the detection of cardenolides of *T. peruviana*, thevetin A, thevetin B and neriifolin could be detected in the leaf extracts of *T. peruviana*.

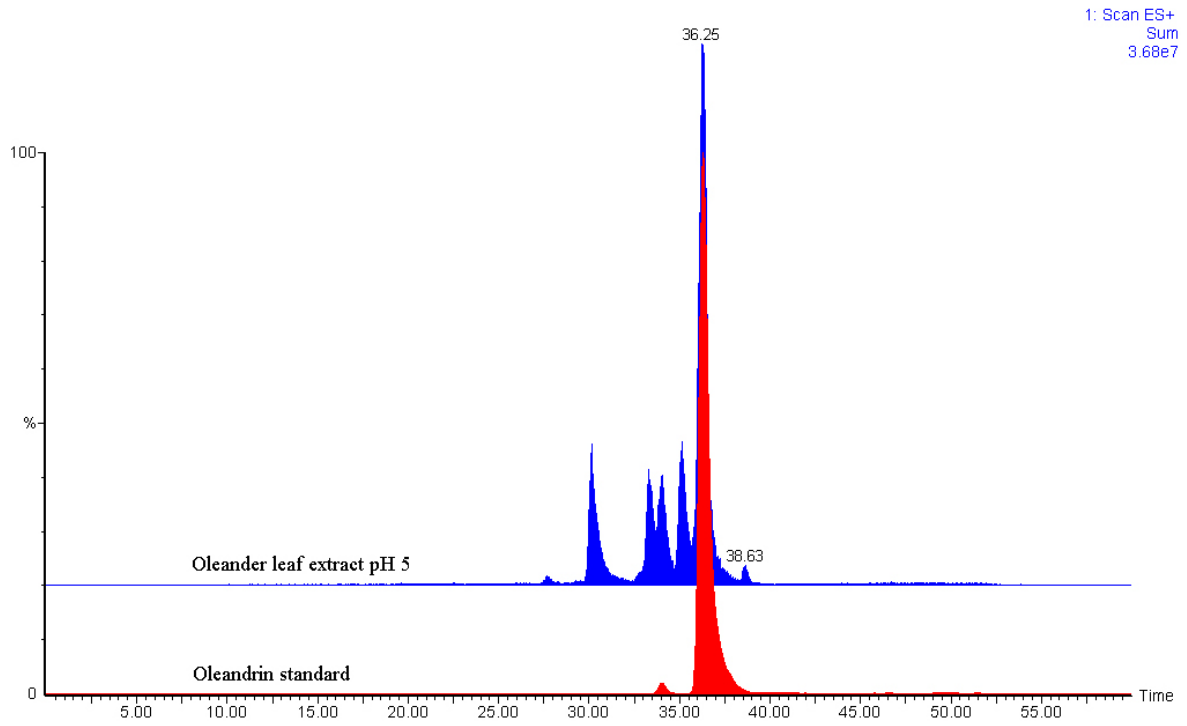


Figure 9.11

Overlaid ESI$^+$ chromatograms of the leaf extract of *Nerium oleander* and oleandrin standard (ZQ system)

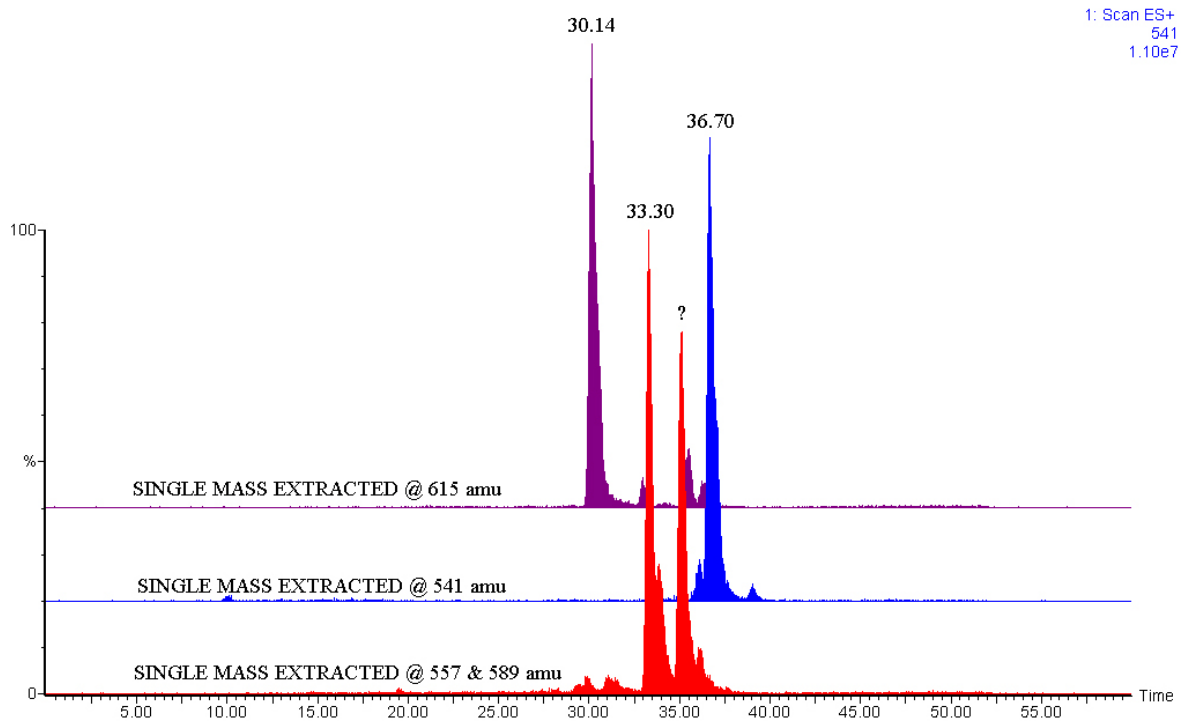


Figure 9.12

SIM ESI$^+$ chromatograms of the other cardiac glycosides detected in the leaf extract of *Nerium oleander* (ZQ system)

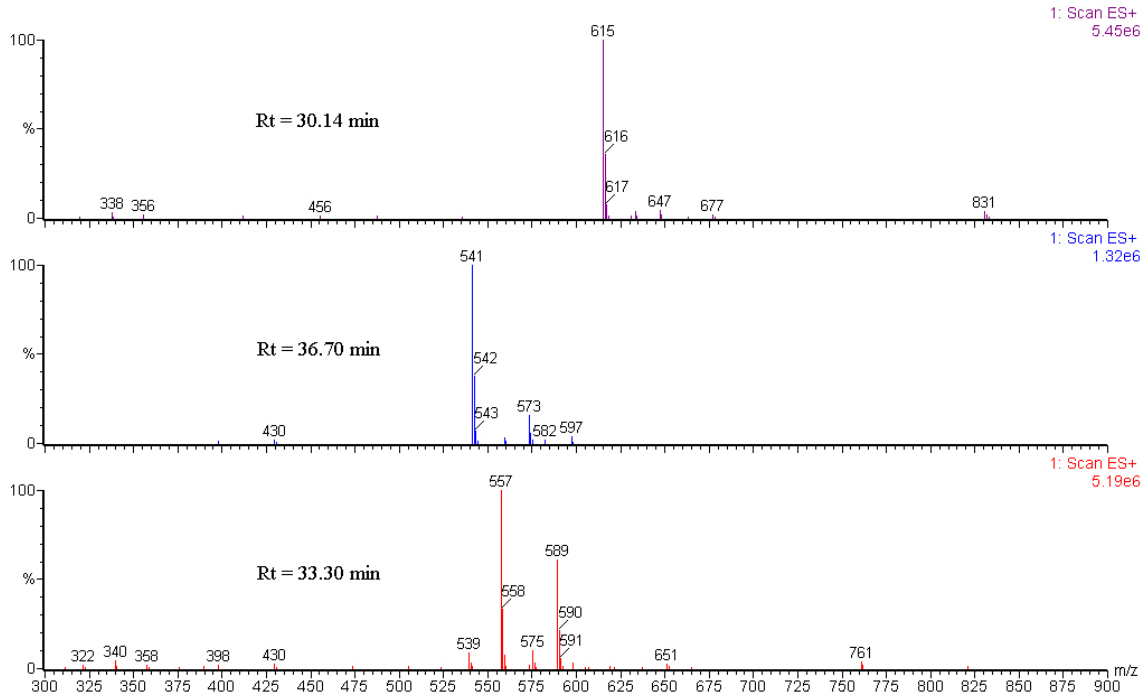


Figure 9.13

ESI$^+$ mass spectra of the other cardiac glycosides detected in the leaf extract of *Nerium oleander* (ZQ system)

The PBS-HCl SPE extracts of the five colour varieties were compared (Figure 9.17). A comparison of the chromatograms (Figure 9.18) revealed that the various colour forms have similar profiles, the main components were present in all the colour forms but the concentration of the various components varied between the colour forms.

9.9.3 Chemical extraction and analysis of viscera and case exhibits

The extraction and positive identification of cardiac glycosides have always been a problematic procedure at the FCL JHB. In the past solvent extraction with methanol or chloroform produced positive results only for case exhibits (*muti* or plant material), but no cardiac glycosides could be detected in the viscera samples. The viscera extracts were very complex and easily contaminated the instruments due to the large amount of putrifacts and fat that were co-extracted.

A forensic case (Case 995) was received of a person that went to a traditional healer to obtain a *muti* to cleanse his system. The person administered the *muti* as an enema, became seriously ill and died the next day. The request from the investigating officer was to identify the herbal toxin(s) present. The *muti* consisted of a mixture of liquid and plant material and was extracted with the optimised SPE method.

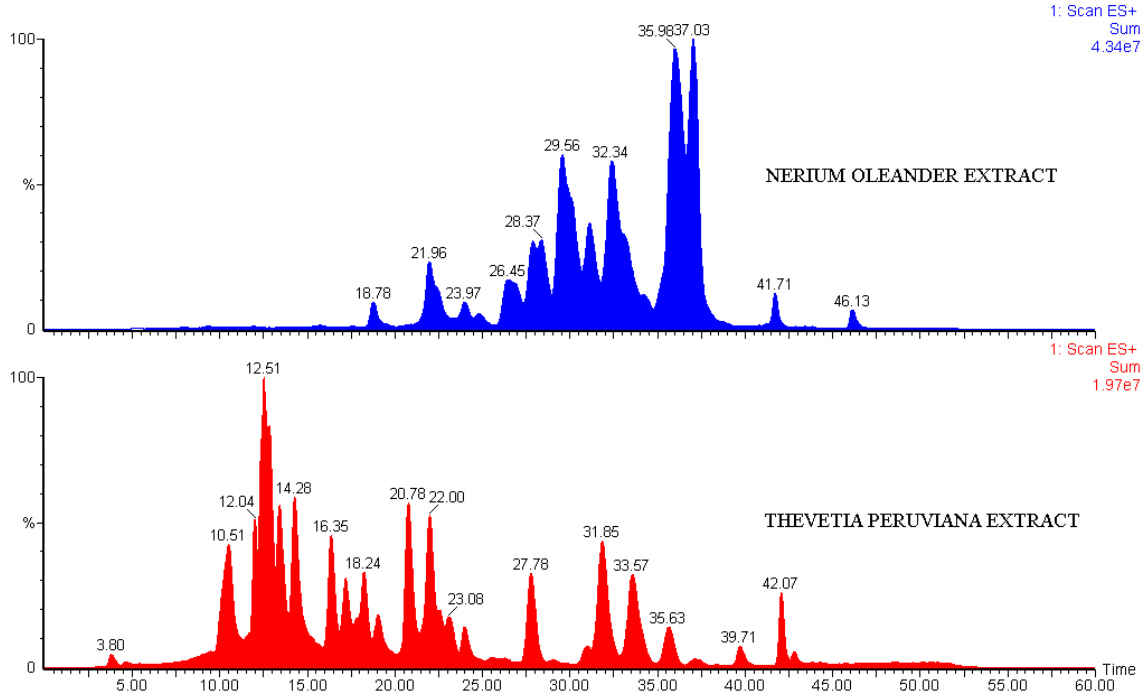


Figure 9.14

ESI$^+$ chromatographic profiles of the *Nerium oleander* and *Thevetia peruviana* leaf extracts (ZQ system)

