Analysis of pyrrolizidine alkaloids in *Crotalaria* species by HPLC-MS/MS in order to evaluate related food health risks

GM Rösemann

A thesis submitted in fulfilment of the requirements for the degree of

Philosophiae Doctor

Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria

Promoter: Professor JN Eloff
Co-Promoter: Professor CJ Botha

DECLARATION

The work described in this thesis was conducted on a part time basis at FARMOVS-Paraxel, Bloemfontein between January 2004 and March 2005, and at Drs DuBuisson, Bruinette and Kramer, Pretoria, between April 2005 and December 2006.

I hereby declare that the data included in this thesis are the results of my investigations and that the thesis was written by me. References made to published literature have been duly acknowledged.

Magda Rösemann

30 December 2006

University of Pretoria

Abstract

Analysis of Pyrrolizidine Alkaloids in *Crotalaria* species by HPLC-MS/MS in order to evaluate related Food Health Risks

By Magda Rösemann

Promoter: Professor JN Eloff

Co-Promoter: Professor CJ Botha

Phytomedicine Programme

Department of Paraclinical Sciences

Pyrrolizidine alkaloids (PAs) are one of the most significant groups of plant toxins in the world and are an important cause of poisoning in livestock, resulting in significant financial and production losses each year (Kellerman *et al.* 1996). Pyrrolizidine alkaloids may also enter the human food chain as contaminants of grains, via animal products such as milk, eggs and honey or may be consumed as constituents of herbal medicines (ANZFA 2001).

Not all PAs are toxic. Pyrrolizidine alkaloids affecting human health are the esters of 1,2-unsaturated hydroxymethyl dehydropyrrolizidines (DHP). Before it can be converted to DHP, PAs need to have certain essential features, which include an unsaturated 3-pyrrole ring, one or two hydroxyl groups attached to the ring, one or two ester groups and a branched acid moiety (Mattocks 1986). These compounds can be metabolized in the liver to nucleophillic pyrroles which cause damage to hepatocytes (Winter and Segall 1989).

Although the involvement of PAs in the development of hepatic veno-occlusive disease is well established (Bras *et al.* 1961), there is still uncertainty concerning the consequences of long-term, low-dose exposure in humans. Exposure to PAs through the use of herbal remedies

may also be a contributing factor to the high rates of liver cancer and cirrhosis seen in Africa (Steenkamp *et al*. 2000).

Crotalaria spp. are known to contain toxic PAs and various incidences of human poisoning through contaminated grains have been recorded in the scientific literature (IPCS 1989). Legislation controlling the allowable levels of toxic seeds in grains in South Africa is generally much stricter than in many other grain producing countries. The Soybean and Sunflower Forum recently commissioned a study (Eloff *et al.* 2003) to review published and unpublished information on toxic seed that could affect human health in South Africa and to make recommendations accordingly.

Crotalaria sphaerocarpa is one of the problem plants discussed in the review and is apparently the only species which regularly contaminate grain in certain areas in South Africa. There is uncertainty at present about the number of these seeds that should be allowed in grains and the threat that this may pose to human health. Based on the review a provisional recommended level of 10 seeds of *C*. *sphaerocarpa* per 10 kg of grain was proposed as an approximated safe level in the report. As emphasized by the authors (Eloff *et al.* 2003), this absolute level is based on assumptions that must still be tested.

As a follow-up on the report, a sensitive LC-MS/MS method for the determination of toxic PAs in plants was developed in this study. The characteristic fragments produced by 1,2-unsaturated necine bases under specific MS/MS conditions were used to discriminate between the toxic and non-toxic PAs. The concentration of these PAs were then determined using multi-reaction-mode experiments. Quantitative results were calculated against a retrorsine calibration curve and expressed as µg retrorsine equivalents per gram plant material.

Various extraction methods described in the literature were investigated. A final liquid-liquid extraction method was used to extract unsaturated PAs from small amounts (about one gram) of milled plant samples. Recoveries from spiked lucerne samples were 98% for retrorsine and 105% for monocrotaline.

To determine the applicability of the LC-MS/MS method the unsaturated PA content of *C. laburnifolia* and *C. dura* were investigated. *Crotalaria laburnifolia,* which is regarded as non-toxic, contained low concentrations (< 20 $\mu g.g^{-1}$) of unsaturated PAs. *Crotalaria dura,* on the other hand, is known to be toxic to livestock and the concentration of unsaturated PAs was

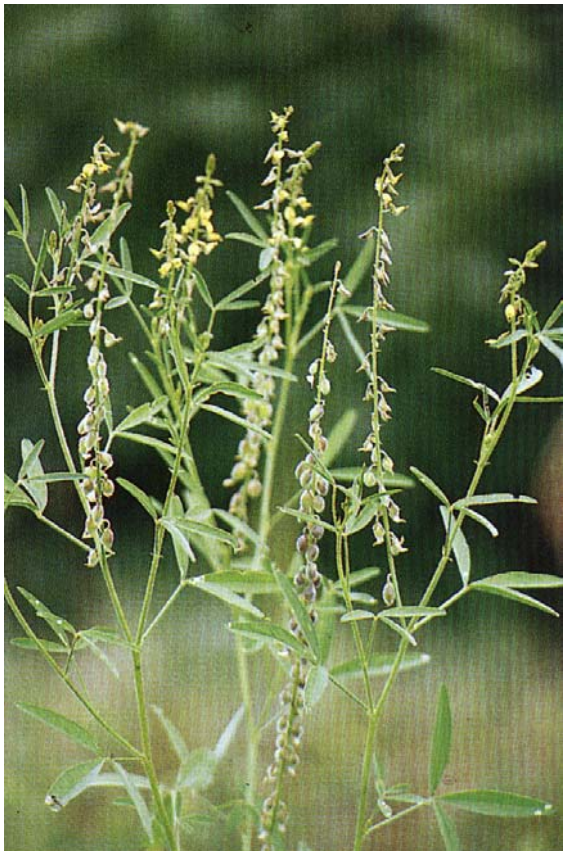
significantly higher (585 µg.g^{-1}). The toxic PA content of *Senecio inaequidens* was also determined after an incident of livestock poisoning. The plant material contained very high concentrations of retrorsine (11.5 mg.g^{-1}) and senecionine (0.5 mg.g^{-1}) which were also present in the rumen content collected post-motally. These results confirmed the suspected toxicity of *S. inaequidens.*

The LC-MS/MS method was also used to follow variations in unsaturated PA content in *C. sphaerocarpa* plants during the growing season. Pyrrolizidine alkaloids were present in the roots of the growing plants as *N*-oxides and also found in the mature aerial parts, where it was present mainly as the basic alkaloids.

The method was used to determine the concentration of unsaturated PAs, in various *C. sphaerocarpa* seeds from different locations, in order to calculate the allowable level of *C sphaerocarpa* seed in maize. Of all the seed samples analyzed, the highest unsaturated PA concentration found was 150 µg.g^{-1}. The allowable level of seed was calculated using this result and was found to be 656 seeds per 10 kg maize, based on the Australian and New Zealand Food Authority level of 0.1 µg.kg^{-1}.day^{-1}. If these results are confirmed with systematic statistical samples of *C. sphaerocarpa* seed from different grain production areas, the allowable level could be increased substantially. This may have an economic benefit to grain producers.

ACKNOWLEDGEMENTS

The author wishes to thank everyone involved in this project:
Prof. Kobus Eloff, Phytomedicine Programme, Onderstepoort, for identifying the project and for his support and encouragement.
Prof. Christo Botha, Faculty of Veterinary Science, Onderstepoort, for his support and for the *Senecio inaequidens* specimens.
Riaan Bruitendag, Bothaville, for providing *Crotalaria sphaerocarpa* plant specimens.
Prof. Theuns Naudé, Faculty of Veterinary Science, Onderstepoort for *C. laburnifolia* and *C. dura* samples.
Dr. Leentjie van Niekerk, AMPATH, and Dr. Ken Swart, Parexel, for the use of their LC-MS/MS instruments.



Crotalaria sphaerocarpa

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADI	Acceptable daily intake
amu	atomic mass unit
C.	*Crotalaria*
CI	Chemical ionization
CID	Collision induced dissociation
DHP	Dehydropyrrolizidine alkaloid
EI	Electron impact
ESI	Electrospray ionization
FMO	Flavin-containing monooxygenase
GC	Gas chromatography
GSH	Glutathione
HPLC	High performance liquid chromatography
LLE	Liquid-liquid extraction
LLOD	Lowest limit of detection
$[M]^+$	Molecular ion (equal to the molecular mass of the compound)
$[M+H]^+$	Pseudo-molecular ion (molecular mass + 1)
MRM	Multi reaction mode
MS	Mass spectrometer
m/z	Mass to charge ratio of the fragment
NMR	Nuclear magnetic resonance
NOAEL	No-observed-adverse-effect-level
PA	Pyrrolizidine alkaloid
PCI	Positive chemical ionization
RI	Retention indices
SCX	Strong cation exchange
SPE	Solid phase extraction
Spp.	Species
TIC	Total ion chromatogram
TLC	Thin layer chromatography
UV	Ultra violet

CHAPTER 1: LITERATURE REVIEW

1.1 Pyrrolizidine alkaloids

1.1.1 Introduction

Alkaloids are natural plant products that contain a heterocyclic nitrogen atom, are basic in character and are sometimes toxic to animals when eaten (Holstege *et al.* 1995). The biological role of alkaloids in plants is largely unknown, but is thought to have evolved as a feeding deterrent (Ober and Hartmann 1999). Direct evidence of this protective role in the plants is rare, but it is well known that many insect herbivores have developed various adaptations, even utilizing alkaloids for defense against predators (Boppre 1990; Lindigkeit *et al.* 1997).

Pyrrolizidine alkaloids are mainly present in the families Boraginaceae (many genera), Asteraceae (tribes Senecioneae and Eupatorieae), Orchidaceae (nine genera) and Fabaceae (mainly the genus *Crotalaria*) (Dharmananda 2002). More than 95% of the PA containing plants investigated thus far belonged to these four families (Ober and Hartmann 1999).

Pyrrolizidine alkaloids are a major cause of livestock losses each year and can also enter the human food chain as contaminants of grain. Contamination of grain is particularly likely to occur in parts of the world with arid climates and poor rainfall, which promote the growth of PA-containing plants, growing as weeds among cultivated crops. The first recorded instance of PA poisoning in humans was in 1920 in South Africa when many people in the Western Cape suffered from liver cirrhosis after eating bread made with wheat, probably contaminated with *Senecio burchellii* (Willmot and Robertson 1920). To date the largest reported outbreak of human intoxication by PAs was in Afghanistan in 1974 when an estimated 35 000 people were affected after grain was contaminated with *Heliotropium* plant material. Among 7 200 cases examined, 1 600 were affected and many died 3-9 months after the onset of clinical signs (Mohabbat *et al.* 1976).

Pyrrolizidine alkaloid contaminated animal products can also enter the human food chain and although these exposure incidences may not cause immediate toxic effects in humans, the effect of long-term, low-level, chronic exposure is still uncertain. The most frequently encountered source of residues is the milk of animals that have ingested PA-containing plants (Dickinson *et al.* 1976). Chickens can also transfer PAs to their eggs after eating contaminated grain (Edgar and Smith 2000) and honey has been found to contain high PA levels, up to 1 $mg.kg^{-1}$, causing a risk to those who consume large amounts of honey (Deinzer *et al.* 1977). Pyrrolizidine alkaloid residues are, however, unlikely to be present in meat from animals ingesting plants containing PAs, since the compounds are rapidly cleared from the tissues and slaughter would have to occur within a few hours after grazing on contaminated pastures (Mattocks *et al.* 1988).

1.1.2 Structures

Generally, PAs are esters of hydroxylated methyl pyrrolizidines, consisting of a necine base- and necic acid moiety. The necine base can either be 1,2-unsaturated or saturated. The unsaturated necine bases are further classified as two types; retronecine-type (or heliotridine-type, a 7(S)-isomer of 7(*R*)-retronecine) and otonecine-type alkaloids (Mattocks 1986). Pyrrolizidine alkaloid bases can also exist as *N*-oxides, which are often found together with the basic alkaloids in plants (Fig 1-1).

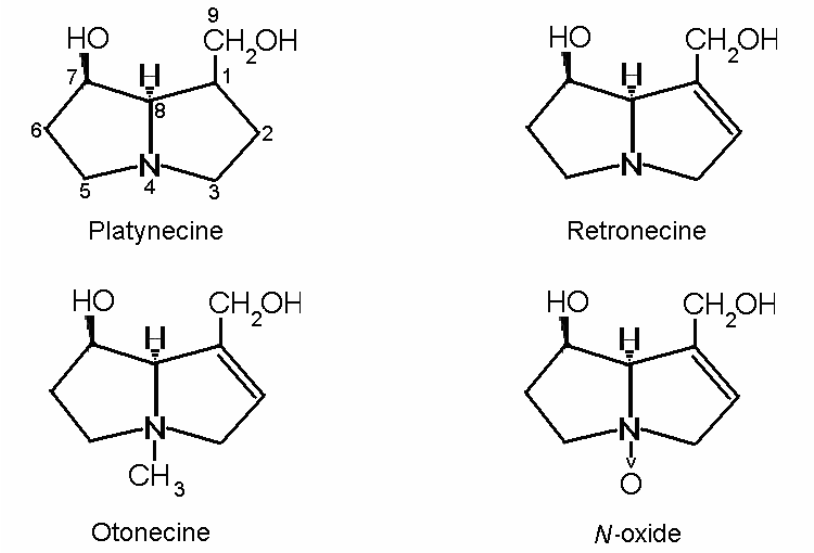


Figure 1-1: Typical structures of the different types of necine bases of PAs

Together with the *N*-oxides more than 640 pyrrolizidine alkaloid structures are possible, of which at least 350 types have already been found in nature and their structures elucidated (Röder 1995).

The acids with which the necines are esterified are called necic acids. Acid moieties often contain mono- or diester groups, with the diesters cyclic or acyclic (Fig 1-2) (Röder 1995).

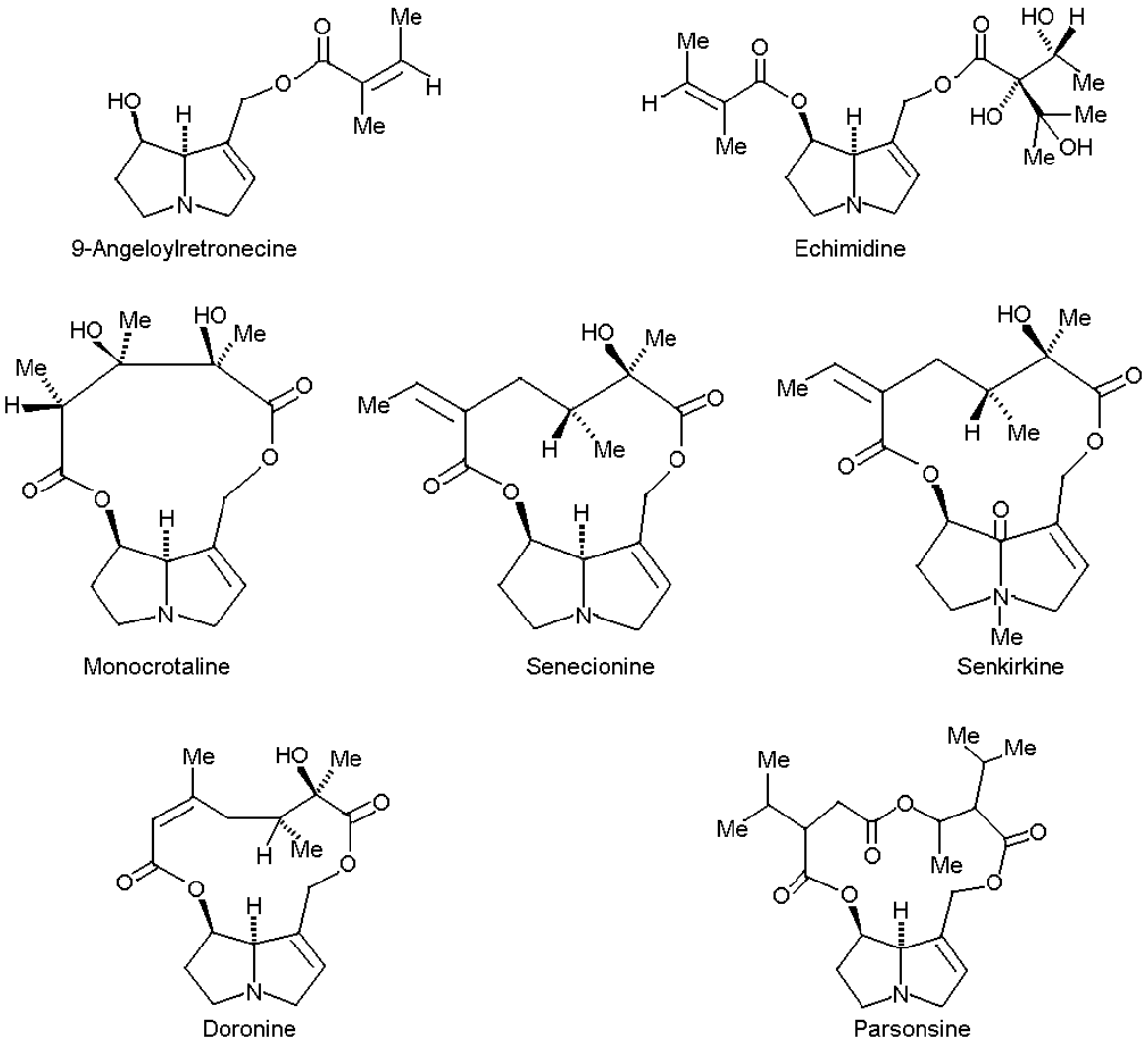


Figure 1-2: Unsaturated PAs bearing typical necic acid moieties

1.1.3 Synthesis

Pyrrolizidine alkaloids are synthesized in plants during amino acid metabolism. The necine base is derived from ornithine and arginine via putrescine and homospermidine metabolism while the acid portion is mainly derived from valine and leucine although acids from isoleucine and other compounds are known (Hartmann *et al.* 1988). Pyrrolizidine alkaloids are synthesized as *N*-oxides in the roots of most of the PA producing plants and are translocated to the aerial parts where they are converted into the species-specific alkaloids (Ober and Hartmann 1999). The conversion reactions include position-specific dehydrogenations, hydroxylations, epoxydations and *O*-acetylations. It is suggested that evolutionary variations in the activities of these enzymes, caused by mutations of the underlying genes, led to the variation in PA patterns between species (Hartmann 1997).

1.2 Absorption and biotransformation

1.2.1 Absorption

Information regarding the absorption of PAs originates from experimental work done by Swick *et al.* (1982). They measured the transfer of a mixture of PAs across isolated segments of the rabbit gastro-intestinal tract and found that PAs were transferred across ileum and jejunum, but not the stomach. Once absorbed from the small intestine PAs are transported to the liver where they are metabolized.

1.2.2 Liver activation

Saturated PAs are not converted to toxic metabolites and are quickly excreted in the urine without any adverse effects. Depending on the structure of the acid moiety, the parent alkaloids of many PAs are also chemically unreactive and much of the dose is excreted unchanged. The remainder of the PAs may become cytotoxic through its metabolism to pyrroles in the hepatic parenchymal cells (Prakash *et al.* 1999). The major metabolic routes of unsaturated PAs in the liver are (Fig 1-3): (a) hydrolysis of the ester group to the corresponding necine base, (b) oxidation of necine base to form the corresponding *N*-oxide and (c) dehydrogenation of the necine base to the corresponding didehydropyrrole. Other minor

routes are known, but these three pathways account for the major known effects of the alkaloids (Winter and Segall 1989).

1.2.2.1 Hydrolysis

Unsaturated PAs that are susceptible to hydrolysis (a in Fig 1-3) have low toxicities. These PAs are hydrolyzed by tissue esterase to highly water-soluble necine bases and acid moieties, which are readily excreted in urine within the first 24 hours.

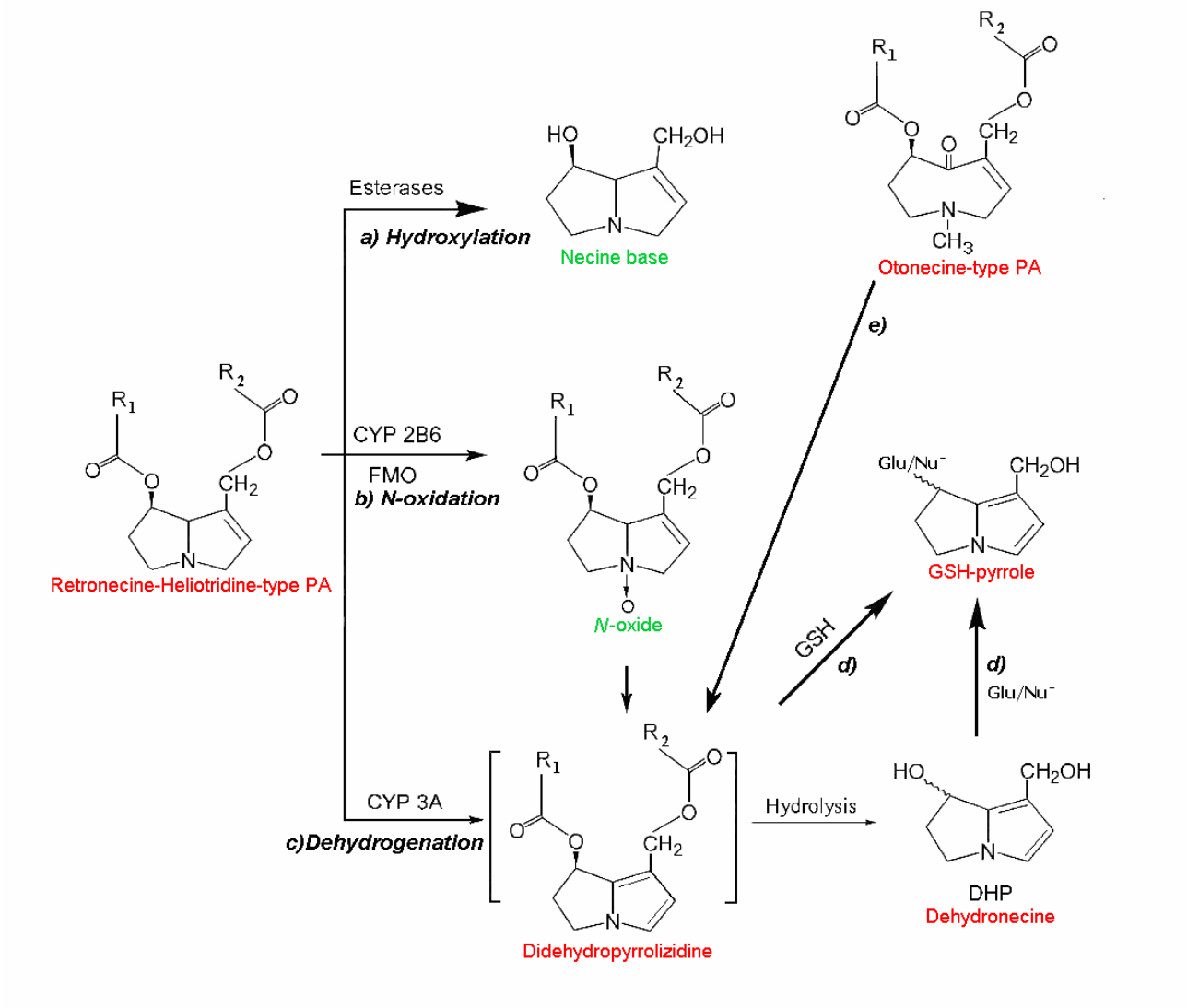


Figure 1-3: Metabolic pathway of 1,2-unsaturated PAs to toxic and non-toxic bases (adapted from Fu *et al.* 2002a)

The susceptibility of the PA molecule to hydrolysis is directly related to the structure of the acid moiety, with short and unbranched acid chain esters more easily hydrolyzed than those where

hydrolysis is sterically hindered (Mattocks 1992). Esterase activity towards monocrotaline is high in guinea pig liver and may account for guinea pig resistance towards monocrotaline poisoning (Deuker *et al.* 1992).

1.2.2.2 N-Oxidation

N-oxidation (b in Fig 1-3) of unsaturated PAs leads to the formation of non-toxic or less toxic *N*-oxides. The pathway is catalyzed by flavin-containing monooxygenase (FMO) enzymes and by cytochrome P450 FMO (Craig 2002). This pathway is also a detoxification route that produces highly water-soluble metabolites. *N*-oxides are often present in plants together with the basic alkaloids. There is no evidence that the *N*-oxides are toxic unless first converted to basic alkaloids by microbes in the gut of some animals (Mattocks 1986).

1.2.2.3 Dehydrogenation

Dehydrogenation by cytochrome P450 (c in Fig 1-3) leads to the formation of reactive dehydropyrrolizidines, which are the primary toxic metabolites responsible for acute toxicity (Castagnoli *et al.* 1997). Dehydropyrrolizidine alkaloids (DHP) may undergo further hydrolysis in the liver cells to form corresponding pyrrolic alcohols. These are also active alkylating agents, less reactive than the pyrrolic esters, but far more persistent and are referred to as the secondary toxic metabolites known to cause extensive extra-hepatic injury (Winter and Segall 1989).

1.2.2.4 Conjugation

Dehydropyrrolizidine alkaloids can in addition also react with glutathione (GSH) to form DHP-GSH conjugates (d in Fig 1-3) which are more water-soluble and subsequently excreted into the bile or sinusoidal blood and ultimately into the urine (Yan and Huxtable 1995). Alternatively the soluble GSH-pyrrole can serve as a transport vehicle to other organs such as the lungs in which toxicity can be elicited if the pyrrole is released from GSH (Cheeke 1989).

1.2.2.5 N-demethylation

Alkaloids of the otonecine-type PAs undergo *N*-demethylation with the eventual formation of a dehydropyrrolizidine alkaloid (e in Fig 1-3). Otonecine-type PAs can also be conjugated with GSH and are about 7 times less toxic than the corresponding necine, as it must first be *N*-demethylated, leading to a less effective conversion (Ge Lin 1998a).

1.2.3 Experimental evidence of liver activation

The relationship between liver activation and toxicity is well established in the literature e.g.:

- Pyrrolizidine alkaloids do not produce localized toxicity when applied to the skin or injected subcutaneously. The major site of PA metabolism is the liver and it is also the site of most damage (Schoental and Head 1955).
- Bull *et al.* (1968) injected a high dose of heliotrine into the tails of young rats. Heliotrine was present in the liver after two minutes and it was also the site with the most damage in the post-mortem specimens.
- Mattocks (1977) studied the distribution of a radioactive labeled PA analogue in rats. The highest concentration of radioactivity was seen in the liver (3.9%), spleen (0.27%) lungs (0.19%) and kidneys (0.18%). About 69% of the dose was excreted in the urine in the first day. The binding of radioactivity in the liver and lungs was more persistent than in the other organs.

Although the main site of damage is the liver, some PAs also affect other organs. There is no evidence that PAs are metabolized in tissue other than the liver, thus, damage to other organs is probably due to metabolites transported from the liver (Culvenor *et al.* 1976). To damage tissues other than the cells in which they are formed, active metabolites must cross the cell membranes and must not be metabolized while transported in the bloodstream. Estep *et al*. (1992) injected ^{14}C-labelled monocrotaline into rats and found high residual radioactivity in the red blood cells. They concluded that red blood cells may be the transport agent for hepatic-generated reactive metabolites to other organs.

1.3 Toxicity of pyrrolizidine alkaloids

1.3.1 Toxicity and structure

Primary toxic metabolites of PAs are highly electrophillic and react with nucleophillic constituents in the cell to exert their effect (Hincks *et al.* 1991). The structure of the individual unsaturated PA determines the metabolic route, which will in turn determine the toxicity of the metabolite, once formed (Fig 1-3). The rate of bio-activation also depends on substrate (PA) concentration and on the metabolizing enzyme activity of the host animal.

Among the PAs, cyclic diesters are the most toxic, with non-cyclic diesters of intermediate toxicity and the monoesters the least toxic. The amino alcohols are not toxic. The toxicity of the *N*-oxides, when first reduced to the basic alkaloid by bacteria in the gut, is of the same order as that of the basic alkaloid (Mattocks *et al.* 1988). *N*-oxides are, however, much more water-soluble and are subject to different pharmacokinetics when absorbed unchanged from the gut (see LD_{50} results listed in Table 1-1).

The steric orientation of the PA molecules and the degree of hydrophilicity appear to be the major factors governing the relative amounts metabolized through the different pathways. Factors preventing hydrolysis include a branching in proximity to the carbonyl groups and rigidity of the acid chain due to cyclic diester rings or unsaturation. Hydrophilic PAs are also more accessible to hepatic microsomal enzymes, which facilitate their conversion to pyrroles and *N*-oxides. The ratio of *N*-oxide to pyrrole varies depending on the type of ester and the enzyme activity of the animal (Winter and Segall 1989).