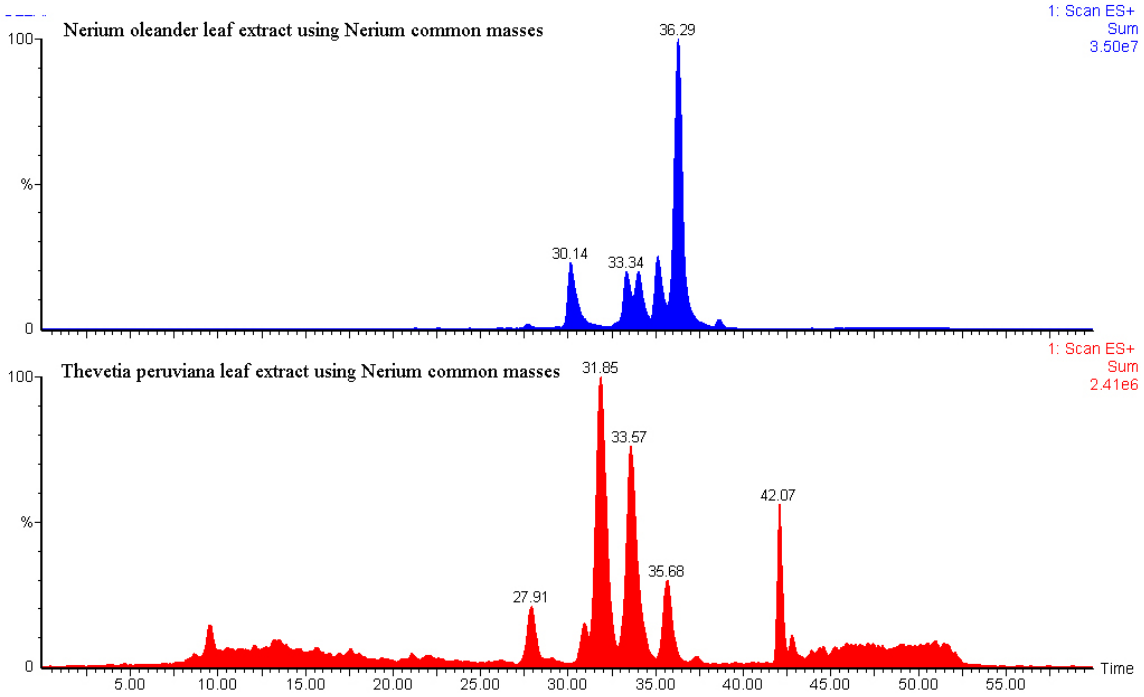


Figure 9.15

Comparison of *Nerium* and *Thevetia* leaf extracts using the common masses of *Nerium oleander* (ESI$^+$ chromatograms on ZQ system)

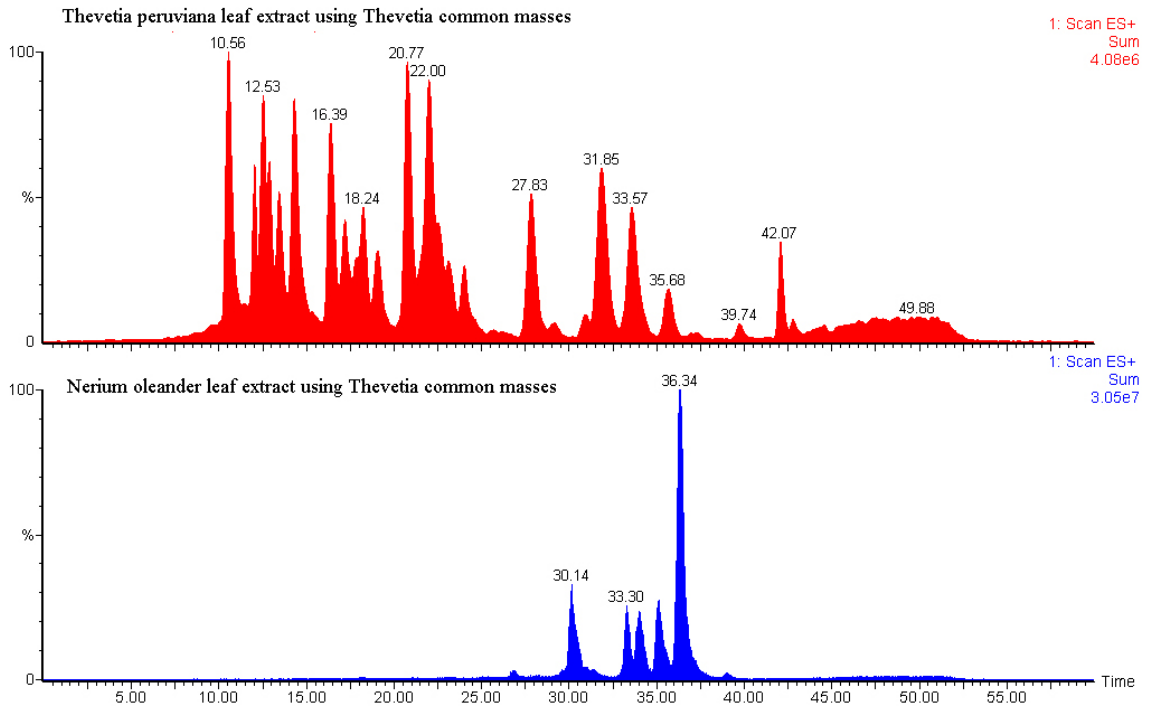


Figure 9.16

Comparison of *Nerium* and *Thevetia* leaf extracts using the common masses of *Thevetia peruviana* (ESI^+ chromatograms on ZQ system)

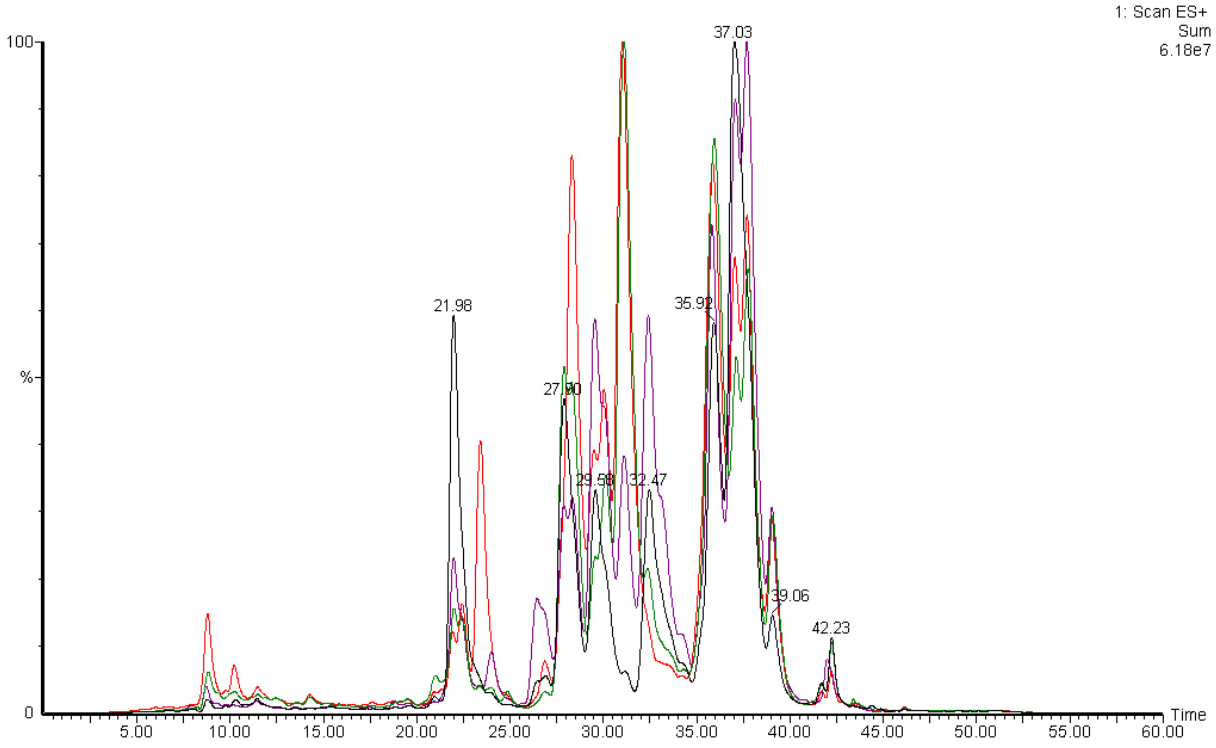


Figure 9.17

Comparison of the ESI^+ chromatographic profiles of five colour forms of *Nerium oleander* (Full analysis run on ZQ system))

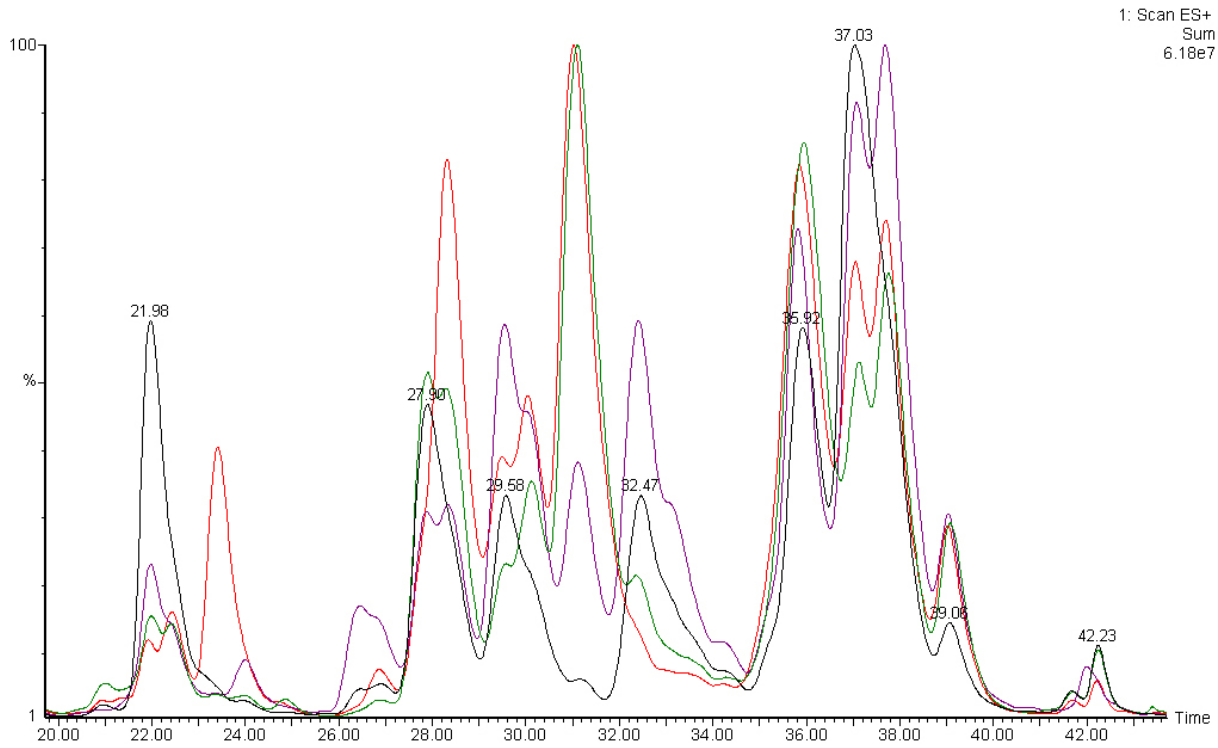


Figure 9.18

Comparison of the ESI$^+$ chromatographic profiles of five colour forms of *Nerium oleander* (Zoomed – ZQ system)

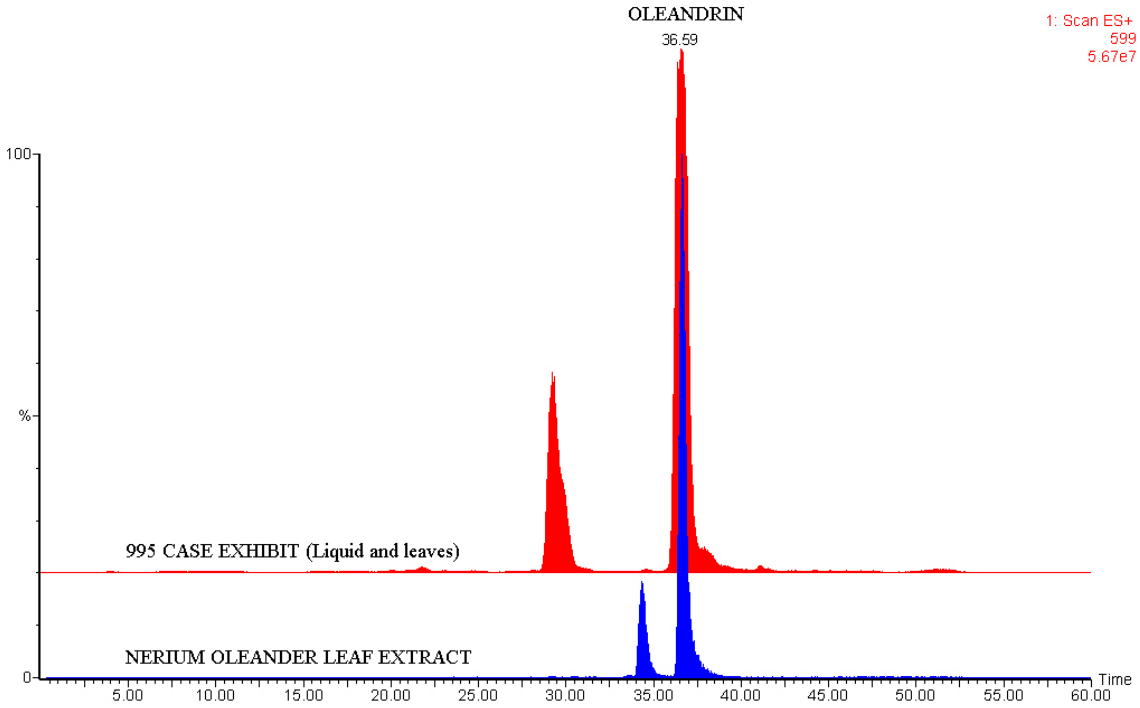


Figure 9.19

ESI$^+$ chromatographic comparison of the extracts of the exhibit of Case 995 and a *Nerium oleander* leaf extract (ZQ system)

The extract was analysed on the ZQ ESI system and produced a chromatographic profile similar to that of the *N. oleander* leaf extract (Figure 9.19). A SIM analysis of the data at 599 amu ($[M+Na]^+$ of oleandrin) revealed a large peak that was consistent with that of oleandrin and a library match (MassLynx user library) confirmed the presence of oleandrin (Forward match 92%, Reverse match 99%).

The viscera samples of Case 995 were extracted with the PBS-HCl buffer and analysed on the ZQ system. The TIC chromatograms did not display profiles compatible with *N. oleander*, but a SIM analysis of the data revealed the presence of a possible oleandrin peak in all the samples (Rt 36.7 ± 0.2 min) (Figure 9.20). The spectra of the compounds (Figure 9.21) were compared with the MassLynx user library and the results are summarised in Table 9.5.

Table 9.5 Analysis results of the samples pertaining to Case 995

SAMPLE	Rt (min)	FORWARD MATCH	REVERSE MATCH	IDENTITY
Stomach	37.17	29%	98%	Oleandrin
Liver	36.92	66%	99%	Oleandrin
Kidney	36.95	58%	99%	Oleandrin
Blood	36.90	76%	99%	Oleandrin
Exhibit	36.60	92%	99%	Oleandrin

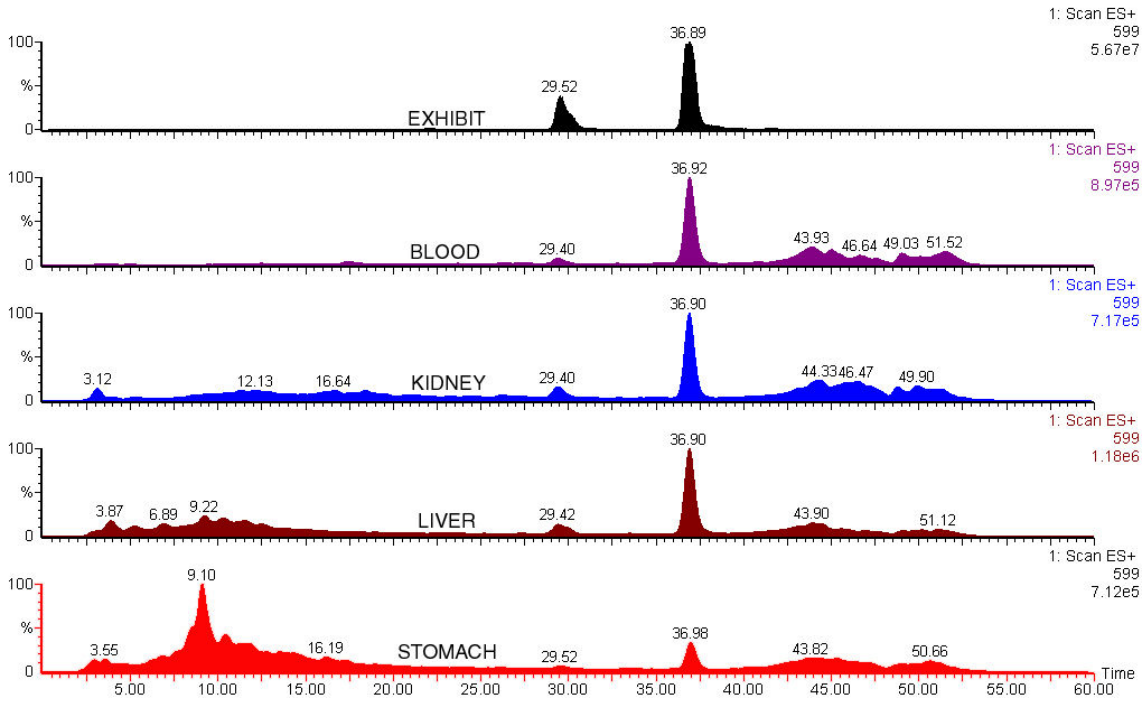


Figure 9.20

ESI^+ SIM chromatograms at 599 amu of the various samples of Case 995 (ZQ system)

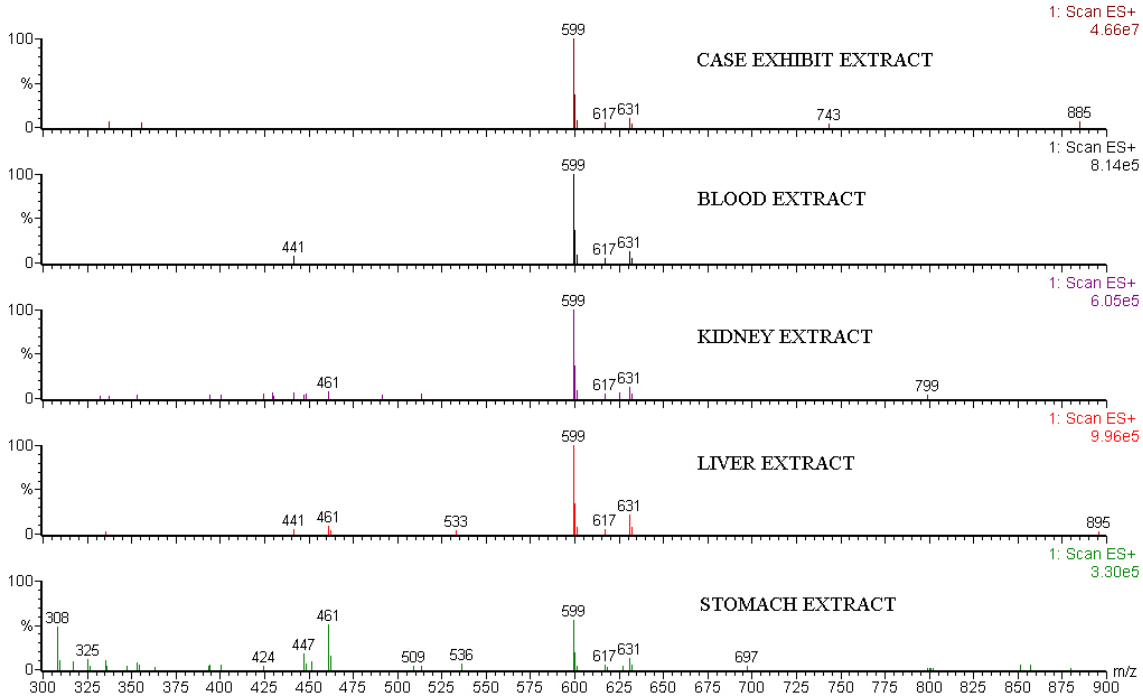


Figure 9.21

Spectra obtained from the ESI$^+$ SIM analysis at 599 amu of the samples of Case 995 (ZQ system)

Oleandrin was detected in all the viscera samples and confirmed the use of the *muti* that contained large amounts of oleandrin. The variation in match quality can be attributed to the varying levels of oleandrin in the samples – the stomach contained the least and had the worst match qualities while the case exhibit contained the most and had the best match qualities (Table 9.5).

The success of this method sparked renewed interest in a previous case (Case 234) of suspected oleander self-poisoning that happened in 2003. The person was found dead in his room after boiling up some leaves and drinking the extract. A small amount of the extract (3 ml) and a blood sample (2 ml) were submitted to the FCL JHB and was extracted with the normal SPE - PBS buffer method and analysed on the TMD system. Oleandrin was detected and confirmed in the *muti* (NIST library matches) but not in the blood sample. The samples were frozen after analysis but partially freeze-dried during storage. The samples were thawed and re-extracted with the SPE – PBS-HCl method and analysed on the ZQ system. The initial analysis was done in full scan mode and presence of oleandrin was indicated in the *muti* extract but not in the blood sample. A SIM analysis of the blood sample indicated the presence of oleandrin but was not conclusive. The SIM analysis method was expanded to monitor the six common ions of oleandrin (ion clusters at 599 and 631 amu).

The oleandrin standard was re-analysed with the adapted SIM method and the spectrum appended to the MassLynx user library. The *muti* sample (Figure 9.22) and the blood sample (Figure 9.23) were re-analysed. The spectra of the suspected oleandrin peaks of the *muti* and blood sample (Figures 9.24 and 9.25) were compared with those in the user library and the presence of oleandrin was confirmed in both samples (Table 9.6).

Table 9.6 Analysis results of the samples pertaining to Case 234

SAMPLE	Rt (min)	FORWARD MATCH	REVERSE MATCH	IDENTITY
Blood	37.40	99%	99%	Oleandrin
Exhibit	37.36	99%	99%	Oleandrin

9.10 FORENSIC APPLICABILITY

The FCL JHB dearly needed a good screening method for cardiac glycosides. The method developed is capable of detecting various cardiac glycosides – cardenolides as well as bufadienolides.