It is also possible that other metabolites, such as 4-hydroxy-2,3-unsaturated aldehydes may contribute to the acute hepatotoxicity of some PAs (Segall *et al*. 1985). However, this has still to be confirmed.

Table 1-1: Acute toxicity data for unsaturated pyrrolizidine alkaloids (adapted from Mattocks 1986)

Type	Alkaloid	Animal	LD_{50} mg/kg
Cyclic diesters:	Monocrotaline	Rat male	109
	Monocrotaline	Rat female	230
	Monocrotaline	Mouse female	259
	Retrorsine	Rat male	34
	Retrorsine	Rat female	153
	Retrorsine	Mouse female	69
	Retrorsine	Guinea pig	>800
	Senecionine	Rat male	50
	Senecionine	Mouse	64
	Integerrimine	Mouse	78
Non cyclic diesters:	Heliotrine	Rat female	478
	Heliotrine-*N* -oxide	Rat female	2500

1.3.2 Essential features for toxicity

Toxic PAs must firstly have a structure that can be converted to toxic metabolites, and secondly the animals` enzymes must be able to bring about the conversion. It became clear from extensive research done by Mattocks (1986) that PAs need to have certain essential features before they can be toxic. These are:

1. An unsaturated 1-2-pyrrole ring. The other ring is not essential for toxicity and can even be absent.
2. One/preferably two hydroxyl groups or substituted hydroxyl groups attached to the pyrrole ring via one carbon atom.
3. At least one of the hydroxyls must be esterified and diesters are more toxic than monoesters.
4. The acid moiety must have a branched chain.

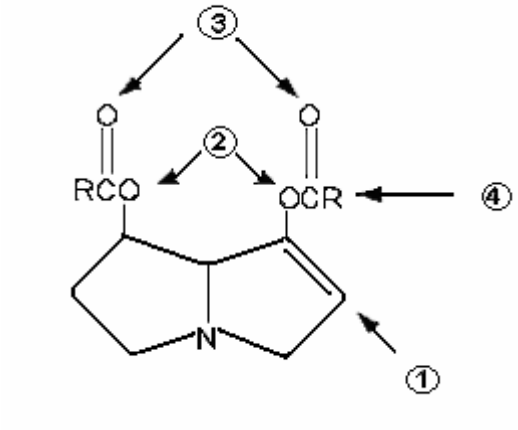


Figure 1-4: Essential structures for hepatotoxicity (Prakash *et al*. 1999)

1.3.3 Reactivity of active metabolite

The amount of didehydropyrrolizidines formed in tissues depends on the availability of substrate (PA) for activation, on the activities of cytochrome P450 enzymes (mainly members of the CYP3A and CYP2B6 subfamilies in the target site) and on the activation of detoxification pathways.

Dehydropyrrolizidine alkaloids are bi-functional intermediates with reactive electrophilic centers at C-7 and C-9 which can react with a variety of nucleophilic cellular macromolecules. Hydroxy-, mercapto- or amino groups of enzymes, globulins, haemoglobin and purine- or pyrimidine bases of DNA and RNA may function as nucleophiles (Fu *et al.* 2002a). The bi-functionality of the pyrrolic metabolites may cause cross-linkages in DNA and RNA, leading to modifications of genetic material (Thomas *et al*. 1998). These adducts may persist in tissues and generate chronic injury. The less stable metabolites are the most reactive and tend to react with nucleophiles close to the point of formation, while more stable metabolites can disperse more before exerting an effect. Thus, monocrotaline frequently damages lung tissue, whereas retrorsine, which yields more reactive pyrrolic metabolites, causes only liver damage (Mattocks 1992).

Detoxification of the pyrrolic metabolites is possible via different mechanisms (Fu *et al*. 2002a):

- Alkylation of PA metabolites with constituents such as glutathione which renders more polar products that are easily excreted in urine (d in Fig 1-3).
- Polymerization – it has been shown that metabolites that polymerase easily are also less toxic.
- Hydrolysis – pyrrolic alcohols, rather than DHP, are formed in an aqueous environment.

Monocrotaline was shown to arrest activation of cdc2 kinase in the target cells (Thomas *et al.* 1998). This inhibits the G2 phase of cell division, which is the normal checkpoint activated in the presence of DNA damage, allowing time for DNA repair. Progress to mitosis is therefore not possible, and this leads to the antimitotic affect seen in PA toxicosis.

1.3.4 Comparative responses

Animal species have different susceptibilities to the toxic effects of PAs. Horses and cattle are often poisoned after consuming moderate amounts of PA-containing plants e.g. consuming about 5% of their total body weight of *S. jacobaea* over time will cause death in cattle. Sheep and goats can consume quantities up to 300% of their body weight without showing any adverse effects (Craig 2002). These differences are partly due to variations in effectiveness of liver enzyme conversion of PAs to toxic metabolites (Prakash *et al.* 1999). The resistance of sheep and goats to PA poisoning has also been linked to detoxification by ruminal microorganisms (Craig 1995). On the other hand, differences in resistance may, however, also

be due to the balance between the formation of toxic pyrrolic metabolites and the detoxification pathway producing non-toxic *N*-oxides and DHP-GSH conjugates (Yan and Huxtable 1995).

1.4 Clinical signs and pathology of toxicosis in livestock

1.4.1 Peracute mortality

High doses of PAs can cause rapid death - within minutes of ingestion. This type of peracute toxicity is not due to cytotoxic actions, but is associated with pharmacological actions and death may be preceded by convulsions or by coma. The acute toxicity for some PAs, e.g. supine cannot be determined due to rapid mortalities (IPCS 1989).

1.4.2 Acute, subacute and chronic toxicity

The rat LD_{50} of most of the alkaloids known to be significant for human health is in the range of 34-300 $mg.kg^{-1}$ (Mattocks 1986). Factors such as sex, age and nutritional status play a role in susceptibility of the individuals.

Pyrroles act preferentially on the hepatocytes and endothelium of blood vessels during the acute phase (Prakash *et al.* 1999). In the hepatocytes the immediate action is a rapid fall in cytoplasmic protein synthesis, reaching 30% of control levels after 15 minutes (Harris *et al.* 1969). This is manifested as disaggregation of polyribosomes, and is followed by failure of pyruvate oxidation, loss of glycogen, structural damage to the mitochondria, lysosomal activity, failure of mitochondrial NAD systems and nuclear NAD synthesis, and finally necrosis (Mattocks *et al.* 1986). Some of the toxic metabolites escape and damage the endothelium of central veins, causing cell proliferation and veno-occlusive disease. More stable metabolites may once again escape via the blood stream and induce damage in other organs, especially the lungs.

Consequently, typical hepatotoxic lesions of PA toxicosis are swelling of hepatocytes, centrilobular necrosis, megalocytosis of the parenchymal cells, fibrosis, bile duct proliferation, veno-occlusion with consequently loss of liver function. These effects are mainly due to the alkylation of pyrroles with DNA, causing impaired cell division (Chojkier 2003). In longer

surviving cases the liver becomes hard, fibrotic and shrunken with subsequent signs of liver failure such as hyperbilirubinaemia, jaundice, hypoalbuminaemia, oedema and ascites.

In acute toxicity studies using laboratory animals, death commonly ensues 1- 4 days after a fatal dose. The liver is firm, congested, with deep-red, granular appearance and ascitic fluid is often present. In addition to necrosis, the sinusoids may become dilated with blood, causing compression of surrounding hepatocytes. Veins are occluded by cells of uncertain origin which may become replaced by fibrous tissue contributing to loss of function (Prakash *et al.* 1999).

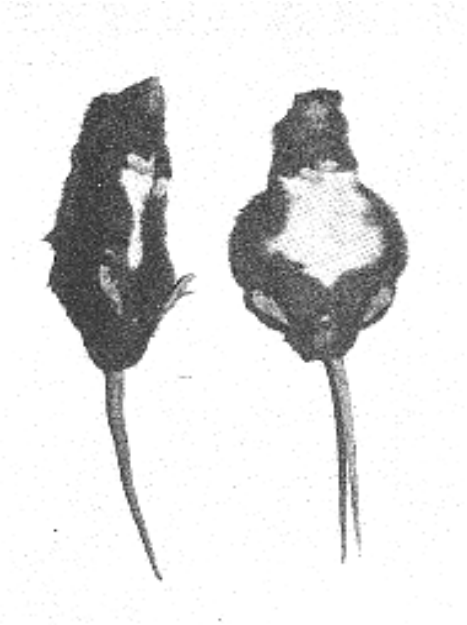


Figure 1-5: Ascites in a rat (right) due to PA intoxication, compared to a control rat (left) (Cheeke 1989)

With livestock poisonings the toxic effects of PAs are often delayed as the signs of poisoning become apparent some time after the animals have eaten PAs containing plants. In acute poisoning, death occurs within about seven days, due to severe liver damage and clinical signs include jaundice, wasting and sometimes photosensitization (Cheeke 1989).

A special feature of chronic PA-induced pathological changes is that after low level single/multiple exposure and well after the alkaloid and its soluble metabolites have been eliminated from the body, the disease is commonly progressive. Liver failure may occur suddenly, months to years after the last episode of PA exposure (Schoental and Magee 1959). It has been shown that the binding of pyrrolic metabolites to various nucleophiles is reversible,

suggesting that this constitutes a reservoir of secondary metabolites from which the active molecules could be released in a continuing or intermittent manner and maintain the progress of cell damage (ANZFA 2001).

Liver lesions associated with chronic PA toxicity in laboratory animals include the proliferation of bile ducts, various degrees of fibrosis and diffuse infiltration of the parenchyma with cells of unknown origins. Many of these changes persist after exposure to PAs is stopped and the liver never returns to normal even though the animal may appear to be in good health. The progression of chronic liver lesions is the same, whether the animal received a single sub-lethal dose, or a succession of smaller doses. Post-necrotic fibrosis is present in all the laboratory animals by day 10 with progressive enlargement of the parenchymal cells (Schoental and Magee 1959).

Ascites is often attributed to low serum albumin levels (oncotic pressure) (Cheeke 1989). Elevated serum billirubin concentration is normally only present in terminal stages and elevated activities of liver enzymes are transient i.e. increase only when actual tissue necrosis occurs. Aspartate aminotransferase can sometimes be an indicator of *Crotalaria* poisoning in livestock (Sippel 1964). Craig (2002) found that gamma glutamyltransferase increases and remains high during low level, chronic exposure. Bile ducts may account for almost half the weight of the liver in the terminal stages of chronic toxicosis. Other clinical signs include a rough hair coat, diarrhoea, prolapsed rectum, dullness, photosensitization and abnormal behavior (Cheeke 1989).

1.4.3 Other clinical effects

Certain PAs, e.g. monocrotaline, produce veno-occlusive disease of the liver as well as a sequence of changes in the lungs and heart that result in pulmonary arterial hypertension and right ventricular hypertrophy (Shubat and Huxtable 1992). Early changes in the lung include alveolar oedema and haemorrhage, causing progressive proliferation of alveolar walls and pulmonary hypertension.

Chronic lung lesions have been produced by most PAs that produce chronic liver lesions, though higher doses were required in some instances (Culvenor *et al.* 1976). Shubat and Huxtable (1992) gave various low doses of monocrotaline to rats over different time periods. They found that the threshold for monocrotaline toxicity is a function of the cumulative dose

received (>14mg.kg^{-1}) and is independent of the period over which it was administered. This study provides evidence for the cumulative effects due to chronic or intermittent consumption of PA contaminated products.

In South Africa many *Crotalaria* species cause “jaagsiekte” in equidae (Kellerman *et al.* 1988). This chronic respiratory disease in horses and mules is characterized by fever, polypnoea, dyspnoea, pulmonary emphysema, pneumonia and sometimes fibrosis or cirrhosis of the liver. Small amounts of the plant must be ingested for many weeks for “jaagsiekte” to develop. Respiratory lesions have also been reported in cattle poisoned by *C. spartioides* and sheep drenched with *C. dura*. The neurological abnormalities sometimes seen in horses (head-pressing) are attributed to the elevated blood ammonia levels, associated with hepatic encephalopathy (Cheeke 1989).

In sheep the consumption of PA-containing plants often leads to excessive liver copper concentrations, followed by a haemolytic crisis associated with chronic copper poisoning. This condition also occurs in horses, rats and rabbits. Increased copper levels are attributed to aberrations in copper absorption, copper-binding proteins or decreased excretion.

PA intoxication also affects iron metabolism and may cause decreased haematopoiesis due to an inability to incorporate iron into red blood cells. The spleen is greatly enlarged in PA poisoning, causing deposition of haemosiderin in various tissues (Anon. 1997).

PA intoxication also affects vitamin A metabolism, leading to an overall reduction of vitamin A levels. This is most likely due to suppression of synthesis by the liver of retinol binding protein (Cheeke1989).

There are also reports of kidney damage due to PA poisoning in animals, mostly attributed to monocrotaline (Hooper and Scalan 1977).

1.4.4 Clinical effects in humans

The reversibility of liver damage following PA exposure is unpredictable in man. It is reported that following an outbreak of acute PA intoxication, some fifty percent of patients will recover completely and about twenty percent will die rapidly. Some of the survivors will appear to

recover clinically, but may go on to develop cirrhosis and liver failure year's later (Stuart and Bras 1957).

Pyrrolizidine alkaloid poisonings in humans presents with nausea, acute upper-gastric pain, fever and elevated liver enzymes. This may lead to abdominal distention with prominent dilated veins on the abdomen (Fig 1-6). Mortality is usually high in the acute phase due to hepatic failure.