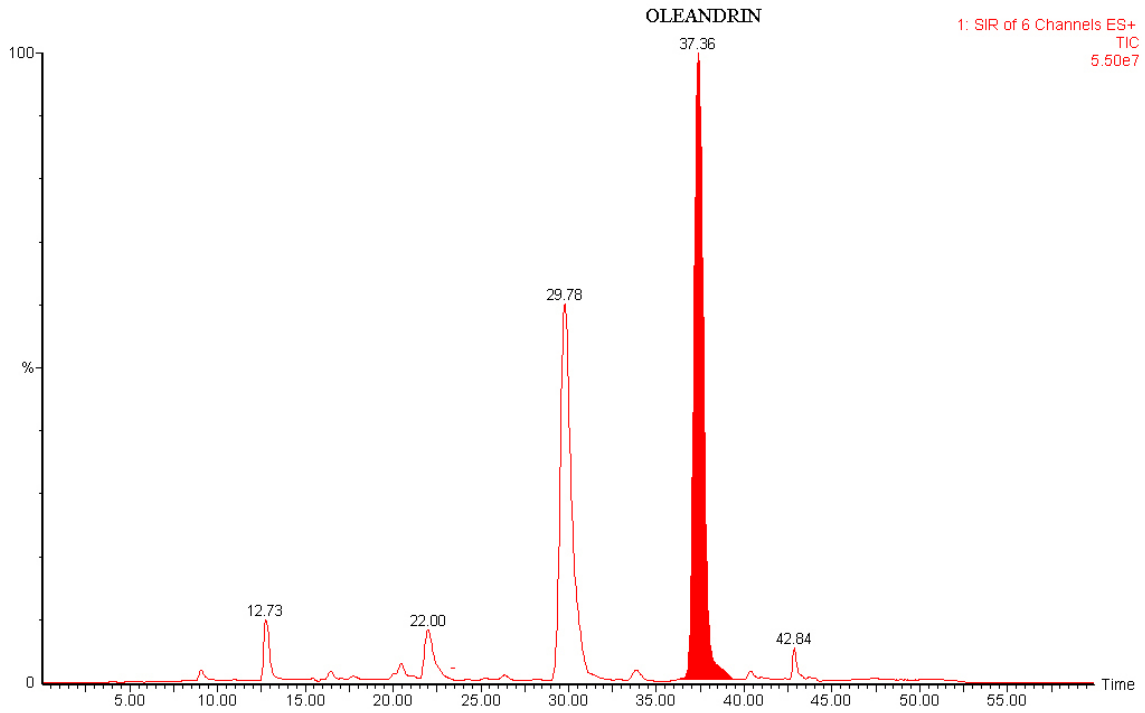


Figure 9.22

ESI$^+$ chromatogram of the SIM analysis (six masses) of the *muti* sample of Case 234 (ZQ system)

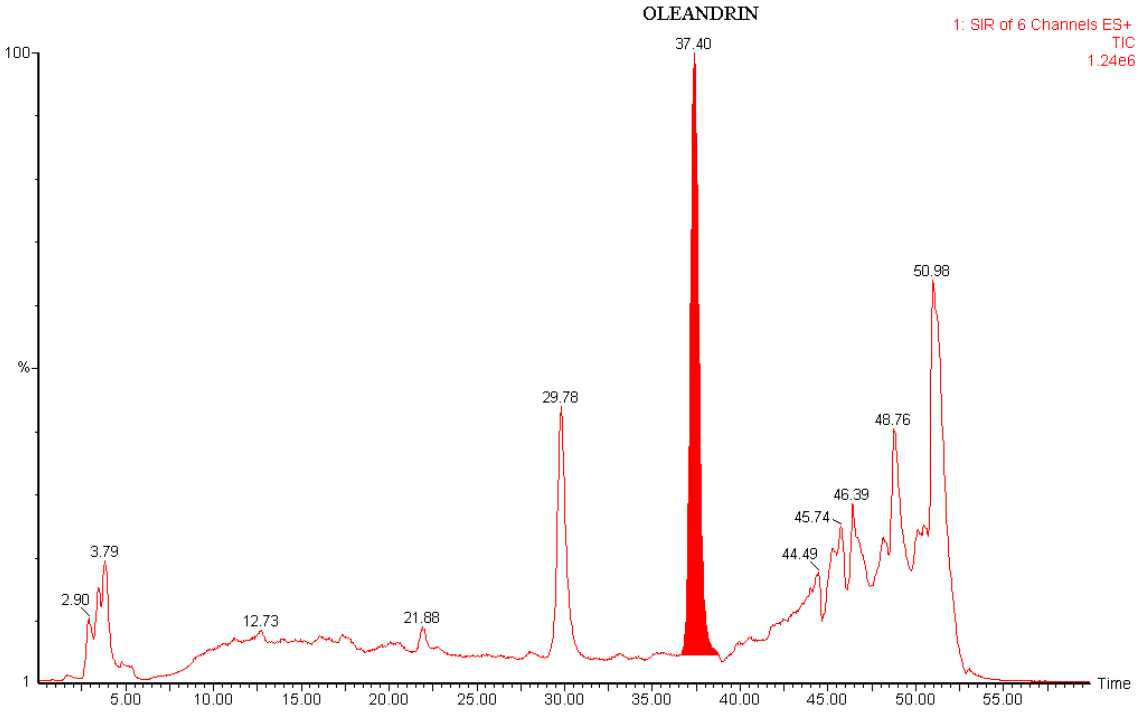


Figure 9.23

ESI$^+$ chromatogram of the SIM analysis (six masses) of the blood sample of Case 234 (ZQ system)

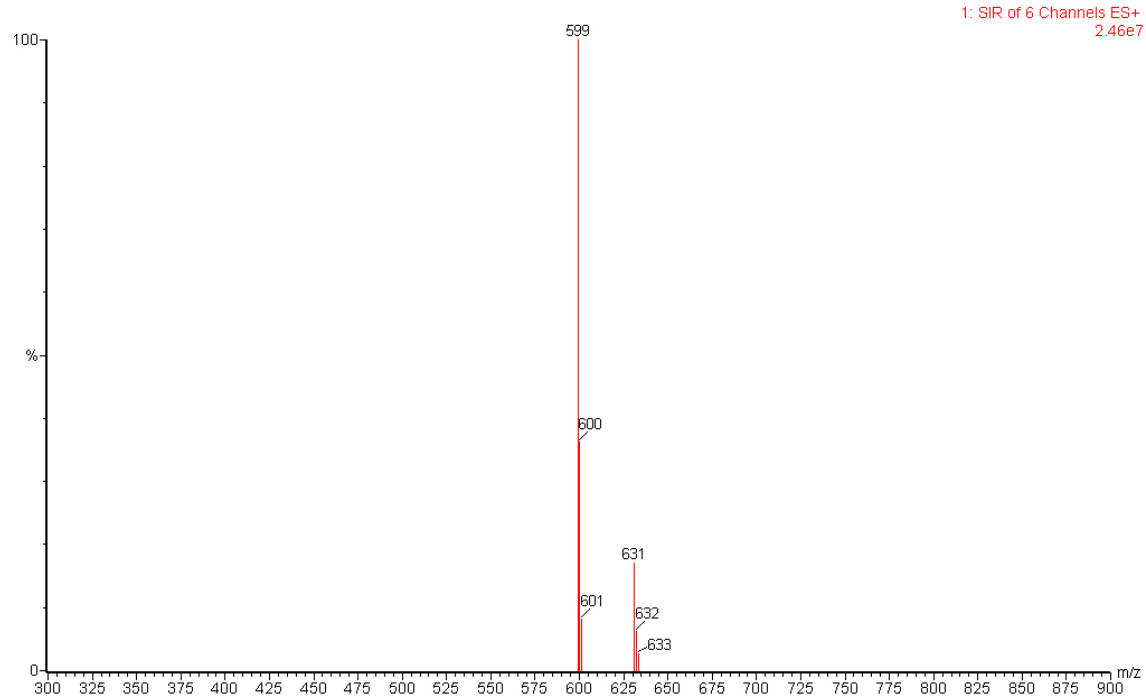


Figure 9.24

ESI$^+$ spectrum of the SIM analysis (six masses) of the *muti* sample of Case 234 (ZQ system)

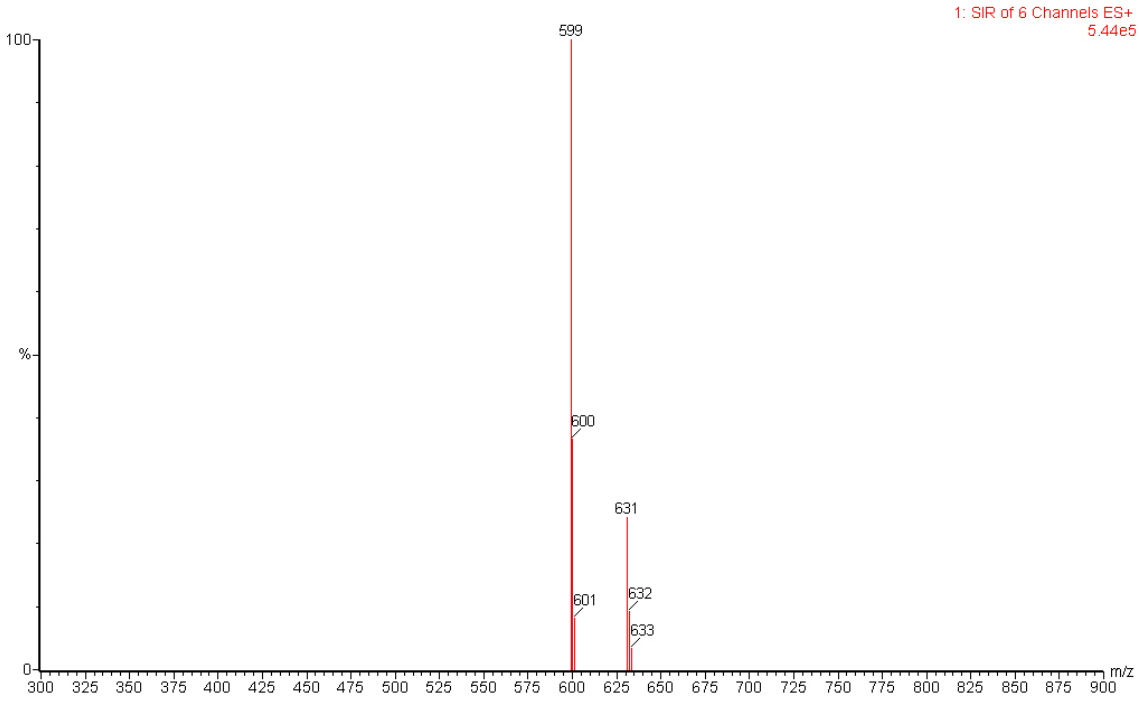


Figure 9.25

ESI$^+$ spectrum of the SIM analysis (six masses) of the blood sample of Case 234 (ZQ system)

Although some of the test compounds might not occur in forensic samples submitted to the FCL JHB, the versatility of the method was clearly demonstrated by separating and detecting 11 cardiac glycosides. The method is ideally suited for the detection of oleandrin in high concentrations (full scan mode), low concentrations (selected masses) or trace levels (SIM analysis of ion clusters).

The method is able to distinguish between extracts derived from *N. oleander* and *T. peruviana* and was able to detect and confirm neriifolin, odoroside and neritaloside in *N. oleander* leaf extracts. Analysis of forensic case exhibits were also successfully done with this method and performed well with liquid and solid matrices. Forensic analysis of viscera samples did not produce positive results in the past, but with the new method oleandrin could be detected at trace levels. The analysis of oleandrin on the blood sample was done on a sample size of less than 100 µl and still produced excellent results.

Although this method could function as a primary screening method, especially to detect known cardiac glycosides (previously analysed and included in the MassLynx user library) at very low concentration levels, it is ideally suited as a secondary screening method to confirm the presence of possible cardiac glycosides detected on the TMD system.

CHAPTER 10

Bowiea volubilis

10.1 BOTANICAL DESCRIPTION

Bowiea volubilis Harv. ex Hook. f. (Hyacinthaceae) is an interesting plant with a greenish-white, fleshy bulb that grows partly above ground (Figure 10.1). The bulb scales are fleshy in contrast to the papery or fibrous scales of *B. disticha*. The main above-ground part of the plant is the succulent, twining, leafless flowering stems (Figure 10.2). The flowers are small and greenish in colour, and eventually turn into small, two-locular capsules with numerous black seeds [171]. It is commonly known as the climbing potato, *umagaqana* (Xhosa) and *igibisila* (Zulu).

FIGURE 10.1

The fleshy bulb and stems of *Bowiea volubilis*

FIGURE 10.2

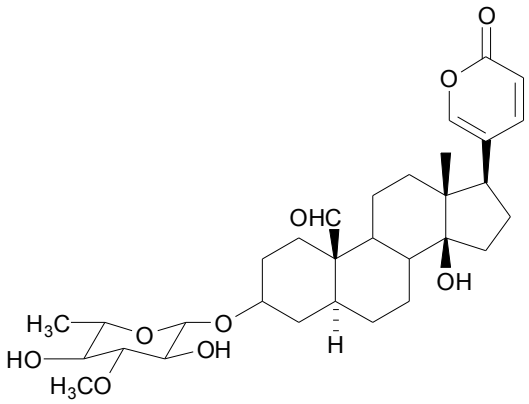
Flowers of *Bowiea volubilis*

10.2 ORIGIN AND DISTRIBUTION

Bowiea volubilis is indigenous to South Africa and is widely distributed through the eastern parts of the country [171].

10.3 TYPE OF TOXIN

Bowiea volubilis contain cardiac glycosides of the bufadienolide type. The main component is bovoside A with a molecular formula of $C_{31}H_{44}O_9$ and a molecular mass of 560.7 [69].



BOVOSIDE A

FIGURE 10.3

Chemical structure of bovoside A

10.4 ACTIVE COMPONENTS AND PHARMACOLOGICAL EFFECTS

The main pharmacological effects are caused by the cardiac glycosides including bovoside A. Their action as cardiac tonics, which increase the cardiac output through increased muscular contractions, are well known and have found application in the treatment of congestive heart failure. Bovoside A is, however, extremely toxic with a LD_{50} (cat, ivn) of 0.13 mg/kg [40]. Bufadienolides are also inhibitors of the enzyme Na,K-ATPase, and will cause similar physiological abnormalities as the cardenolides. The first signs are gastrointestinal discomfort, nausea and vomiting. These are followed by neurological symptoms that usually include weakness, mental confusion and visual disturbances. Next to appear are cardiac irregularities, usually bradycardia due to conduction problems and the onset of an A-V block resulting in fibrillation [172, 173].

10.5 ETHNOPHARMACOLOGICAL IMPORTANCE

All the parts of the plant are extremely toxic. Due to the very high LD_{50} value, the difference between therapeutic and toxic doses becomes very small, making the use of this plant for medicinal purposes very risky. However, the plant is widely used in traditional medicine and freely available on the *muti* markets of South Africa. It is used to treat headaches [174], hot water extracts of the roasted bulb is taken as a purgative and the fresh bulb is used to treat oedema and infertility in women. The fresh juice of the bulb is applied to the skin of a sick person or a decoction may be applied as a lotion to treat sore eyes [45]. It is a powerful emetic, working rapidly in small doses, and is also used as a purgative [175]. Bulbs are used in potions taken by

men as love charm emetics. Infusions are taken during pregnancy to facilitate delivery and are also used to induce abortions. Bulb decoctions are taken for bladder pains associated with venereal disease [176]. It is reported that the bulb also possesses magical properties [177, 178] and is sprinkled on *impi's* to ward off enemies.

10.6 POISONING

10.6.1 Accidental

Since the early 1900's numerous cases of poisoning have been reported that involved the bulb of *B. volubilis*. It was initially thought that the toxic principle was an alkaloid, but Katz made an in-depth study of the plant and isolated three highly active cardiac glycosides [45]. It is the cause of numerous fatalities in humans and animals and may be fatal within a couple of minutes [45, 179, 180, 181].

10.6.2 Deliberate

No reference in literature could be found of any deliberate attempt to kill or commit suicide by using *B. volubilis*.

10.7 FCL JHB INVESTIGATION

Bufadienolide-containing plants is a major problem in agriculture in South Africa [182, 183] and Australia [184] due to their toxicity to livestock. According to Kellerman et al. [185], bufadienolide glycosides represent the most important cause of mortality due to plant poisoning among cattle in South Africa and it is estimated that 33% of plant-related mortality may be attributed to bufadienolides, leading to loss of 12 000 head of cattle per year. Prompted by these statistics, a literature study was done on the use of bufadienolide-containing plants in traditional medicine and *B. volubilis* was identified as a potential candidate for human poisonings. *Bowiea volubilis* is widely used and freely available, but has not been detected by any of the screening procedures of the FCL JHB.

No reference could be found in the FCL JHB data base or any of the forensic case files submitted to the laboratory regarding the use of *B. volubilis*. The lack of a standard and a sensitive screening method for bovoside A is probably the main reasons why this compound has not been detected before. Another reason could be the lack of a good extraction method for the bufadienolides and in particular bovoside A.

References were found to a few LC-MS methods for the detection of oleandrin [186, 187]. These were all electrospray techniques using acidic eluents (formic acid or ammonium formate) and

acetonitrile as organic phase. In Chapter 9, a versatile and robust extraction method was developed for the extraction of oleandrin from plant material and viscera and a very sensitive and versatile ESI positive detection method was developed for the detection of ten cardenolides and one bufadienolide. The aim of this study was:

(a) To apply the developed methods to the analysis of the dried bulb of *B. volubilis* for the detection of bovoside A.

(b) To determine whether this protocol can distinguish among extracts of *B. volubilis* and other common South African plants containing cardiac glycosides, e.g. *Nerium oleander*, *Thevetia peruviana* and *Gomphocarpus fruticosus*.

10.8 EXPERIMENTAL PROCEDURE

10.8.1 Standards and reagents

No standard is commercially available for bovoside A. The standards and reagents, as described in §9.8.1 of chapter 9, were used.

10.8.2 Instruments and conditions

The optimised instrumental conditions, as described in §9.8.2 of chapter 9, were used. These conditions can be summarised as follows:

TMD conditions:

Table 10.1 HPLC Gradient profile of the general screening method (TMD system)

	TIME min	FLOW ml/min	%A H_2O	%B MeOH	%C Buffer	%D ACN	CURVE
1		0.20	0	0	95	5	6
2	1.00	0.20	0	0	95	5	4
3	10.00	0.20	0	0	80	20	6
4	12.00	0.20	0	0	80	20	4
5	20.00	0.20	0	30	50	20	5
6	35.00	0.20	0	10	0	90	6
7	37.00	0.20	0	10	0	90	6
8	40.00	0.25	0	10	0	90	6
9	45.00	0.25	0	10	0	90	6
10	50.00	0.20	0	0	95	5	5

This general screening method was developed to give optimal retention of polar compounds on the Phenomenex Aqua C18 column but also functions well with the Phenomenex Synergi Fusion C18 column. The buffer is 0.1% (v/v) formic acid in HPLC grade water.

TABLE 10.2 Optimised MS conditions for the detection of bovoside A on the TMD system

TMD SYSTEM CONDITIONS	BOVOSIDE A
Mode	EI positive
Ionisation energy (eV)	70
Gain	10
Nebuliser temperature (°C)	80
Expansion temperature (°C)	90
Source temperature (°C)	225
Capillary tip temperature (^{o}C)	160
Desolvation gas flow (L/h)	30
Sampling rate	1.0
Scan range (amu)	50 - 550
Ion Energy	1.0
Multiplier gain (V)	1250
PDA scan range (nm)	200 – 600
PDA sampling rate (points/s)	1.0
PDA resolution (nm)	1.2

The conditions used for the detectors of the TMD system are to a certain extent fixed (mode and ionisation energy) while the other parameters are variable and user adjustable. It must be stressed that the various adjustable settings will affect ionisation, nebulisation and sensitivity, and must be carefully selected and controlled.

Another factor that must be kept in mind is whether the data collected can be searched when added to a database. Strict control must be implemented over experimental conditions and detector settings when analysing standards that will be incorporated into searchable libraries.

The same is true for the analysis of the actual samples as deviation from the specified conditions and settings might either render data unsearchable or result in poor match qualities.

ZQ conditions:

TABLE 10.3 Optimised HPLC conditions for the separation of cardiac glycosides on the ZQ system

VARIABLE	OPTIMISED
Column	Phenomenex Synergi Fusion C18 250 x 2.1 mm (4 µm)
Column temperature (°C)	40 ± 1
Mobile phase components	Methanol H_2O (0.1% Formic acid pH 2.3)
Gradient profile	Initial conditions of 70:30 H_2O:MeOH Gradient to 40:60 H_2O:MeOH in 20 minutes (G3) Gradiet to 10:90 H_2O:MeOH at 40 minutes (G9) Isocratic conditions of 10:90 H_2O:MeOH for 5 minutes Return to initial conditions within 5 minutes (G3) Equilibrate for 10 minutes
Mobile phase flow rate	0.2 ml/min
Degassing	In-line (normal mode)
Sample temperature (°C)	5 ± 1

10.8.3 Chemical extraction of *B. volubilis* bulb

Fresh bulbs were purchased from the Soms Traditional Healer's market in Johannesburg, cut into slices and air-dried at room temperature for 48 hours. The bulb slices were blended to a pulp using an Ika Werk A10 water-cooled mill (Janke & Kunkel, Straufen, Germany) and the pulp dried at 40 ^{o}C to constant weight. Powdered pulp (5 g) was placed into a 50 ml polypropylene screw-top centrifuge sample tube. PBS-HCl buffer (pH 5) (30 ml) was added to the tube, capped and shaken on a Heidolph rotating sample mixer for thirty minutes. Oasis HLB SPE cartridges were prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used. After centrifugation of the centrifuge sample tube on a Hermle Z 300 centrifuge at 4000 rpm for 15 minutes, the aqueous phases were passed through the conditioned Oasis HLB cartridges. The cartridges were washed with 3 ml extracting buffer and dried under vacuum for 10 minutes. The cartridges were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml

formic acid per liter methanol), 3 ml basic methanol (10 ml ammonia per liter methanol) and 3 ml acetonitrile and the eluates combined into a single collection tube.

TABLE 10.4 Optimised ESI^+-MS conditions for the detection of bovoside A (ZQ system)

CONDITION	BOVOSIDE A
Mode and polarity	ESI positive
Capillary voltage (KV)	2.50
Cone voltage (V)	50
Desolvation temperature (°C)	500
Extractor voltage (V)	1.0
RF lens (V)	0.1
Source temperature (°C)	120
Cone gas flow (L/h)	0
Desolvation gas flow (L/h)	450
Ion Energy	1.1
Multiplier (V)	-650
Resolution (LMR & HMR)	15.2

The collection tube was placed into a Zymark Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residue was reconstituted with 1 ml of a 80:20 methanol: water mixture.

10.8.4 Purification of bovoside A

A portion of the powdered bulb (see §10.8.3) (20 g) was extracted with the acidic PBS-HCl buffer as described above but the aqueous phase was passed through a preparative Oasis HLB cartridge (20 ml/ 1g). The SPE cartridge was prepared by washing consecutively with 20 ml of methanol, water and the extracting buffer used. The cartridge was washed with 20 ml extracting buffer and dried under vacuum for 10 minutes. The cartridge was then eluted with 20 ml methanol, 20 ml acidic methanol (10 ml formic acid per liter methanol), 20 ml basic methanol (10 ml ammonia per liter methanol) and 20 ml acetonitrile and the eluates combined into one collection flask and evaporated to dryness at 40 °C under reduced pressure. The dry residue was reconstituted using the minimum amount of a 80:20 methanol: water mixture.

Bovoside A could be successfully separated from the sample matrix on the Phenomenex Synergi Fusion column, but the preparative fractionation had to be done on the only available

preparative column, an Xterra MS C18 column (300 x 7.8 mm). The optimised conditions of the Phenomenex Fusion column was tested on the Xterra MS C18 analytical column, but could not resolve bovoside A from the sample matrix. The analytical conditions for the Phenomenex Synergi Fusion analytical column were tested on a Waters Xterra MS C18 analytical column and adequate separation could not be obtained to fractionate bovoside A. The chromatographic conditions were adapted for the preparative fractionation and purification of bovoside A on the Waters Xterra MS C18 analytical column. The preparative conditions were obtained as described in §8.8.4 and calculated the preparative chromatographic conditions for the Waters Xterra MS C18 column (300 mm x 7.8 mm, 10 µm). The UV spectrum of the PDA detector was collected between 200 and 600 nm and a single wavelength at 300 nm was monitored to observe eluding bufadienolides.

10.9 RESULTS AND DISCUSSION

10.9.1 Chromatography and detection of bufadienolides

The chromatography and detection of cardiac glycosides have been discussed in §9.9.1. The chromatography of bufalin, a bufadienolide, was quite acceptable (Figure 10.4) but the ESI positive fragmentation (Figure 9.9) was atypical for cardiac glycosides with the formation of numerous adducts. The main adducts observed were $[M+H]^+$ *m/z* 388, $[M-H+Na]^+$ *m/z* 410 and $[M-H+Na+MeOH]^+$ *m/z* 442 (Figure 10.5). Under ESI negative conditions bufalin produced only the methanol adduct $[M-H+MeOH]^-$ *m/z* 418 (Figure 9.9).

10.9.2 Chemical extraction of the *B. volubilis* bulb

The extract derived from the bulb of *B. volubilis* was injected into the ZQ system and analysed with the previously optimised conditions. The chromatogram was quite complex but after the exclusion of compounds not displaying a bufadienolide-type UV spectrum, the chromatogram became relatively simple (Figure 10.6). The UV spectrum of the main compound was consistent with the typical bufadienolide-type UV spectrum (Figure 10.7) and the ESI positive spectrum (Figure 10.8) suggested that the compound might be bovoside A. The compound did not form numerous adducts like bufalin, but displayed the typical adducts observed for cardenolides, namely $[M+H]^+$ *m/z* 561 and $[M+Na]^+$ *m/z* 583.