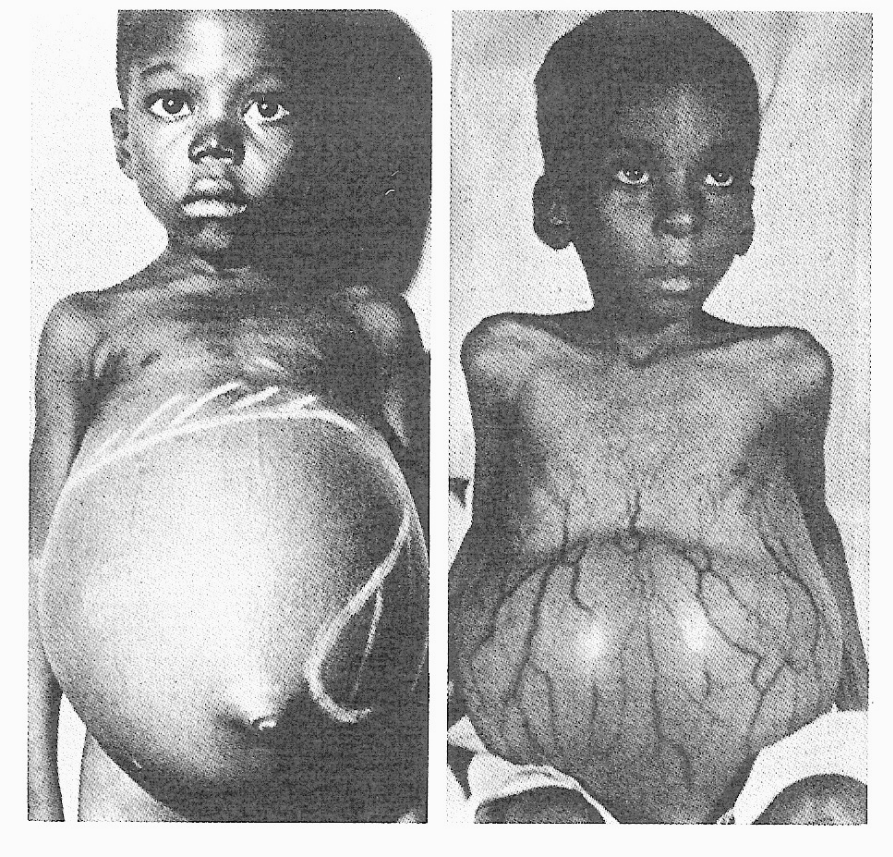


Figure 1-6: Ascites associated with veno-occlusive disease in the West Indies in infants 5 and 7 years old (Huxtable 1989)

Chronic toxic effects in humans are difficult to detect and usually require long-term epidemiological studies. There are no substantial long-term follow-up data to assess whether exposure to PAs resulted in increased incidence of chronic liver disease or cancer in populations where poisonings have occurred (IPCS 1989). With chronic ingestion of small amounts of PAs, or in survivors of acute toxicity, the disease proceeds through fibrosis to cirrhosis, that is indistinguishable from cirrhosis of other causes (Dharmananda 2002). An

unexpected finding in humans is the absence of megalocytes, which is a striking feature of chronic PA poisoning in animals. Removal of PA exposure will usually alleviate the disorder, but liver cirrhosis is not readily reversible. No incidents of primary pulmonary hypertension due to PA poisoning have yet been reported in humans (IPCS 1989).

Veno-occlusive disease is the most common cause of cirrhosis in infants in Jamaica (Bras *et al.* 1961) and the use of PA-containing plants as herbal teas is believed to be a significant aetiological factor. Chronic, low-level exposure of humans to PAs is likely to increase as alternative herbal remedies become more popular. It has been suggested that consumption of PA-containing medicinal herbs might contribute to the high incidence of chronic liver disease and primary liver cancer in Asia and Africa, especially as it may act synergistically with aflatoxins and the hepatitis B virus (Steenkamp *et al*, 2000). The risk of toxic effects due to these PAs may be particularly high in children as they are more susceptible to the effects of PAs, yet are more likely to recover than adults (Stegelmeier *et al.* 1999).

1.4.5 Carcinogenicity and teratogenicity

To date, PAs have been shown to cause cancer only in rodents and there is no conclusive evidence for malignant neoplasia in humans (IARC 1976). Epidemiological studies on survivors of large-scale human intoxications have not indicated any abnormal incidence of cancer and there are also no reports of cancer in domestic animals caused by exposure to PAs in their diet (Prakash *et al.* 1999).

Cancer due to ingestion of large quantities of PAs has, however, been reported in various laboratory experiments. Some examples are:

- Monocrotaline, lasiocarpine and heliotridine had a high mutagenic activity in the *Drosophila melanogaster* assay (Clark, 1959).
- Schoental and Bensted (1963) demonstrated that rats receiving a single large dose of PA may develop chronic liver disease and finally hepatocellular carcinoma more than 13 months after receiving the initial dose.
- More recent evidence presented by the National Toxicology Program showed that some PAs can be carcinogenic. Mice and rats were dosed with riddelline (up to 3 $mg.kg^{-1}$) by gavage for a period of up to two years. The study indicated that riddelline caused liver neoplasms and leukemia in rats and lung neoplasms in female mice (Anon 2003b).

- Fu *et al*. (2002b) lists 15 PAs known to induce tumors in experimental animals, all of which are also present in plants commonly used as Chinese herbal medicines.

In addition, teratogenic effects were produced at very high doses e.g. 50–200 mg.kg^{-1} via intraperitoneal injection of heliotrine in pregnant rats (Röder 1995).

1.5 *Crotalaria* species (rattle pods)

1.5.1 Introduction

Over 600 *Crotalaria* species are known, of which many were used as soil enrichers in the early development of farming lands all over the world (Damron and Jacob 1998). Over 300 *Crotalaria* spp. occur naturally in Africa (Arnold and De Wet 1993) and about 80 indigenous species of *Crotalaria* have been recorded in South Africa (Bromilow 1996). Mattocks (1986) list 43 South African *Crotalaria* species with unsaturated PAs including *C. juncea, C. dura, C. globifera* and *C. spartioides.*

Crotalaria spartioides (duinebos) appears to be the most hepatotoxic *Crotalaria* in SA and is regularly associated with liver failure in cattle. In 1969 a farmer used *C. spartioides* to supplement the diet of about 40 cows. Nineteen of the cows died due to fibrosis or cirrhosis of the liver (Kellerman *et al*. 1988).

Crotalaria burkeana, C. barkae, possibly *C. rhodesiae, C. steudneri* and other annual species result in transient laminitis, colloquially known as ‘stywesiekte’ in cattle and equines (Naudé *et al.* 1992). Due to the difference in clinical presentation this disease may be due to other toxins that have not yet been identified.

Feeding experiments with *C. dura* and *C. globifera* caused “jaagsiekte”, a respiratory disease, in horses at high doses (90 -180 g per day for 116 days) (Kellerman *et al*. 1988). A similar disease was induced in sheep after drenching with *C. dura* (Steyn and De Kock 1932). Marais (1944) extracted large quantities of dicrotaline from both *C. dura* (0.27%) and *C. globifera* (0.18%).

The PA content of *Crotalaria* species from other parts of the world is also known: *C. grahamiana* and *C. pallida* often cause poisoning of horses on Easter Island. The PAs found

in these plants are grahamine (2800 µg.g^{-1}), monocrotaline (100 µg.g^{-1}) and an agrahamine analogue (40 µg.g^{-1}) in *C. grahamiana.* A retrorsine analogue (13 µg.g^{-1}) and a senecionine analogue (2 µg.g^{-1}) are found in *C. pallida* (Anon. 1999).

Crotalaria spectabilis consumption by laying hens has been reported to cause a rapid decrease in egg production, with increased mortality. Feeding trials demonstrated that the adverse effect level was between 0.01 – 0.1% of the diet. Concentrations exceeding 0.3 % were fatal within 18 days (Damron 2001).

Crotalaria spectabilis and *C. retusa* contain retronecine and turneforcidine as well as monocrotaline, spectabiline and retusine (Damron and Jacob 1998). Alkaloids of trichodesmine and senecionine were identified in *C. juncea* after accidental poisoning with herbal medicine in Ecuador (IPCS 1989). Crotananine and cronaburmine were isolated from *C. nana* after grain contamination in India (Tandon *et al.* 1976). Monocrotaline and fulvine were isolated from *C. fulva* after herbal poisoning in the West Indies (Mattocks *et al.* 1988). The two main *Crotalaria* alkaloids involved in human toxicity in Australia are cronburmine and cronaburmine from *C. nana* (ANZFA 2001).

A total of 49 plant species used in Chinese herbal medicine are known to contain PAs (Fu *et al*. 2002b). It includes five *Crotalaria* spp. namely *C. albida, C. assamica, C. mucronata, C. sesseliflora* and *C. tetragona.* These species are known to contain monocrotaline and/or retrorsine.

Other *Crotalaria* species, used as medicines, have also caused toxicity in East Africa *(C. brevidens, C. incan, C. laburnifolia, C. mucronata, C. recta, C. retusa),* in Jamaica *(C. brevidens),* Asia *(C. laburnifolia, C. retusa)* and Sri Lanka *(C. verrucosa)* (Huxtable 1989).

Huxtable (1989) lists various *Crotalaria* species used as medicinal herbs in the developing world. They include *C. fulva* and *C. spectabilis* used as medicinal teas in Jamaica, *C. retusa* as herbal tea and as vegetable in Barbados, *C. laburnoides* used as herbs in Tanzania and *C. juncea* used as herbal tea in Ecuador. He suggests that PA poisoning should be considered in these countries in all human cases presenting with veno-occlusive disease.

Two epidemics of *C. nana* poisoning via contaminated grain have occurred in Mahya Pradesh in India in the 1970's, of 67 cases studied 28 died. There was, however, a poor correlation between the contamination level of the grain eaten by the affected households and the presence of veno-occlusive disease in the affected individuals (Huxtable 1989).

1.5.2 *Crotalaria sphaerocarpa*

Several of the *Crotalaria* spp. are minor weeds, but only *C. sphaerocarpa* (maize crotalaria; mielie crotalaria) poses a serious threat as it grows in close association with grain (Fig 1-7). *Crotalaria sphaerocarpa* is difficult to control as it germinates over the whole season; deep germinating plants are generally difficult to control with pre-emergence herbicides and the plant is tolerant to most post-emergence herbicides once it is past the seedling stage. The plants grow to a height of up to 1.5 m and produce large numbers of seeds (Fig 1-8).

Figure 1-7: *Crotalaria sphaerocarpa* growing between maize in the Bothaville district

Figure 1-8: Large numbers of seeds are produced by *C. sphaerocarpa*

Seed dimensions (2 x 3 mm) should allow easy removal by normal sieving, although the seedpods (3-4 x 4-6 mm) containing two seeds may not be removed that easily (Fig 1-9). Contaminated grain is downgraded due to the alleged toxicity of the seed (Eloff *et al*. 2003). *Crotalaria sphaerocarpa* is included in the poisonous plant database of the US Food and Drug Administration, Center for Food Safety & Applied Nutrition Data as a poisonous plant (Anon. 2003a). All the references used for this listing, however, originate from South Africa, the latest being Kellerman *et al.* (1988).

Figure 1-9: Seed dimensions of *C. sphaerocarpa*

Various *Crotalaria* spp. e.g. *C. spectabilis, C. retusa, C. sagittalis* and *C. mucronata* are listed by Beasley (1999) as hepatotoxic plants in the USA, but *C. sphaerocarpa* is not included in this list. Other poisonous plant databases e.g. GRIN and AGRICOLA lists *C. sphaerocarpa* as native to Africa and it is unclear whether this plant occurs in countries outside of Africa. The IPCS report (Mattocks *et al.*1988) lists plants known to contain hepatotoxic alkaloids as well as the alkaloids isolated from each source. Although the list was updated in 1988, *C. sphaerocarpa* was also not included in this list.

In the 1969 Annual Report of Onderstepoort, feeding experiments with *C. sphaerocarpa* revealed that large amounts were required to induce histological liver lesions in cattle. The investigators concluded that the risks in practice were insignificant (Anon. 1969). In an unrelated more recent study in neighbouring Mozambique, Faftine and coworkers (2000) studied forage selected by cattle on communal land over a two year period. Although *C. sphaerocarpa* was present and probably grazed by cattle, no adverse effects were reported.

Crotalaria sphaerocarpa growing in South Africa has apparently not yet been chemically analyzed and no evidence of acute or chronic toxicity has yet been found (Eloff *et al*. 2003).

1.6 Risks

1.6.1 Introduction

The risk of chemical contaminants in food should reflect the toxicity of the chemical compound as well as the potential daily intake in a normal diet. While the hazardous nature of a compound may be well known, the risk it poses to public life may be negligible if the quantity in a normal diet is below the threshold of toxicity for that substance. The exception is carcinogenic substances, which should be reduced to as low as reasonably achievable (Anon. 1995).

Determination of acute toxicity through establishing LD_{50} levels are not generally performed any more. This has to a large extent been replaced by the no-observed-adverse-effect-level (NOAEL); the highest dose administered to the animal that does not produce any adverse effects. An additional safety factor of 10 is normally added to compensate for inter-individual

variations. This is again multiplied by 10 to compensate for inter-species variations with the extrapolation to man. Once the NOAEL has been determined, an acceptable daily intake (ADI) can be calculated.

1.6.2 Grain contamination

Outbreaks of PA toxicosis typically occur when large numbers of people ingest contaminated food grains and develop veno-occlusive disease. Estimates of PA intakes during these epidemics are approximate and the amounts ingested as well as the toxicities of the different alkaloids vary considerably. Analytical methods are not standardized and it is difficult to compare results between laboratories from different parts of the world.

According to the ANZFA report (2001) levels of PAs found in various grains in Australia have ranged between <50 – 6000 $\mu g.kg^{-1}$. There has, however, never been a systematic analysis of grains entering the food supply.

Altee *et al.* (1998) reported on an episode of PA poisoning in 1994 with *Senecio* contaminated wheat in Mosul, Northern Iraq. During this outbreak 14 people were hospitalized, and two died. The other 12 patients recovered and were symptom free at a 12 month follow-up examination. Seeds were identified as *S. vulgaris.* The seeds gave a positive Ehrlich's test for PAs, but quantitative analysis was not performed.

The estimated PA intake during some *Crotalaria* outbreaks has been calculated (IPCS 1989). In an outbreak in India, millet contaminated with *C. nana* seed had an average PA content of 0.5 $g.kg^{-1}$ and the estimated daily intake by the population was 0.66 $mg.kg^{-1}$ body weight, sustained for approximately two months before disease became apparent. The PA content of wheat in an outbreak in Afghanistan, due to *Heliotropium popovii* seed contamination was 0.04 $g.kg^{-1}$. The estimated daily intake in this case was 0.03 $mg.kg^{-1}$ body weight and was sustained for approximately six months before diagnosis of veno-occlusive disease (Chauvin *et al*. 1994).

No reports could be traced on estimations of chronic exposures through grain contamination over longer periods.

1.6.3 Indirect sources

The exposure to the same toxicant in different food commodities would result in a possible market-basket effect. Related toxins or other toxins present may additively or synergistically increase toxicity. Systematic analysis of the exposure levels of PAs entering the human food chain has never been done and due to the lack of available data, it is not possible to estimate the potential dietary exposure of the general population to PAs (ANFZA 2001).

Pyrrolizidine alkaloids have been found in a variety of foods. Alkaloid levels of up to 1 $mg.kg^{-1}$ have been recorded in honey from certain hives in Australia (Culvenor *et al.* 1981). No reports of poisoning through contaminated honey could be found and it is assumed that blending and bulking reduced these PA level to non-toxic ranges.