10.9.3 Purification of bovoside A

Bovoside A could be successfully separated from the sample matrix on the Phenomenex Synergi Fusion column, but the analysis on the Xterra MS C18 analytical column did not resolve bovoside A from the sample matrix. The solvent composition was adjusted to separate the target compound successfully from the sample matrix. The scale-up of this method was done as described for ricinine in §8.8.4. The method however produced excessive back pressure at the

specified flow rate of 2.76 ml/min, and the method was adapted to lower the operating back pressure by replacing the methanol with acetonitrile. Although acetonitrile severely suppressed the ionisation of cardiac glycosides, it could be incorporated into the eluent as the PDA detector was used for the fractionation and not the ZQ detector. The optimised HPLC conditions for the fractionation of bovoside A is summarised in Table 10.5. As a precaution the temperature of the sample compartment was raised to 40 ^{o}C to prevent excessive precipitation of the crude sample.

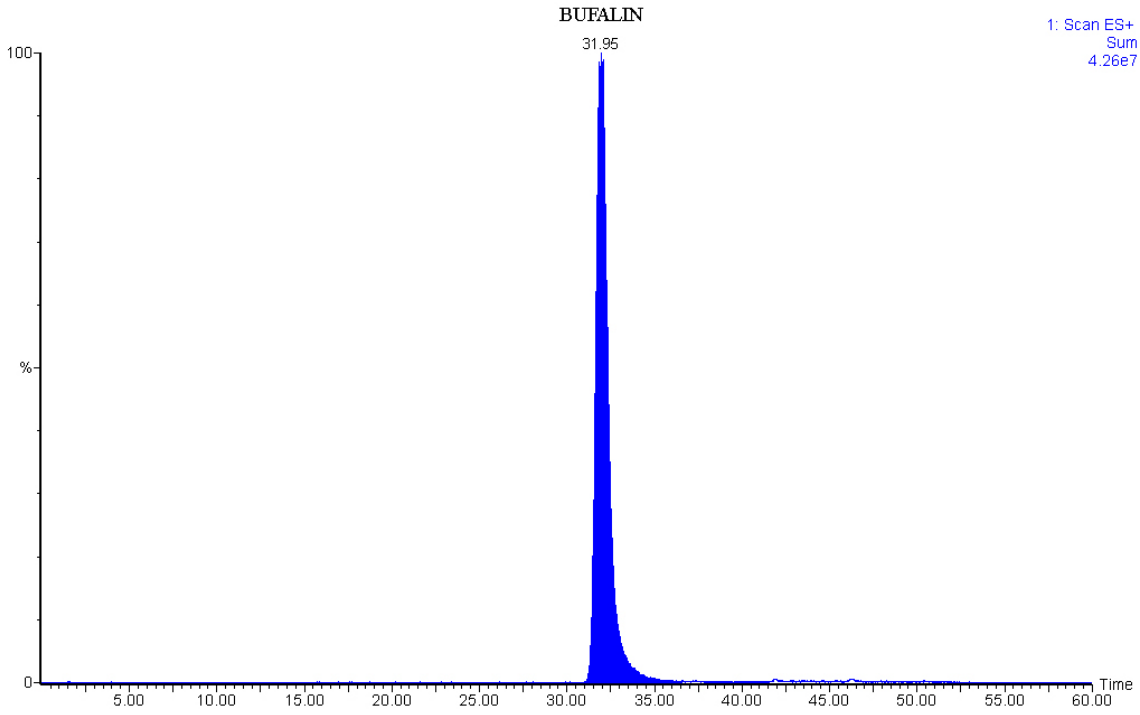


Figure 10.4

ESI^{+} chromatogram of a bufalin standard on the ZQ system

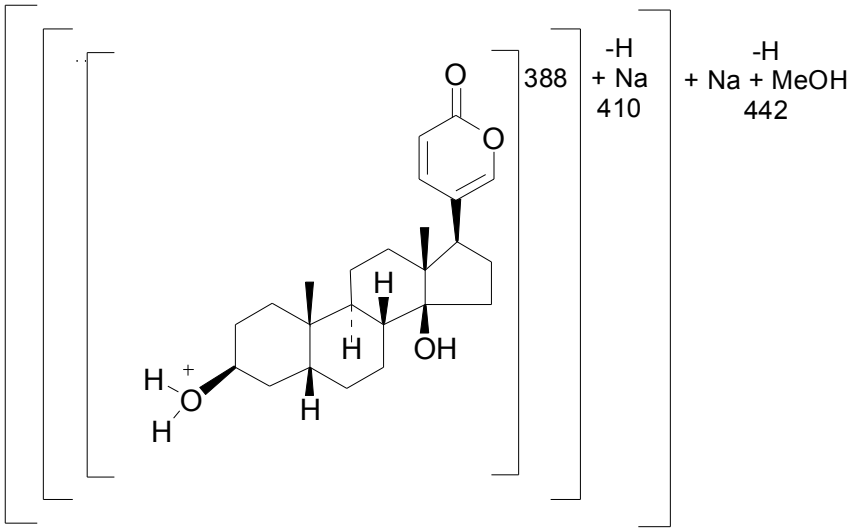


Figure 10.5

Adducts of bufalin under ESI^{+} conditions on the ZQ system

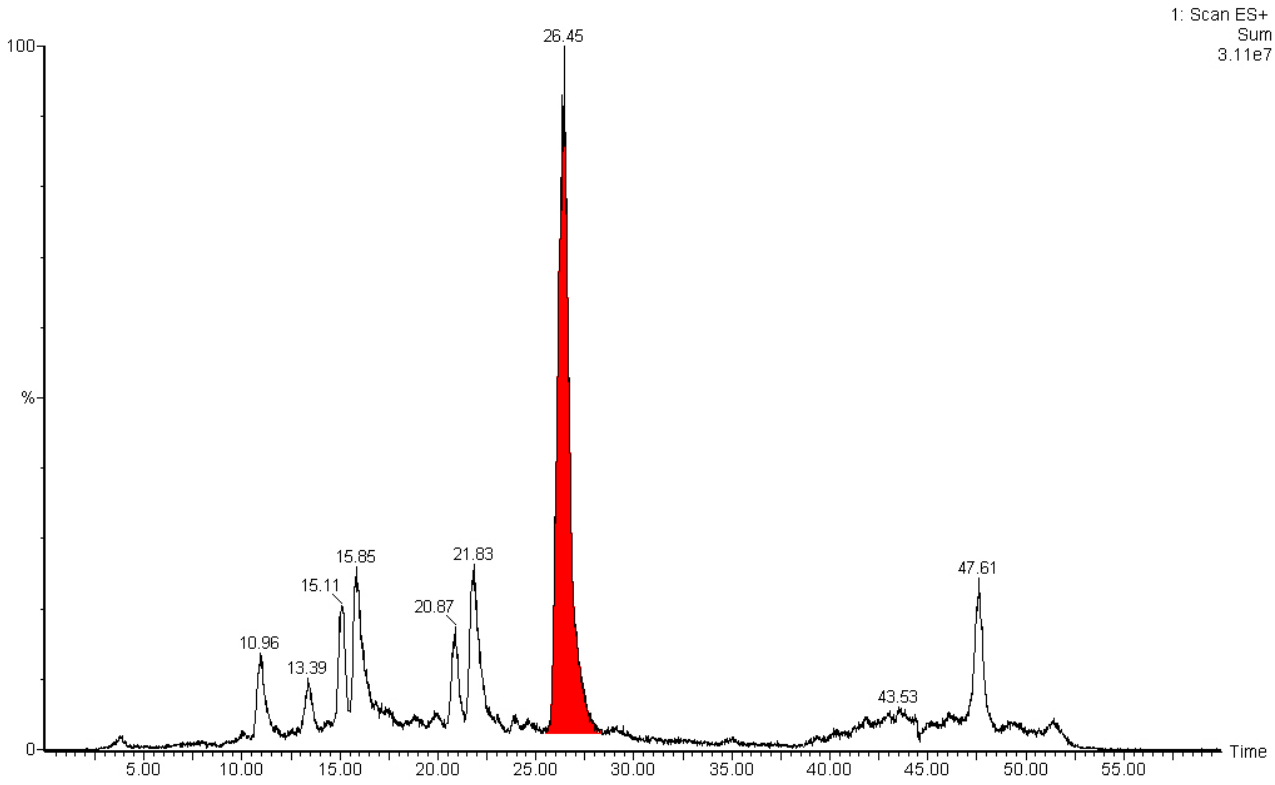


Figure 10.6

ESI$^+$ chromatogram (selected masses) of the bulb extract of *Bowiea volubilis* (ZQ system)

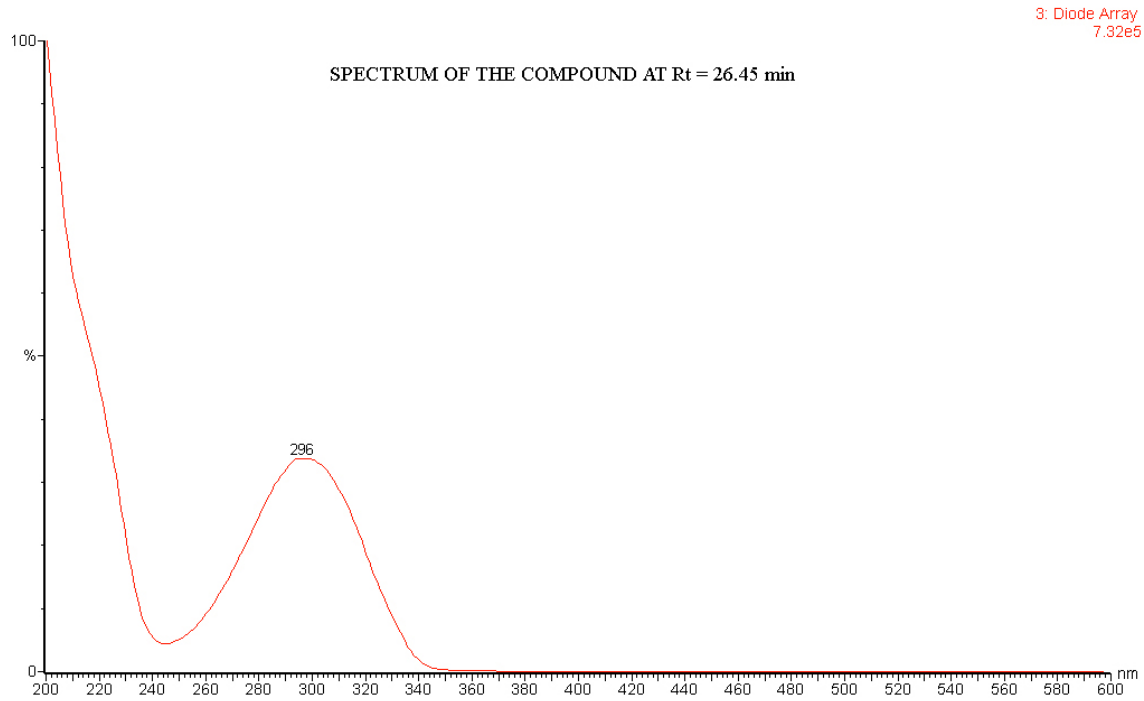


Figure 10.7

UV spectrum of the compound at R_t = 26.45 min (Figure 10.6)

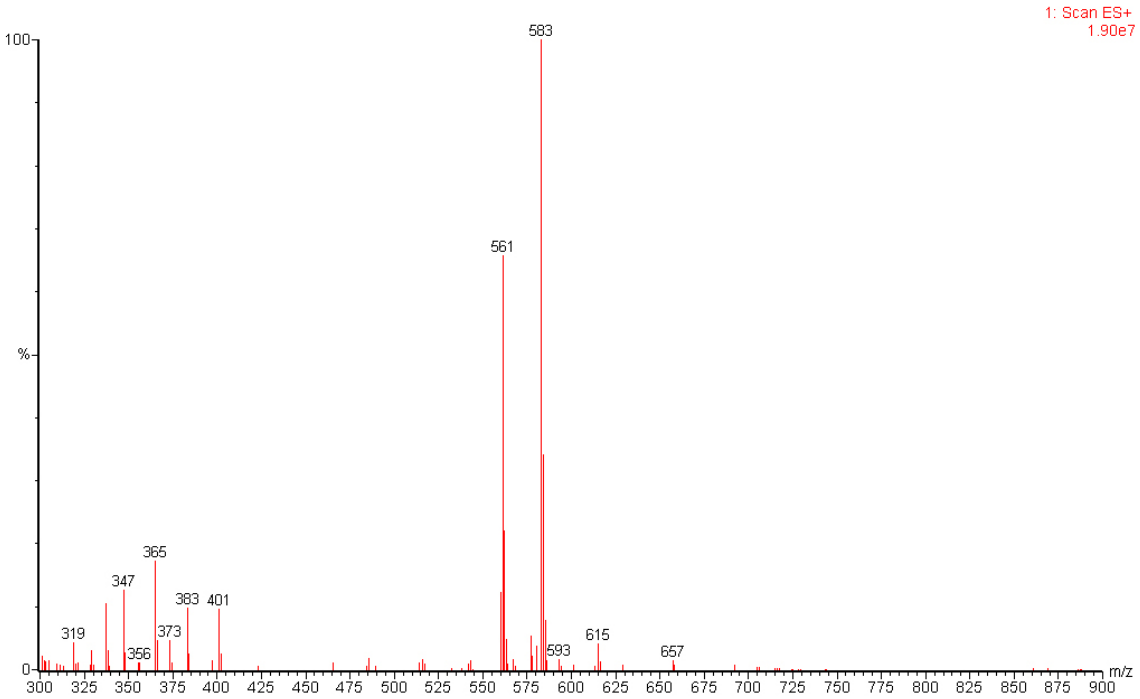


Figure 10.8

ESI^+ spectrum of the compound at R_t = 26.45 min (Figure 10.6)

The purified fraction was injected into the TMD system and displayed a major component at R_t 30.82 min on the PDA detector (Figure 10.9) and at R_t 31.42 min on the TMD detector (Figure 10.10). The UV spectrum was identical to that of the compound detected at R_t 26.45 min in the crude extract of *B. volubilis* (Figure 10.7).

TABLE 10.5 Optimised HPLC conditions for fractionating bovoside A

VARIABLE	OPTIMISED
Column	Xterra MS C18 300 x 7.8 mm (10 µm)
Column temperature (°C)	40 ± 1
Mobile phase components	Acetonitrile H_2O (0.1% Formic acid pH 2.3)
Gradient profile	Initial conditions of 70:30 H_2O:ACN Linear gradient to 40:60 H_2O:ACN in 20 minutes (G6) Gradient to 10:90 H_2O:ACN at 25 minutes (G3) Isocratic conditions of 10:90 H_2O:ACN for 2 minutes Return to initial conditions within 5 minutes (G3) Equilibrate for 8 minutes
Mobile phase flow rate	2.8 ml/min
Degassing	In-line (normal mode)
Sample temperature (°C)	40 ± 1

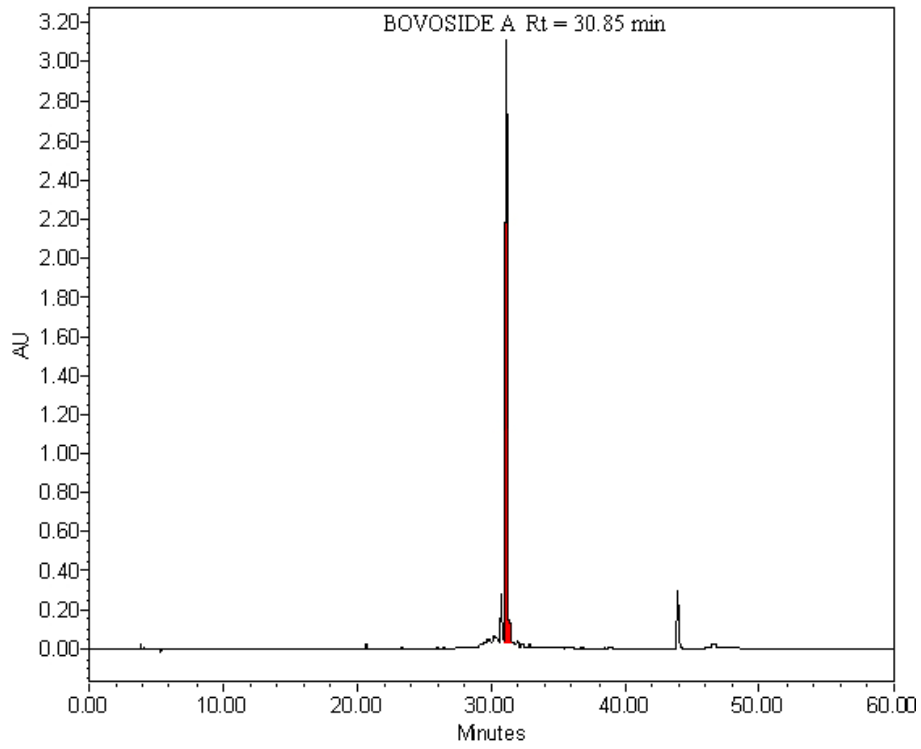


Figure 10.9

MaxPlot UV chromatogram of the purified bovoside A fraction on the TMD system

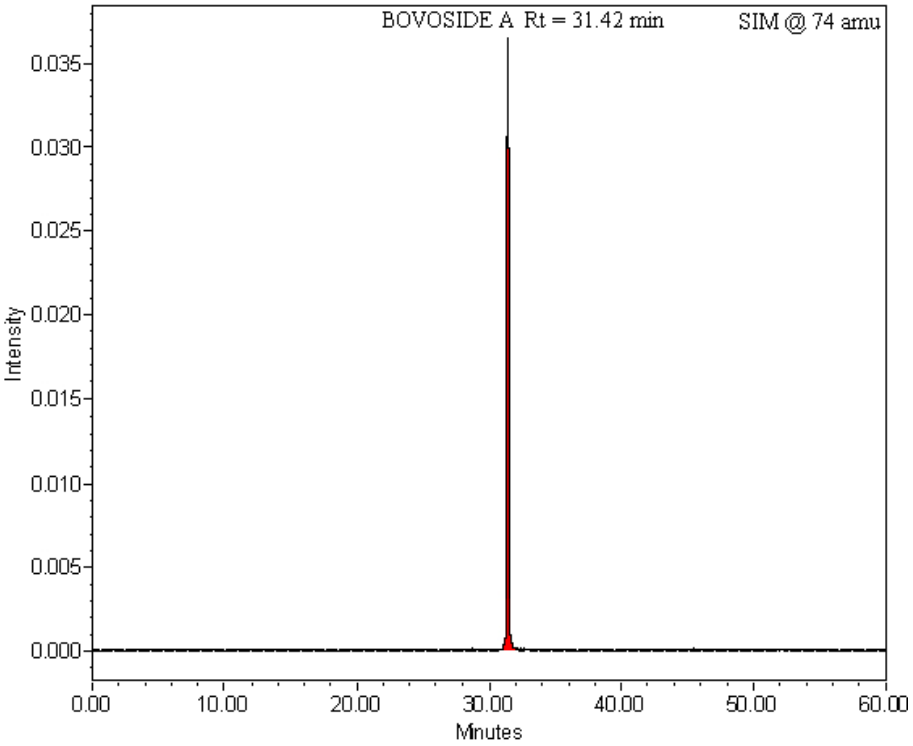


Figure 10.10

EI SIM chromatogram of the purified bovoside A fraction on the TMD system

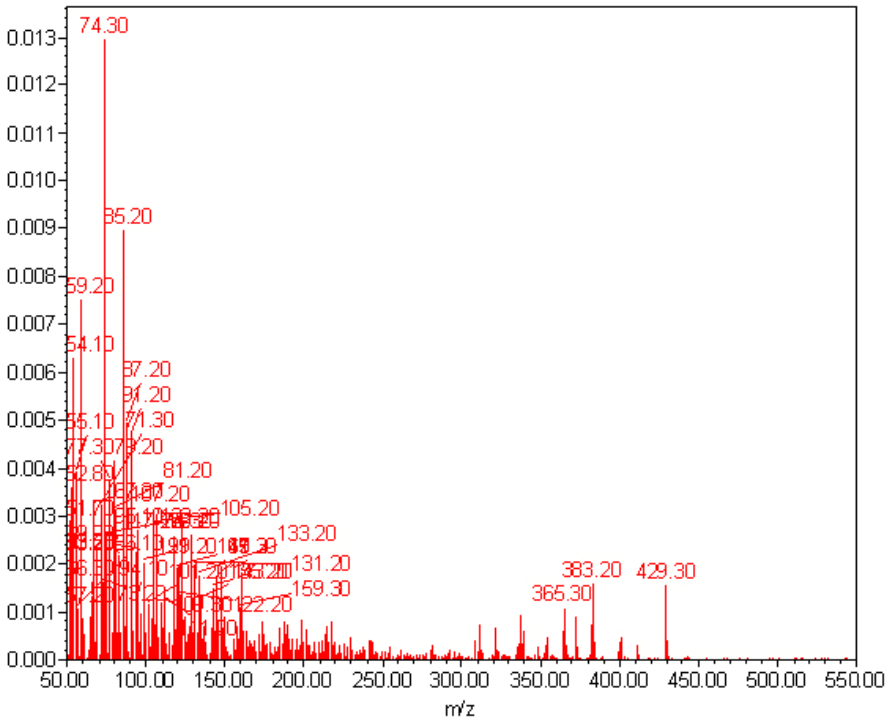


Figure 10.11

EI spectrum of the purified bovoside A fraction on the TMD system

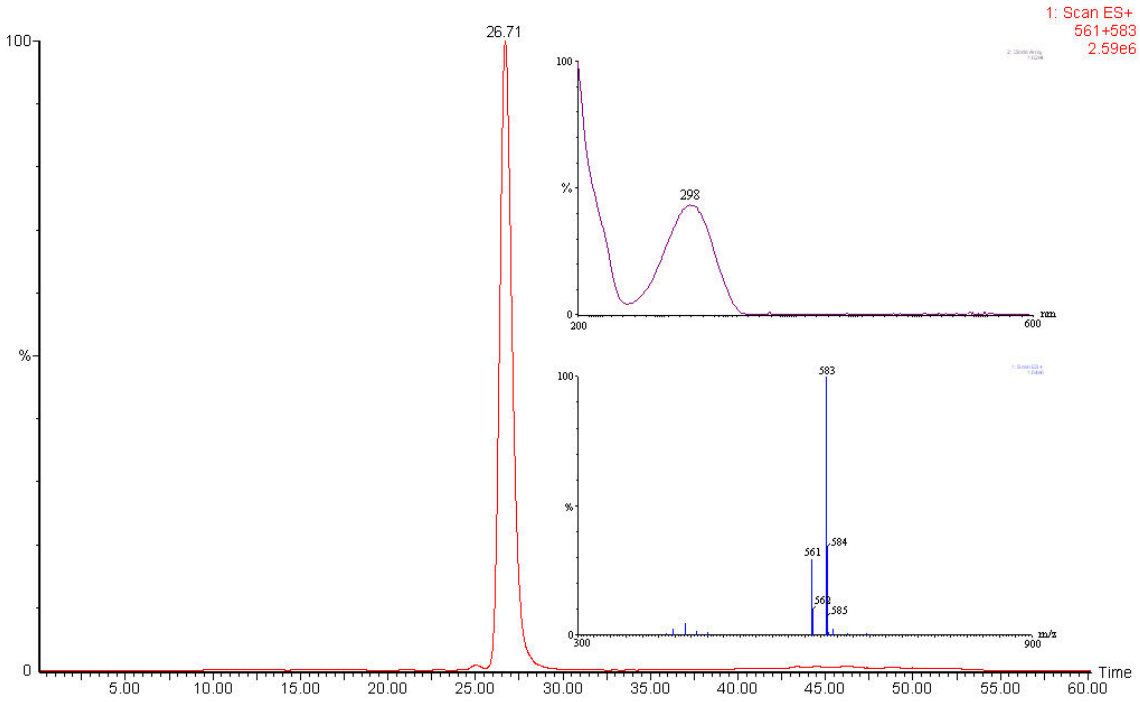


Figure 10.12

ESI$^+$ chromatogram of the purified bovoside A fraction on the ZQ detector.