Edgar and Smith (2000) determined PA quantities in eggs after contamination of wheat, used in chicken feed, with *H. europaeum* seeds. The PA levels in contaminated eggs ranged from 5 -168 $\mu g.kg^{-1}$ after feed grain was contaminated. The total PA concentration in the seeds was 26 $\mu g.g^{-1}$ and contained heliotrine, europine and lasiocarpine. The same PAs as well as their secondary metabolites were present in the eggs. The highest concentration of PAs in eggs was 38 µg per egg.

Pyrrolizidine alkaloids have been detected in human breast milk during PA poisoning epidemics, causing veno-occlusive disease in babies (ANFZA 2001). De Medeiros *et al.* (1999) fed *C. spectabilis* seeds to a lactating dairy goat. Milk from the goat was given to growing rats over an eight week period. The seeds had no clinical effect on the goat as goats are known to be relatively resistant to PA poisonings. The rats developed interstitial pneumonia, as well as liver and kidney damage, indicating that the monocrotaline or toxic metabolites were excreted in the milk.

In cows, experimental exposure to *S. jacobaea* led to high levels (470 – 835 $\mu g.L^{-1}$) of PAs in milk (Dickinson *et al.* 1976), but it had no adverse affects when fed to calves. It appears from this study that only the more water soluble metabolites are excreted in milk, which will directly affect the final toxicity of the milk consumed.

Although PA levels in milk, honey and eggs are often higher than the recommended safety levels, no evidence of chronic health problems in humans associated with these edible products could be found.

1.6.4 Herbal or medicinal consumption

Apart from accidental poisonings there is also increasing concern about the use of plants containing PAs in herbal medicinal preparations.

Dharmananda (2002) published an extensive review of herbal intoxications, which led to liver damage and even death in humans. The dose and duration were estimated in some of these cases e.g. for heliotrine a dose of 4-10 mg.kg^{-1} per day for 3 to 7 weeks led to veno-occlusive disease. In another case, a combination of crotanine and cronaburmine at less than 1 mg per day for several months led to the same disorder. Liver necrosis was caused by retrorsine and riddelline consumption at 0.7-1.5 mg.kg^{-1} per day for 2 weeks. From these cases the toxic dose range in humans appears to be between 0.1-10 mg PA.kg^{-1} per day.

In South Africa many *Senecio* and *Crotalaria* species are sometimes used to prepare traditional herbal medicines. Steenkamp *et al* (2000) identified 20 children at two South African hospitals suffering from veno-occlusive disease after administration of traditional remedies. In four of the cases a simple colourimetric screening test confirmed the presence of PAs in on-admission urine samples. PA poisoning by traditional medicines may be a huge problem in South Africa as these cases are often not diagnosed or reported.

1.6.5 Other factors to consider

Little research has been conducted on the effect that environmental factors have on PA concentration in plants. It is known that in the Asteraceae, biosynthesis of PAs is strictly controlled by root growth, and production ceases when root growth stops (Ober and Hartmann 1999).

Hol and co-workers (2003) investigated the effect of environment on the PA content in *S. jacobaea* plants when grown in a climate chamber. They reported that increasing nutrient levels led to a significant reduction in the PA concentration of the roots and shoots of the plant, but that the total amount of PA produced in the plant was not affected. They concluded that

change in biomass, rather than production rate, was the reason for the changes induced by nutrients.

Crotalaria juncea, known to contain toxic PAs in Equador, is regarded as a useful fodder plant in Asia and is often fed to cattle without any adverse effects (Srungbomee and Makasame, 1981). It may be possible that the PA content in a specific plant may be entirely different when growing in different areas.

Pyrrolizidine alkaloids can also be changed by heat or by enzymatic action when harvested plants are stored (Bull *et al.* 1968). Changes were also observed during the drying process in the ratios of *N*-oxide to the free base. However, the stability of the unsaturated pyrrolizidine alkaloids and *N*-oxides at high temperatures, for example, during cooking, is not known.

Other toxins may be present in plants containing PAs. In one case of *C. mucronata* poisoning of sheep in Australia, animals started dying within the first day due to acute lung damage (Laws 1968). The rapidity of onset and the atypical lung lesions found in this case suggest that toxins other than PAs were causing the mortalities (IPCS 1989).

Although evidence is still conflicting, health risks in some population groups may also be higher. Factors such as age, gender, nutritional status, as well as other synergistic effects need to be incorporated into future NOAEL.

1.7 Regulation criteria

1.7.1 Aim of regulation

Because of their known involvement in human poisoning and their potential carcinogenicity, exposure to PAs should be kept as low as practically achievable. Prevention of exposure is the only effective method of limiting toxicity and the setting of regulatory tolerance levels for certain grains may be the best method to prevent exposure.

Specific information is needed before NOAEL can be established. International standards, of allowable levels of toxins in food, are drawn up by the Codex Alimentarius Commission, under the auspices of the Food and Agriculture Organization (FAO) and the World Health

Organization (WHO). The current standards do not specify the concentrations allowed, but state that levels should not pose a threat to human health. Specific information is therefore needed in each case to set the standards. The toxicity of the substance, the mass of the seed and the concentration of the toxin must be known. The effect of environmental factors on the toxin content and the stability of the toxin during processing should also be taken into account. A safety factor should be built in to accommodate inter-individual variations and possible synergistic effects due to other compounds (Eloff *et al.* 2003).

Ideally, the levels of contaminating toxins in processed food consumed by the South African population, particularly children who are more susceptible, would be the only justifiable indicator for determining the acceptable tolerances in grains and other food commodities.

1.7.2 Regulations in other countries

The NOAEL of PAs has not yet been established in experimental animal studies. Estimates of intake causing toxic effects in humans indicate that they are more sensitive than rats and domestic animals (IPCS report 1989). The lowest known dose that caused veno-occlusive disease in a human was estimated to be 15 $\mu g.kg^{-1}$ per day, and was the result of self-medication with a comfrey (*Symphytum officinale*) preparation (Ridker *et al.* 1985).

Due to the hazards that may arise, the Federal Health Department of Germany has drastically restricted the sale of pharmaceuticals containing PAs and *N*-oxides with a 1,2-unsaturated necine skeleton. PA-containing pharmaceuticals are exempted from this restriction if a daily oral administration of 0.1 µg per day, and 10 µg per day with external application is not exceeded (Röder 1995). Similar regulations have been proposed in other countries such as Britain and Australia (Stegelmeier *et al.* 1999).

Regulations in Australia and New Zeeland are discussed in the ANZFA report (2001). The main alkaloids involved in human poisoning in Australia until 1988 were heliotrine from *Heliotropium*, echimidine from *Symphytum*, riddelline from *Senecio longilobus* and crotanine from *Crotalaria nana*. The approximate rat oral LD_{50} for these alkaloids are 300, 500, 50 and 100 $mg.kg^{-1}$, respectively. The collective data from these incidents suggest that the daily PA intake were cumulative in doses down to 33 $\mu g.kg^{-1}$ (expressed as heliotridine equivalents). From this data a tentative NOEL of 10 $\mu g.kg^{-1}$ per day is calculated. Applying a factor of 10 for

human variability sets the provisional tolerable daily intake (PTDI) for humans at 1 µg.kg^{-1} body weight per day.

Current USA guidelines restrict allowable seed levels of certain PA containing plants: *Crotalaria spectabilis, C. sagittalis* and *C. striata* require less than one whole seed per pound of product (Mattocks *et al*. 1988).

1.7.3 Regulation in South Africa

In South Africa, prior to 2002, regulation guidelines, set by the Department of Health, suggested a limit of one seed of *C. sphaerocarpa* per 10 kg of maize (Anon. 1987). The toxic seed regulation, on the other hand, allowed tolerances to be determined by the Agricultural Boards and a level of three seeds per 10 kg grain was consequently accepted. After the demise of the marketing boards in 1990, the allowable level reverted back to the regulations set by the Department of Health. This level was, however, more stringent than those of other trading partners. Due to subsequent pressure from producers, the Department of Health changed the allowable level to 10 *C. sphaerocarpa* seeds per 10 kg grain (Anon. 2002), as an interim measure, based on recommendations made by Eloff and co-workers (2003). This is the interim allowable level, provided that research confirms the validity of the assumptions made in the report.

Eloff *et al.* (2003) calculated the risk posed by grain contamination using 500 g of dry maize as the normal daily intake (two meals). They reasoned that a dose of 5 mg.kg^{-1} per day of monocrotaline (the most abundant PA in *Crotalaria* spp.) led to cancer in approximately a third of the rats in a feeding trial by Shumaker *et al*., 1976. By applying a safety factor of 10 to achieve non-toxic levels, and an additional 100 fold decrease for species differences, they estimated that 5 -10 µg.kg^{-1} per day would probably be equivalent to a NOAEL for humans. Based on findings of Marais (1944) it was calculated that PAs can contribute up to 0.05 % of the seed mass. For an average *C. sphaerocarpa* seed of 3.6 mg this calculates as 1.8 µg PA per seed. For acute toxicity a 70 kg person would have to eat 194 seeds per day (3880 seeds per 10 kg) to reach this intake. If the daily intake limit is reduced to 1 µg.kg^{-1} per day (Australian criteria for chronic exposure) the level should be set at 777 seeds per 10 kg maize. To comply with European regulation of 0.1 µg per day the level should drop to 11 seeds per 10 kg. The authors stressed that the assumptions on which this recommendation was made, still have to be tested.

1.8 Conclusion

Unsaturated PAs pose a threat to human health especially as the risk of low-level exposure has not yet been determined. Grain contamination with *C. sphaerocarpa* is a real problem in certain areas (e.g. Bothaville district) in South Africa. The toxicity of *C. sphaerocarpa* seed is, however, not known and may be very low. Previous feeding trials to prove toxicity in animals have failed and it is therefore inappropriate to investigate toxic levels via this route. The alternative is to determine the toxic PA content of *C. sphaerocarpa* seeds with an analytical method in a laboratory and to calculate the risk to humans based on daily consumption.

1.9 Aim and objectives

The aim of this study is to determine the toxicity of the PAs present in *C. sphaerocarpa* seed and to ensure that risk assessment is based on systematic, scientifically acceptable criteria. In order to achieve this, the following questions need to be answered:

- How toxic are *Crotalaria* species occurring in SA that may contaminate grain, and which PAs are present in these species?
- Is there a difference in PA levels under different environmental conditions?
- In order to minimize low-level exposure, should government only regulate levels of seed allowed in grain or rather regulate the concentration levels of toxic PAs in food products?
- Will it be possible to determine PA levels in processed grain?
- What is the threat to SA consumers of grain products imported from countries with more toxic PA contaminants?

It appears that the PA content of *C. sphaerocarpa* seeds growing in South Africa has never been chemically analyzed. Furthermore, several unpublished experiments carried out by the Veterinary Institute at Onderstepoort in 1968 could not show acute toxicity in the animals tested (Anon 1969). To repeat the toxicity studies under these circumstances was not a viable option. It is also often difficult to extrapolate toxicity results from animal studies to humans.

The main objective of this study is therefore to develop an analytical method that can distinguish between toxic and non-toxic PAs, in order to determine the toxic PA content in *Crotalaria* spp. that may contaminate grain in South Africa. The levels of toxic alkaloids will be

used as the basis for recommendations on the level of noxious seed that could be allowed in grain in future.

The objectives of the study are the following:

- Develop an HPLC-MS/MS method to distinguish between toxic and non-toxic PAs.
- Identify and quantify the toxic PAs in *C. sphaerocarpa* seed.
- Determine the concentrations of the toxic PAs in seeds from different sites.
- Determine toxic PA levels in other parts of *C. sphaerocarpa* and of different ages.
- Investigate the toxic PA content of other PA-containing plant species.
- Determine the stability of toxic PAs during the food cooking process.
- Determine the lowest level of detection (LLOD) for the determination of toxic PAs in maize meal.

The study will focus on the toxic PAs in the seeds as these are thought to be the reason for the inclusion of *C. sphaerocarpa* in the FDA Poisonous Plants Database (Anon 2003a). Compounds that meet the structural criteria for toxicity will be isolated where possible and identified in subsequent investigations. The toxicity tests of the isolated compounds in animals are beyond the scope of this study especially since many attempts to prove toxicity have already failed (Anon 1969). Other tests may be added to the study depending on the specific PAs found in the selected plants.

1.10 Envisaged results

The research may lead to a better understanding of toxic PAs that cause a variety of human, wildlife and livestock health problems in South Africa. If it could be proven that only non-toxic *Crotalaria* species occur in cultivated lands, the allowable level of seeds could drastically be increased. On the other hand, if products from other grain producing countries have a high risk of PA intoxication; it could give South African producers a competitive edge in the market.

Herbal preparations and traditional medicines containing hepatotoxic PAs may pose a real threat to humans as large quantities are normally consumed and children are often the ones who are treated in this manner. These products are also not controlled by any regulatory guidelines. The screening method for toxic PAs, developed in this study, could therefore also be used to regulate or restrict the use and sale of these products.

The study may furthermore lead to the establishment of a network of reference laboratories that could assist member states in identifying plants and their seeds suspected of containing toxic PAs. This in turn will allow regulation and restrictions on the import and sale of seeds, herbs and herbal preparations that may contain harmful pyrrolizidine alkaloids.

CHAPTER 2: ANALYSIS OF PYRROLIZIDINE ALKALOIDS

2.1 Background

Alkaloids are groups of basic compounds characterized by the presence of a heterocyclic nitrogen atom. They are naturally present in many plant species and are often toxic to animals and humans. Alkaloids include such diverse molecules as strychnine, atropine, cotinine, nicotine, solanidine and pyrrolizidine alkaloids (Holstege *et al.* 1995).

When analyzing for PAs, it is important to recognize that this group consists of many different compounds and that these often occur as very complex mixtures in plants. They may vary in structure, relative molecular mass, response to analytical procedure and in toxicity. Pyrrolizidine alkaloids are also often volatile and the use of extreme evaporation steps must be avoided during preparation. Care should also be taken to prevent hydrolysis of ester groups during the analytical process.

Various analytical techniques have been used for separation, identification and quantification of PAs in plants. These techniques include colourimetric screening using various adaptations of Ehrlich's reactions (Mattocks 1971), separation of the compounds with thin layer chromatography (TLC) (Wagner *et al.* 1981), quantitative analysis using gas chromatography (GC) (Culvenor *et al*. 1981, Mattocks 1986) and high performance liquid chromatography (HPLC) (Tittel *et al*. 1979). For most of these procedures, however, authentic reference materials are needed, of which only a few are currently commercially available. Identification of PAs is mostly achieved using nuclear magnetic resonance (NMR) (Logie 1994; El-Shazly 2002). These techniques are, however, limited in application by sensitivity factors and are often not sensitive enough for the determination of the very low levels of alkaloids that may be present in some foodstuffs.

The methods that have been used and new methods that may be applied is discussed in this chapter.

2.2 Sample preparation procedures

2.2.1 Extraction from plant material

The extraction of PAs from plant material has to some extent been standardized (Mattocks 1986; AOAC 1990). In general, the plant material is first extracted with hot or cold ethanol. The ethanol extracts are dried and the alkaloids taken up in dilute acidic solution. Neutral organic materials like chlorophyll and fats are removed by solvent extraction with dichloromethane or petroleum ether. *N*-oxides, which can also be converted to toxic pyrroles in the host animal, are often present in plants together with the basic PAs. The polar *N*-oxides which are generally more difficult to extract out of the acidic solution, can easily be reduced to the basic alkaloids before extraction. The extract is divided into two fractions and the *N*-oxides in one of the fractions are reduced by addition of zinc. The acidic solutions are filtered, made basic and the alkaloids extracted with dichloromethane or ethyl acetate. The total alkaloid content is determined in the reduced fraction, while the other fraction is used to determine the basic alkaloids content. The *N*-oxide content is represented by the difference between the total and the basic alkaloid fractions.

Mroczek *et al.* (2002) extracted both the unmodified *N*-oxides and the free bases from various plant samples with strong cation exchange (SCX) solid phase extraction columns. PAs were extracted from plant specimens by reflux with methanol. Extracts were filtered and dried and the dried extracts dissolved in dilute acid. Columns were pre-conditioned with distilled water before loading the samples. The PAs and *N*-oxides were eluted with a mixture of methanol-ammonia. Recoveries of 80 – 100% were achieved by this method for both the basic PAs and their *N*-oxides.

2.2.2 Extraction from animal derived samples

Jago *et al.* (1969) extracted heliotrine metabolites from blood samples of sheep after acetone precipitation of blood. The acetone was evaporated and the samples dissolved in dilute sulphuric acid. Fat soluble components were removed with chloroform while the metabolites were retained in the acid layer. Analysis was done using thin layer chromatography.

Ames and Powis (1978) extracted indicine and indicine *N*-oxide from rabbit urine and plasma. The compounds of interest were extracted with chloroform from basic samples. The sample

residues were then acidified and zinc added to convert the *N*-oxides to the basic alkaloids. Analysis was done on GC with electron-capture detection.

Lafranconi *et al*. (1985) extracted metabolites of monocrotaline from bile with solvent extraction. The metabolites were retained in the sodium acetate buffer (pH 8), while the unwanted compounds were removed with ethyl acetate. Separation of the metabolites was achieved with silica column elution and analysis was done on a mass spectrometer (MS).

Ge Lin *et al.* (1998b) extracted PAs from rat serum after dosing trials were conducted. Serum samples were made alkaline with ammonia and PAs extracted with dichloromethane. The organic extracts were evaporated and dissolved in methanol before LC-MS analysis.

It is generally accepted that PAs are rapidly metabolized, so that the amount recovered within a few hours after ingestion may be very small. Analytical methods that can detect the more persistent metabolites like the dehydropyrrolizidines, otonecine bases and secondary pyrrolic alcohols, rather than the primary metabolites (Anon. 2003b) will therefore be more applicable in cases of acute poisoning.

2.2.3 Extraction from food samples

Crews *et al.* (1997) described a HPLC-MS method for the determination of PAs in honey, derived from *Senecio jacobaea.* Solid phase extraction was used and recoveries ranged from 57-70%. Detection was done with MS after atmospheric pressure chemical ionization.

PAs in honey was also determined by Deinzer *et al.* (1977) who used the basic liquid extraction method described above (paragraph 2.2.1). Honey was acidified and the fat soluble matter removed. PAs were then extracted from the basified honey with chloroform and analysis was done on GC-MS.

Dickinson *et al*. (1976) used the same basic liquid extraction method for extraction of PAs from milk. Analysis in this study was done on TLC.

Edgar and Smith (2000) also used the same method to extract PAs from eggs after grain contamination of feed. Analysis was performed on GC-MS and identification of the PAs by fast atom bombardment-mass spectrometry (FAB-MS).

2.3 Analytical techniques

2.3.1 General screening methods

2.3.1.1 Ehrlich's reagent

Due to the low UV absorbance of most PAs, Ehrlich's reagent is often used to visualize the screening results. What makes this procedure valuable is that only compounds with unsaturated pyrrole rings will react with Ehrlich's reagent to give pyrrolizidine derivatives with an intense colour in the region of 565 nm (red-magenta colour is formed).

Mattocks and Jukes (1987) describe a robust method using Ehrlich's reagent for the detection of toxic PAs in plant specimens under field conditions. In this method plant material is crushed with ascorbic acid solution and split into two fractions. Aqueous nitroprusside is added to one fraction to convert the *N*-oxides to pyrrolic derivatives and the solutions are both heated on a water bath. The solutions are heated again with Ehrlich's reagent. Any magenta colour in the nitroprusside fraction will be due to unsaturated *N*-oxides. Colour development in the other fraction is due to the presence of other pyrroles or indoles and further investigations should be done. To test for the presence of basic alkaloids plants are crushed in chloroform. Ortho-chloranil is added and then Ehrlich's reagent. Any magenta colour reaction is due to unsaturated PA bases. Plants with toxic PAs can thus be identified in the field and collected for further analytical investigations.

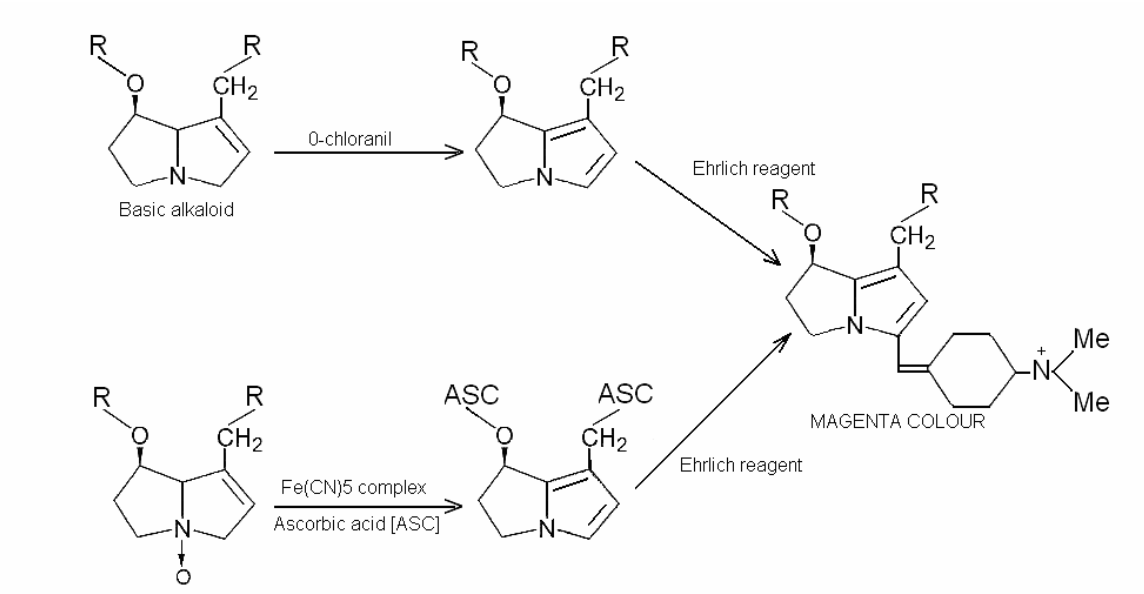


Figure 2-1: Reactions of Ehrlich's reagent with basic PA and *N*-oxide structures

This method has been refined for quantitative application and used by Azadbakht and Talavaki (2003) to determine PAs in wheat and flour samples contaminated with *Senecio spp*. In the quantitative method the intensity of the colour reaction is measured with a UV spectrometer against a reference standard calibration curve.

2.3.1.2 Screening method for PAs in urine

A qualitative screening method for the presence of PAs in urine is described by Steenkamp *et al.* (2000). Negative and positive (retrorsine spiked urine) controls are prepared together with the samples. Urine is applied to preconditioned solid phase extraction columns and washed with water. The alkaloids are eluted with a mixture of methanol, acetonitrile and ethyl acetate. After evaporation the residue is dissolved in chloroform, methyl orange and sulphuric acid are added and the solution is scanned on a spectrophotometer. A peak between 500-550 nm is indicative of the presence of unsaturated PAs in the urine.

General screening methods can provide valuable information on possible toxicity but lack specificity, as many other compounds present in the extract may also give positive reactions.

2.3.2 Separation techniques

2.3.2.1 Thin layer chromatography (TLC)

PA mixtures can be separated using TLC on silica gel plates with a mixture of methanol-chloroform-ammonia. Rf values can vary and monocrotaline is often used as a reference (Mattocks, 1967). Unsaturated PAs can be detected by treating the plates with hydrogen peroxide and then with Ehrlich's reagent. TLC of PAs has to a large extent been replaced by HPLC and GC due to their higher resolution, better sensitivities and quantification.

2.3.2.2 Gas Chromatography (GC)

PAs have been determined by GC with nitrogen-phosphorus detection (Holstege 1995). Identification of the PAs relies on retention time comparisons with reference standards, limiting the application to known PAs where authentic reference standards are available. PAs have also been analyzed on GC-MS as their trimethylsilyl derivatives (Evans *et al.* 1980). Derivatization gives rise to compounds, which are both stable and volatile and will therefore separate better on GC columns. Although derivatization can be applied to answer specific questions, it is often difficult to relate the MS-fragmentation patterns back to the original compound, especially when working with unknown PAs.

Capillary GC coupled to MS is to date the most widely used technique for analysis of PAs in complex mixtures. It is possible to identify most PAs with mass spectral libraries without the need of external standards, provided the specific reference spectrum is included in the library. Witte *et al.* (1993) compared the correlation of GC-MS analysis done on 100 different PAs between two laboratories. This report provides information on retention behaviour on different GC columns, and also lists the retention indices (RI) for all 100 PAs. It also provides a list of the characteristic fragments found in the different types of PAs, which can be valuable when attempting to identify unknown PAs.

2.3.2.3 Systematic toxicological analysis (STA)

Stelljes *et al.* (1992) developed a STA method for 23 different PAs extracted from plants. They used a multiple system approach consisting of TLC and GC and could predict many structures based on the differences in retention behavior between the two techniques. Two TLC methods on silica plates were used: System CMA with chloroform-methanol-ammonia as solvent, where plates were developed once and a second system, (LiCl), with chloroform-methanol-lithium chloride, where the plates were developed twice. Unsaturated PAs were sprayed with ortho-chloranil, heated and sprayed with Ehrlich's reagent (purple spots). Saturated PAs were visible after spraying with Dragendorff reagent followed by $NaNO_2$ (orange/brown spots, depending on the concentration).

For GC-MS a DB-5 column was used with a temperature program from 90 ºC to 250 ºC. The comparative behaviour of PAs on TLC and GC provided insight into the molecular structure. Generally the LiCl in the TLC system acted as an ion pair of sterically unhindered hydroxyl groups and increased the Rf values compared to the CMA system. The position of the acetyl groups could be predicted based on the Rf-value of the compound relative to that of monocrotaline. Acetylation of the 7-hydroxyl group provided much more mobility relative to acetylation of the ester at the 9-position. Thus the positions of the OH-groups had a considerable effect on TLC and could be predicted. In GC on the non-polar column, retention times roughly followed molecular weight. Other trends were noted – within similar groups, saturated PAs were retained longer than the unsaturated compound and 9-substituted esters were retained longer than 7-substituted esters. Unsaturated PAs examined showed a peak at *m/z* 120. The position of the ester group could be determined by the base ion; 7-angelylretronecine gave a base peak *m/z* 80 and 9-angelylretronecine a base peak *m*/z 93.

Using this approach, it was possible to predict many of the molecular structures based on retention behavior. Knowing what structures to expect can simplify deconvolution of the fragmentation patterns found with GC-MS.

2.3.2.4 High Performance Liquid Chromatography (HPLC)

HPLC is a non-destructive, quantitative technique and is mainly used to separate alkaloids in plant extracts where further analysis is needed.

Qualls and Segall (1978) used a µ-Bondapak CN column with a mixture of tetrahydrofuran (THF) and ammonium carbonate to separate PAs from *S. vulgaris.* Fractions were collected and analyzed on MS. Due to the high UV cut-off of THF the eluent was monitored at 235 nm which limited the sensitivity of the method considerably, as most PAs have little UV absorbance above 230 nm.

Segall (1979) used a u-Bondapak C_{18} column with a methanol-phosphate buffer gradient to elute the PAs in *S. longilobus* plant extracts. The method was more sensitive as the eluent could be monitored at 225 nm, which is closer to the UV maximum for most PAs. Ramsdell and Buhler (1981) described a similar reverse phase method using a RP-8 column. Caffeine was used as an internal standard leading to improved repeatability.

Mroczek *et al.* (2002) used a Hypersil BDS column and hexanesulphonic acid as ion-pairing agent to separate *N*-oxides and free bases from various plant samples. Detection was done at 220 nm with a UV detector. Although the more polar *N*-oxides were retained, the limit of detection was high (0.1 µg.ml^{-1}) due to background interference at this wavelength.

Kedzierski and Buhler (1986) developed a gradient HPLC method using a styrene-divinylbenzene column to separate a racemic mixture of necine-DHP-pyrroles formed after incubation of mouse liver with senecionine, seneciphylline and retrorsine. This method led to valuable information about the metabolism of PAs in liver.

The major disadvantage of HPLC is the non-specific detection, especially at the low UV (220 nm) where many other compounds may interfere. The technique also depends heavily on external reference standards, and does not provide much structural information.