Inserted the UV and ESI$^+$ spectra of the peak at R_t = 27.71 min

10.9.4 Identification of unknown plant extracts

The aim of any screening method is to distinguish unambiguously between different components without the need to re-analyse the samples on another method or detection system. This is also the case with a screening method for cardiac glycosides – it must be able to distinguish between the different chromatographic profiles of the plant extracts, but must also be able to confirm the presence or absence of the active compounds.

The crude extracts derived from the leaves of *Nerium oleander*, *Thevetia peruviana* and *Gomphocarpus fruticosus* and the bulb of *Bowiea volubilis* were diluted 1:1 and placed in sample vials marked Sample 1, Sample 2, Sample 3 and Sample 4 without keeping track of which extract was placed into which vial. The samples were analysed with the optimised screening method utilising full scan mode between 300 – 1100 Da. The TIC chromatograms were unusable due to high background signals but the BPI chromatograms could be compared with each other (Figure 10.13). The BPI chromatograms were still complex but certain marker peaks were identified that may be used to identify the individual samples. By monitoring the masses of the main compounds of *N. oleander* (oleandrin: 599 amu), *T. peruviana* (Thevetin A & B: 881 & 897 amu) and *B. volubilis* (bovoside A: 561 & 583 amu) the samples could be identified according to the retention time of the characteristic peaks Figure 10.14).

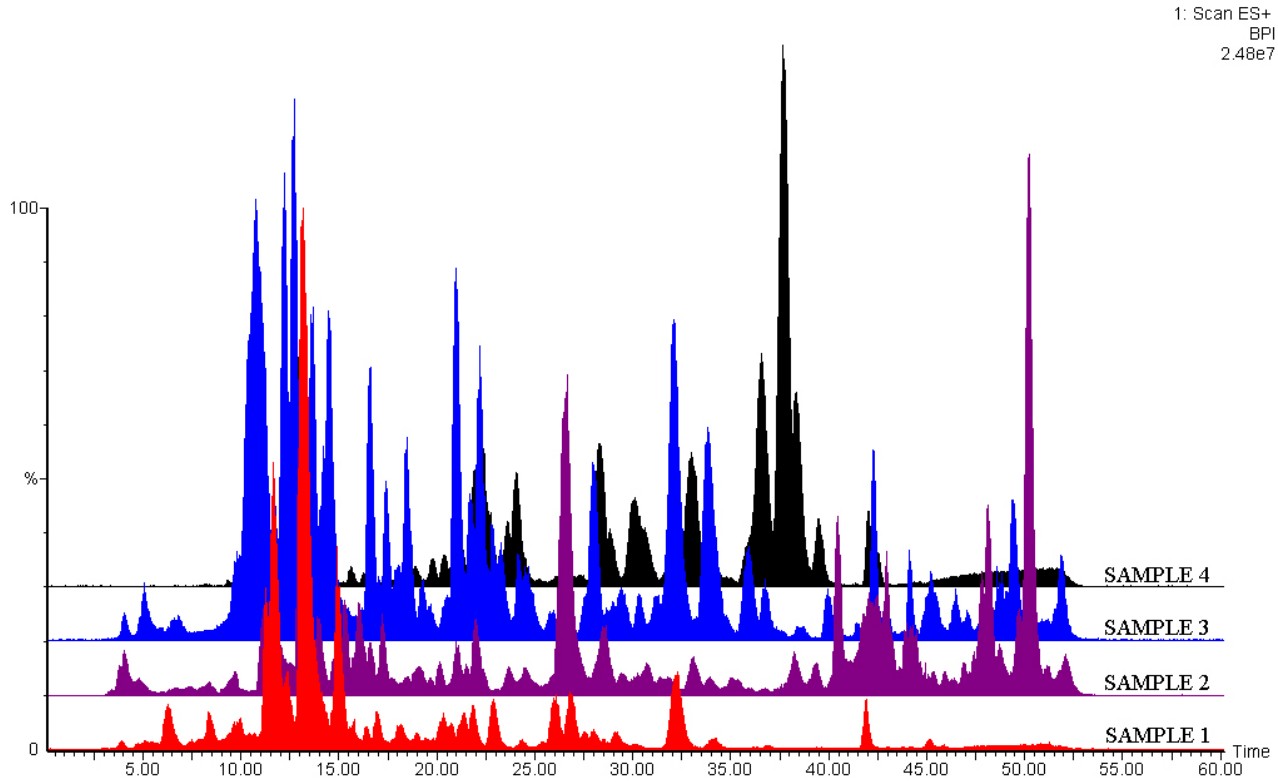


Figure 10.13

Comparison of the ESI$^+$ BPI chromatograms of four plant extracts (ZQ system)

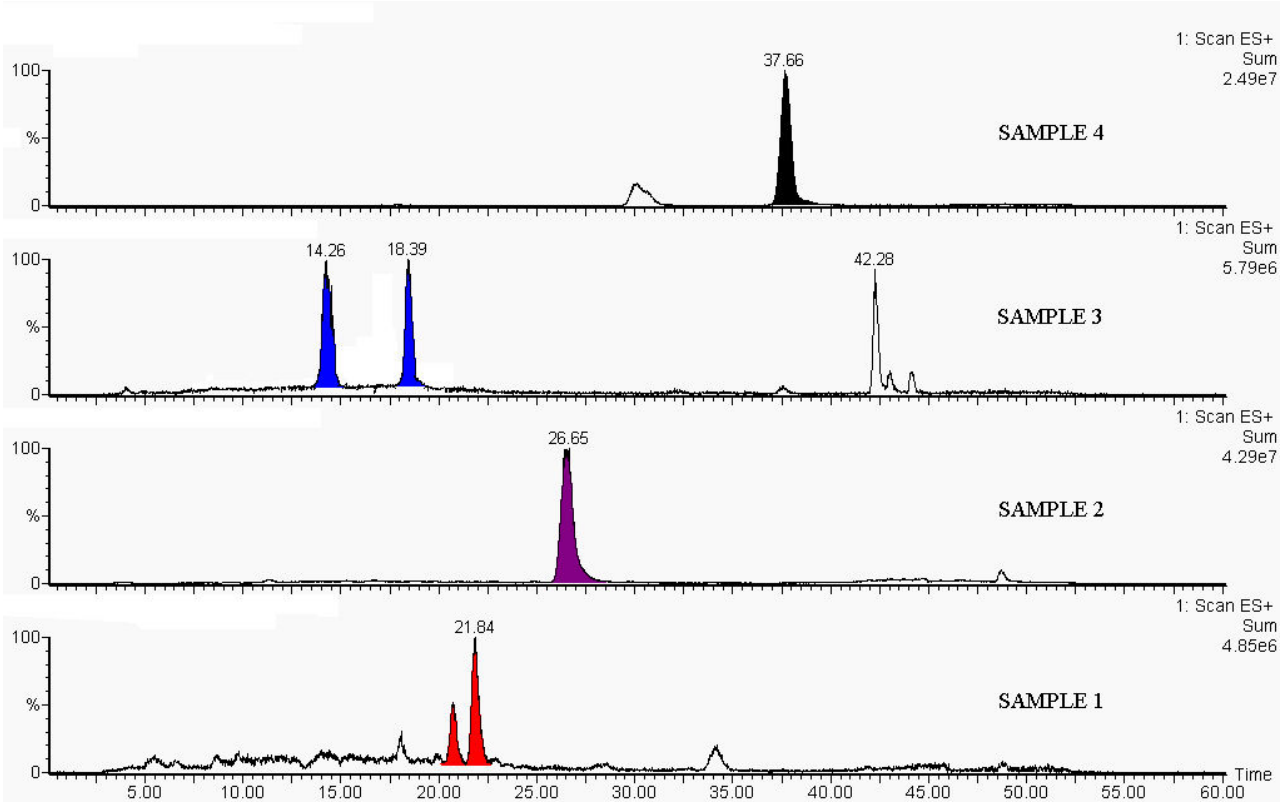


Figure 10.14

Comparison of the chromatograms of the four plant extracts by monitoring 561, 583, 599, 881 and 897 amu (ZQ system)

The identities were confirmed by evaluating the mass spectra of each compound. The identity of the samples was as follows:

Sample 4 : *Gomphocarpus fruticosus*
(Identified by a process of elimination)

Sample 3 : *Bowiea volubilis*
(Bovoside A: mass spectrum contained 561 & 583 amu)

Sample 2 : *Thevetia peruviana*
(Thevetin A & B: mass spectra contained 897 & 881 amu)

Sample 1 : *Nerium oleander*
(Oleandrin: mass spectrum contained 599 amu)

10.10 FORENSIC APPLICABILITY

The chromatographic method developed was capable of separating 11 cardiac glycosides and allowed mass spectral detection of all the compounds. Under these chromatographic conditions, stable cationisation was observed for all the compounds, and methanol adducts were formed with those cardiac glycosides that had a methoxy functional group on the glycoside moiety.

The method was successfully applied to the analysis of the bulb extract of *B. volubilis* and bovoside A was identified as the main bufadienolide present in the bulb. Bovoside A was fractionated and purified and the main peak observed in the extract had an ESI^+ mass spectrum that was consistent with the empirical formula reported in literature [40].

Four extracts of botanical origin could be successfully distinguished from each other by monitoring the main masses of bovoside A, oleandrin, thevetin A and B. These marker compounds were well separated from each other and made the identification of the botanical extracts quite easy, and the identity of each extract was confirmed by the mass spectrum of each peak.

This method is ideally suited as a secondary screening method to confirm or rule out the presence of cardiac glycosides tentatively identified on the TMD system (UV spectrum or NIST library match). In cases where plant material is submitted or the use of cardiac glycoside-containing plants are indicated, the extraction method must be used where after the analytical method can be applied as primary screening method.

The only disadvantage of this method is the lack of commercially available reference standards of the cardenolides and bufadienolides commonly found in the plants of southern Africa. The addition of accurate mass determination will ease the identification process, but the purification and characterisation of the active components might still be needed to identify new compounds unambiguously.

CONCLUSION

The Forensic Chemistry Laboratory of Johannesburg (FCL JHB) was established in 1926 with the sole purpose to supply a chemical analysis service to the customers of the Department of Health. The scope of analysis covers all the pharmaceutical products, all the agricultural products which include all pesticides, herbicides and fungicides, volatile gases (HCN and CO exposure), blood alcohol and herbal products. Most of these compounds received a fair amount of attention as to method development and adaptation of equipment for the optimal detection of the compounds of interest, but the herbal products were badly neglected. The reasons for this were multiple: standards were not commercially available, there was a lack of accurate information as to plants used, plant parts used, dosage, toxicicity and of suitable analytical equipment.

This situation existed for more than 70 years until the purchase of the first LC-MS system, the Waters TMD (EI), which proved very successful in identifying compounds separated by HPLC and even included some plant-derived compounds like alkaloids, flavonoids, and carotenoids. In 2001 I initiated this project to do research and method development in the field of poisonous medicinal plants used in *muti*. The use of LC-EI-MS and GC-EI-MS as initial screening tools proved to be very successful and were quickly adopted as the routine screening tools for the project. However, these systems lacked the specificity and, more importantly, the sensitivity needed to detect *muti* components in viscera.

The introduction of LC-ESI-MS to the analytical arsenal greatly expanded the capabilities not only to detect certain compounds below the detection limit of the EI-systems, but also to confirm these compounds by ISCID. Most of the developed methods functioned well in full scan mode, but if higher sensitivity was needed, the methods were adapted to SIM mode by using at least four significant ions. The SIM methods were specifically useful to monitor the ion clusters of the protonated molecules and to confirm these molecules by also monitoring the ion clusters of the ISCID product(s).

The developed methods were quite unique as no published methods could be found that where applicable in plant and viscera matrices on single quadrupole LC-MS or GC-MS systems. No suitable published methods could be found for the compounds of *B. disticha, A. coranica, C. laureola, A. precatorius, R. communis, T. peruviana* and *B. volubilis*. This research resulted in the publication of three articles in international journals while a fourth article is under review (see Appendix A1).

This study has elevated the analysis of lethal *muti* plants (responsible for most of the recorded human fatalities in South Africa) to a new level. The methods developed covers the main classes

of lethal plant poisons as well as the most commonly available and widely used poisonous *muti* plants. The approach and methods of extraction, detection and analysis developed during this study can be further expanded to cover a wider range of plant species for each of the main classes of compounds (alkaloids, cardiac glycosides and kaurenoid glucosides). In this way, plant-related forensic analysis in South Africa can become more sophisticated, with a higher success rate in terms of the number of cases solved.

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