2.3.3 Detection of pyrrolizidine alkaloids

2.3.3.1 Nuclear magnetic resonance (NMR)

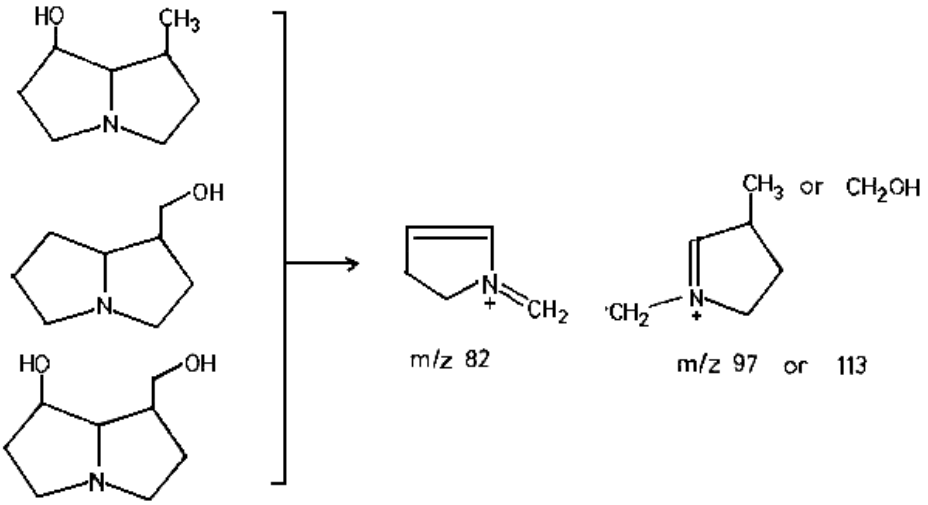
NMR spectroscopy provides detailed information on PA structures and stereo-chemical orientations and can even be used for quantification of compounds. Röder (1990) discussed the role of ^{13}C-NMR in structural elucidation of PAs. The major disadvantage of this technique is the large quantity of purified alkaloid needed to obtain the spectral data. With proton NMR, on the other hand, a useful spectrum can be obtained from a small amount of alkaloid (1 mg). Logie *et al.* (1994) published a review on ^{1}H-NMR of PAs, and described the most useful shift values for the different types of PAs. The ^{1}H-NMR spectral data of more than 350 PAs are listed in this article.

Although valuable information can be obtained with NMR, it is quite often impossible to isolate even milligram amounts of a specific alkaloid needed for this technique.

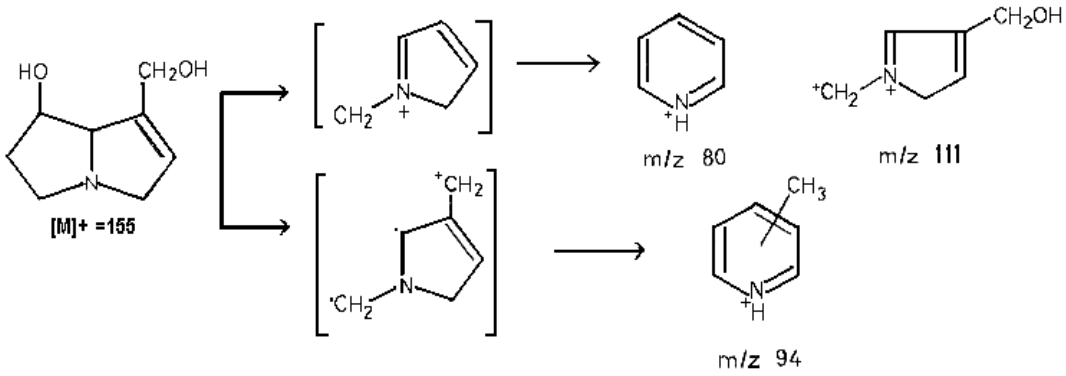
2.3.3.2 Classical electron impact mass spectra (EI-MS)

In classical EI-MS the ionization source energy is always the same, (70 eV), leading to repeatable fragmentation spectra. Spectra can be stored in searchable libraries, allowing identification of unknown compounds based on the fragments and intensities in the mass spectra. Electron impact in combination with capillary GC is a powerful high-resolution method for the identification of underivatized PAs from biological sources. It is possible to identify most PAs if there is some insight into the different fragmentation patterns of the PAs and the retention indices in combination with the molecular ion $[M]^+$ is known. The following summary on fragmentation patterns of PAs is based primarily on work done by Mattocks (1986) and Witte *et al*. (1993).

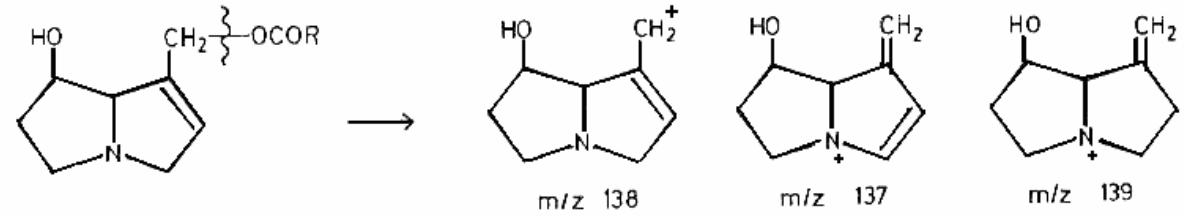
With classical EI fragmentation, saturated necines give typical fragments in the ranges *m/z* 95-97, 113-115, 122-123 and 138-140; with a characteristic base peak at *m/*z 82:



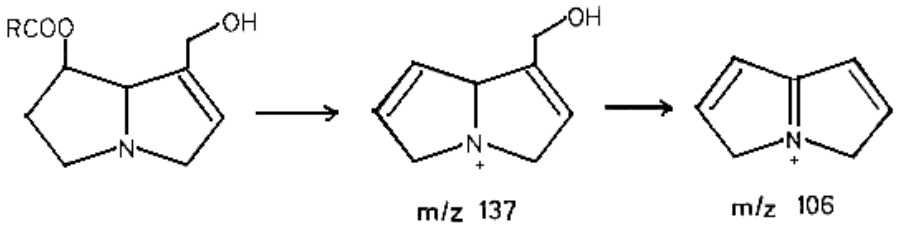
Corresponding fragments from unsaturated necines are two mass units lower with major fragments at *m/z* 80, 94, 111 and 120.



In general, unsaturated pyrrolizidine diesters give groups of fragments at *m/z* 93-95, 119-121 and 136-139. An intense ion at *m/z* 138 is the result of the C-9-O cleavage of the monoester retronecine base or its isomeric form:



Esterification at C-7 results in an intense fragment at *m/z* 137 and 106 due to the loss of the ester:



The presence of strong ions at *m/z* 264 is characteristic of trichodesmine and crotalarine and occurs through the cleavage of the allelic ester bond followed by McLafferty rearrangement.

Otonecine-type necines show an $[M-15]^+$ peak due to the loss of *N*-methyl, and give characteristic fragments at *m/z* 94, 96, 110, 122-123 and 149-151.

The characteristic spectra of *N*-oxides are excluded from the discussion, as the *N*-oxides of interest are converted to the basic alkaloids by zinc reduction during extraction.

In general it is important to combine fragmentation patterns and retention behavior when attempting to identify unknown PAs, as many geometric isomers exist within each group of PAs, and the spectra are often indistinguishable, even when pure standards are available.

2.3.3.3 Chemical ionization mass spectra (CI)

In normal EI spectra of PAs, the acid moiety is greatly fragmented and the molecular ion is often not of detectable intensity, making it impossible to identify the original molecule. Chemical ionization is a softer technique that produces strong $[M-H]^-$ ions as well as weaker $[M+OH]^-$ fragments when negative CI (NCI) is used. When methane is used as reactant gas (positive CI) the fractions obtained with unsaturated PAs are often only the $[M+H]^+$ fragment and two other fragments at *m/z* 138 and 120 (fragmentation of the necine base), simplifying the identification of these compounds. The extent of fragmentation with CI can be manipulated and depends on variations of source temperature and reactant gas flow. These settings are generally optimized according to the compound of interest, and the application of searchable libraries is limited to spectra generated under identical conditions.

2.3.3.4 Tandem LC-MS/MS

Tandem LC-MS/MS is a very sensitive technique, with the advantage that mass spectra can be obtained. Spectral information is dependent on the ionization conditions, allowing much more flexibility when structural elucidation is investigated, but making searchable libraries more complicated. Various groups (Venisse *et al*. 2003, Lips *et al*. 2001 and Hough *et al*. 2000) are

working towards performance based, standard criteria for the generation of collision induced dissociation (CID) spectra to be used for the compilation of searchable libraries. When available, these libraries will allow the identification of unknown compounds, without the need for authenticated reference materials, and will overcome the current disadvantage of LC-MS when compared to GC-MS.

Ge Lin *et al.* (1998b) developed a LC-MS/MS method for the determination of known PAs. Spectra were obtained with in-source collision as well as with CID in the collision cell. All PAs analyzed by electrospray ionization (ESI) in the positive mode exhibited an abundance of the $[M+H]^+$ pseudo-molecular ion. Collision induced spectra of retronecine-type 1,2-unsaturated PAs produced characteristic fragments at *m/z* 138 and 120. Other fragments characteristic of this type of PA was an ion at *m/z* 94 and a fragment corresponding to $[MH - CO]^+$. In the case of otonecine-type PAs, the characteristic fragment ions were *m/z* 168 and *m/z* 150, with two other fragments at *m/z* 110 and *m/z* 122. For saturated necines, characteristic ions appeared at *m/z* 140 and *m/z* 82.

This method should be able to distinguish toxic retronecine- and otonecine type PAs from non-toxic PAs, on the basis of the characteristic fragmentation patterns. Although this method could be developed into a screening method for toxic PAs, no published evidence of such an application could be found in the literature reviewed. Most users of triple quadrupole MS/MS detectors use methods where the pseudo-molecular ion and one or more of the fragments are used to detect and selectively quantify compounds of interest. These instruments can however also be used in precursor scan mode, where the fragments produced after CID can be used to determine the compound of origin. With compounds like toxic PAs, where all the 1,2-unsaturated structures yield such distinguished fragments, this would be the ideal method to develop into a screening method to evaluate PA toxicity in natural products. The development of this screening method is discussed in the next chapter.

CHAPTER 3: LC-MS/MS SCREENING METHOD FOR THE DETECTION OF TOXIC PYRROLIZIDINE ALKALOIDS

3.1 Introduction

One of the conclusions in the IPCS report (1989) was that: "Toxic PAs all possess a 1,2-double bond in the pyrrolizidine nucleus, thus, analytical methods that can selectively detect this feature in complex mixtures will have value in screening for potential toxicity". The primary aim of this study was to develop a sensitive analytical method that could specifically detect 1,2-unsaturated PAs in order to estimate potential toxicity and thereby to calculate the risk to human health. Of all the methods reviewed, the LC-MS/MS method seemed to have the most potential to meet this criterion and was further evaluated in this chapter.

3.2 Method development

3.2.1 Principle

From the data published by Ge Lin *et al.* (1998b) it was clear that unsaturated PAs will produce characteristic fragments under specific MS/MS conditions.

Collision induced fragmentation of the pseudo-molecular ions into specific fragments can be achieved by performing precursor experiments. Using electrospray ionization in the positive mode (ESI+) the $[M+H]^+$ masses of all the pseudo-molecular ions, which produced the specific fragments in the unknown samples, are recorded as peaks in a chromatogram (abundance versus retention time). The software programme can then be used to reveal the molecular masses of the compounds of interest.

The origins of the characteristic fragments seen with unsaturated retronecine (*m/z* 120 and 138) - and otonecine type bases (*m/z* 150 and 168) can be presented schematically as follows:

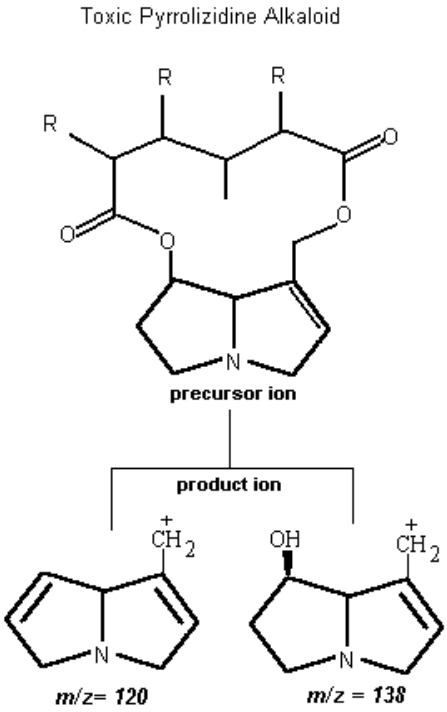


Figure 3-1: Generic structures of a retronecine type toxic PA with the characteristic product ions formed with CID in the collision cell

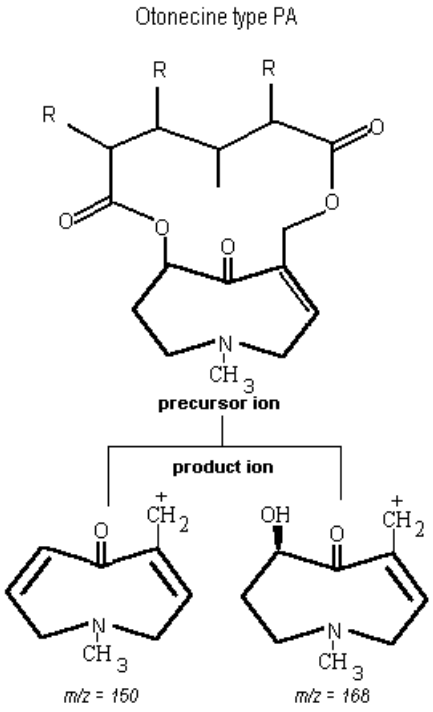


Figure 3-3: Generic structures of an otonecine type toxic PA with characteristic product ions formed with CID in the collision cell

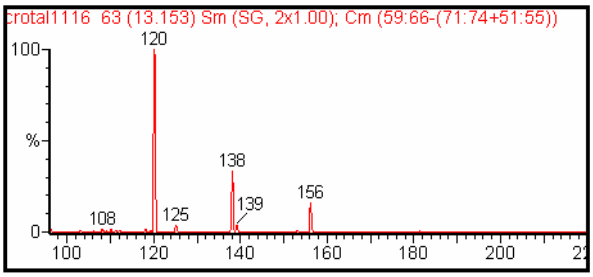


Figure 3-2: LC-MS/MS product ion spectrum of a 1,2-unsaturated necine base showing the characteristic *m/z* 120 and 138 fragments. Precursor ion scans of these fragments will reveal the $[M+H]^+$ mass of the retronecine type PA (Ge Lin 1998)

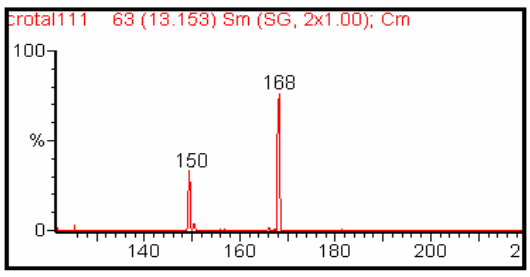


Figure 3-4: LC-MS/MS product ion spectrum of a 1,2-unsaturated otonecine base with characteristic fragments *m/z* 150 and 168. Precursor ion scans of these fragments will reveal the $[M+H]^+$ mass of the otonecine type PA (Ge Lin 1998)

3.2.2 Materials and instrumentation

3.2.2.1 Reagents

Retrorsine (CAS: 480-54-6) and monocrotaline (CAS: 315-22-0) were purchased from Sigma, South Africa. Acetonitrile, ammonium acetate, hydrochloric acid 32%, sulphuric acid, phosphoric acid, ethanol, zinc powder, ethyl acetate, ammonia solution 25%, hexane and methanol were supplied by Merck, South Africa.

3.2.2.2 Samples

Crotalaria sphaerocarpa seed was obtained from Dr J Saaiman Du Toit, Agricultural Research Centre, Potchefstroom (**Sample A,** received April 2004). These seeds were propagated and plant material was collected at various stages over a one year period (**Sample B,** planted September 2004). *Crotalaria sphaerocarpa* plant material was also obtained from two maize farms in the Bothaville district; **Sample C** from a farm where extensive pre-emergence herbicide spraying was practiced, and **Sample D** from a second farm where no herbicide was used during the season. These samples were collected in May 2005. **Sample E** was milled *C. sphaerocarpa* plant material from previous toxicity studies done at Onderstepoort. The last sample (**Sample F**) was *C. sphaerocarpa* plant material, obtained from a soybean farmer in Gauteng, whose crop was rejected due to high levels of contamination, in May 2006.

Figure 3-5: *C. sphaerocarpa* (Sample B) plant growing in a garden in Centurion

.

Crotalaria sphaerocarpa is normally an annual plant, but is also known as an "opportunistic grower" and can complete more than one life cycle per year under favourable weather conditions. For propagation, seeds were removed from the pods and placed in concentrated sulphuric acid for 20 minutes, rinsed and sown in September 2004 (Dr Saaiman Du Toit, personal communication, 2004). These seeds, planted in early spring in a secluded garden area in Centurion (sample B), started to geminate during October. Flowers appeared by January and green seeds were present by early February (1st sample collection). New flowers and green seeds were produced up until March (2nd collection) when new growth ceased. The plants were fully grown at this stage and the seeds matured while the plants started to dry. The plants were almost completely dry by the middle of May (last collection).

Fresh green samples were collected and stored frozen until analysis. Dried plant specimens were collected at the end of the growing season. Samples were collected in large paper bags (0.3 m X 0.6 m) and separated into the various plant parts. Dried samples (250 g to 500 g) were milled before analysis and the fresh/green samples were homogenized during the extraction process. Unless otherwise specified "seed sample" refers to the whole pod together with the 2-3 seeds. Dried lucerne (*Medicago sativa*) plant material was used as a blank matrix for the preparation of the calibration standards.

Senecio inaequidens (DC) plant material was provided by Prof Botha, Faculty of Veterinary Science, Onderstepoort during the course of the method development phase (October 2004). This plant is known to contain toxic PAs and the sample was analyzed at various stages during the method development process as a positive control sample.

Two other *Crotalaria* plants, *C. dura* and *C. laburnifolia* were collected in Kwazulu-Natal during December 2004 and provided by Prof Naudé, Faculty of Veterinary Science, Onderstepoort. These plants were analyzed in later experiments to investigate unsaturated PAs in other *Crotalaria* spp.

3.2.2.3 Equipment

The HPLC instrument was a Waters Alliance 2796 gradient pump system (Microsep, SA). The analytical column was Phenomenex Luna C_{18} 5µm; 250 x 2.0 mm (Separations, SA). Mobile phase A contained 90% 25 mM ammonium acetate buffer (pH 3.84), 2% methanol and 8% acetonitrile. Mobile phase B contained 80% acetonitrile, 10% methanol and10% ammonium acetate buffer (pH 3.84). Gradient elution was 0 - 5 min 98% A : 2% B; 5 - 15 min 40 % A :

60% B (linear); 15 -20 min 98% A : 2% B (linear). The flow rate was 0.2 ml.min^{-1}. Total runtime was 30 minutes with a 5 minute equilibration time at the end of the run. The Mass spectrometer used was a Quattro Micro triple quad instrument (Micromass, Microsep, SA) with ESI in the positive mode. The software used was MassLynx® version 4.0.

3.2.2.4 Instrument optimization

Solutions of pure reference materials retrorsine (FW 351) and monocrotaline (FW 325) in methanol (500 ng.ml^{-1}) were infused to optimize the mass spectrometer settings in the MS, MS/MS and tandem LC-MS/MS modes. LC-MS/MS settings were used for precursor scans as well as for product ion scans performed on all the extracts.

The $[M+H]^+$ pseudo-molecular ions of monocrotaline and retrorsine were obtained by infusing the standard solution in the MS mode.

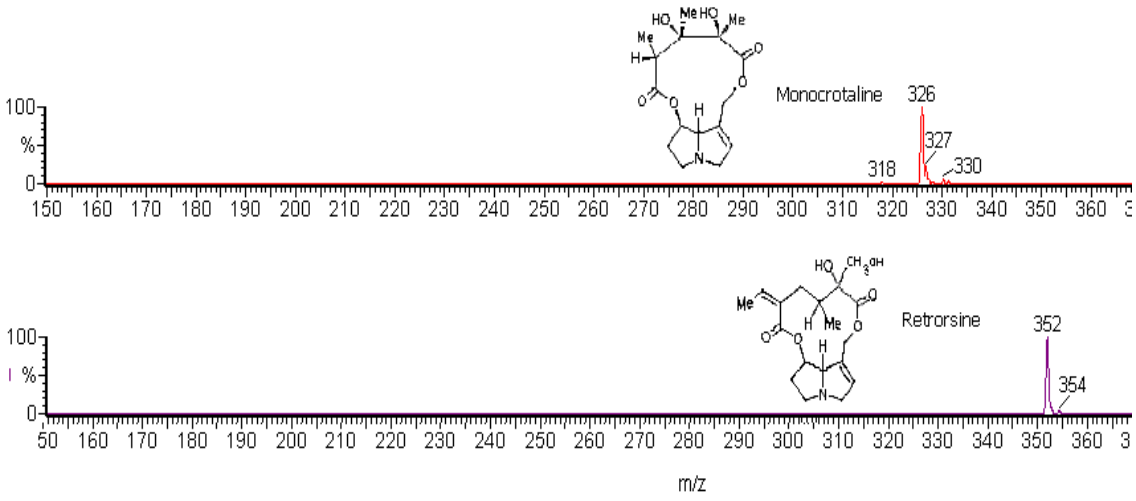


Figure 3-6: ESI$^+$ mass spectra of monocrotaline and retrorsine showing the $[M+H]^+$ ions obtained with the infusion experiments

Product ion scans can be used to obtain a mass spectrum of the peak of interest which can be used to identify compounds. In the product ion mode the first quadrupole is set to allow only a specific mass to pass through, collision of that mass is achieved in the collision cell and the

third quadrupole is set in the full-scan mode to record the mass spectrum of the pseudo-molecular ion.

Conditions in the MS source and the collision cell were optimized with the standard solution to produce the highest abundance of the characteristic fragments of unsaturated PAs (Fig 3-7). These setting were saved and used to investigate all the possible toxic PAs in the samples.

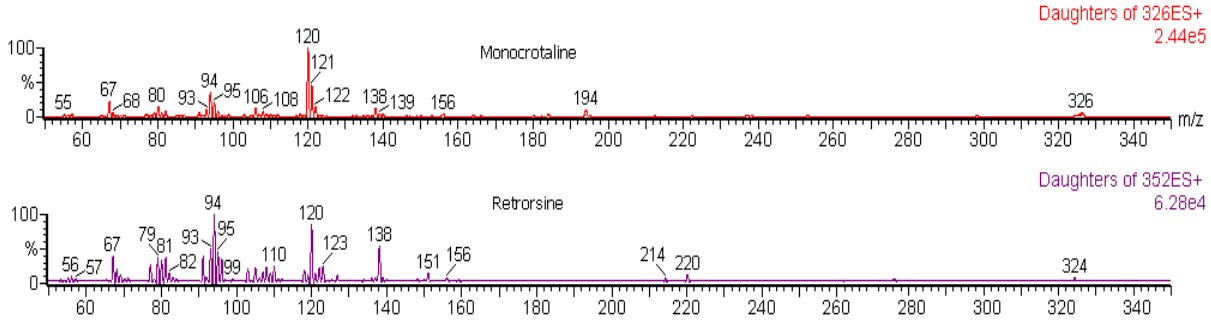


Figure 3-7: MS/MS spectra of monocrotaline and retrorsine with collision induced fragmentation in the collision cell (collision gas = 29) showing the fragments characteristic of unsaturated PAs

In the precursor ion scan mode the first quadrupole is used to scan over a selected mass range (100 – 500 amu), collision of all the masses is achieved in the collision cell and the third quadrupole is set to detect only a specific fragment. Once the specific fragment is detected the mass and abundance of the precursor ion is recorded in the first quadrupole.

Precursor scans were performed on the standard solution and the MS/MS settings were optimized to produce the highest ion count for the *m/z* 120 fragment (Fig 3-8). These settings were saved and used during the precursor ion scans of the samples.

The MS was coupled to a HPLC and the PAs were separated on a C_{18} column with gradient elution as described. The standard solution (500 $ng.ml^{-1}$) was injected into the HPLC with the MS set to perform precursor ion scans of the fragment *m/z* 120.

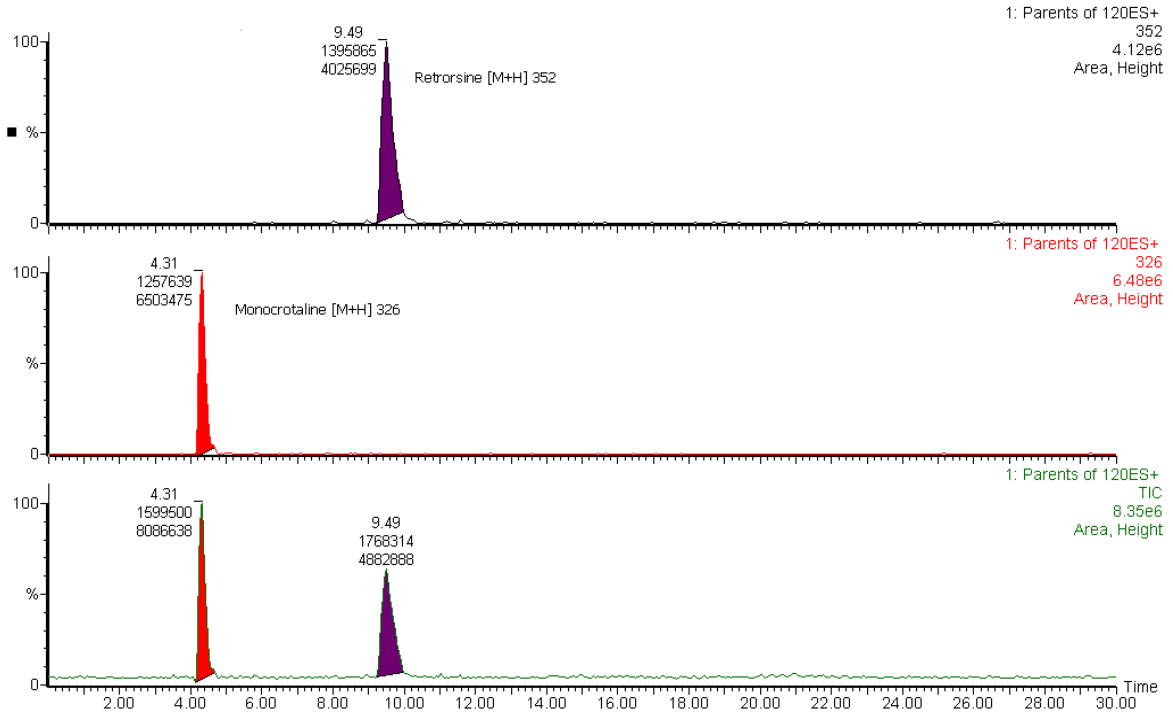


Figure 3-8: The LC-MS/MS total ion chromatogram (TIC) of a mixture of monocrotaline and retrorsine solution and the reconstructed chromatograms of the $[M+H]^+$ precursor ions 326 (monocrotaline) and 352 (retrorsine) which gave rise to the *m/z* 120 fragments

All the pseudo-molecular ions found during the precursor ion experiments were considered as possible 1,2-unsaturated PAs. Quantification of these compounds can be achieved using multireaction mode (MRM) experiments. In this mode the MS/MS is optimized to allow only a specific precursor mass to pass through the first quadrupole, collision is achieved in the collision cell and the third quadrupole is set to record the abundance of a selected fragment. More than one MRM transition can be measured at any time, or the MS can be programmed to measure certain transitions at specific retention times. This mode of detection filters out most of the interfering background compounds normally present in natural extracts, allowing very specific detections at very low concentration levels.

The optimized MS, MS/MS and LC-MS/MS conditions obtained with the infusion experiments are listed in Table 3-1.

Table 3-1: Operating conditions for MS detector used during the MS, MS/MS and MRM experiments

Parameter	MS Scan	LC-MS/MS	MRM
Source (ES+)			
Capillary (V)	3.20	3.20	3.20
Cone (V)	20.0	20.0	20.0
Extractor (V)	3.00	3.00	3.00
RF Lens (V)	0.3	0.3	0.3
Source temp (°C)	120	150	150
Desolvation temp (°C)	200	300	300
Cone gas flow ($L.h^{-1}$)	50	60	60
Desolvation gas ($L.h^{-1}$)	250	300	300
Analyzer			
LM 1 Resolution	15.0	14.0	14.0
HM 1 Resolution	15.0	14.0	14.0
Ion energy 1	0.0	0.2	0.2
Entrance	50	3	3
Collision	0	34	40
Exit	50	1	1
LM 2 Resolution	15.0	13	13
HM 21 Resolution	15.0	13	13
Ion energy 2	0.5	0.2	0.2
Multiplier (V)	650	650	650

3.2.3 Extraction evaluation

Sample size, especially in cases where actual poisoning occurred, can often be a limiting factor and the amount of sample used during the method developing stage was kept to a minimum.

Seven different extraction methods were investigated in order to evaluate the extraction efficiency from the plant matrix. In each experiment 500 µl of an evaluation standard (500 $ng.ml^{-1}$ retrorsine and monocrotaline in lucerne extract) was extracted in triplicate. The extracts were injected on LC-MS/MS and quantified using MRM experiments. The efficiency of the extraction was calculated as the average percentage recovered against a standard solution that was not extracted. The seven methods investigated were the following:

3.2.3.1 Liquid-liquid extraction method (Mattocks 1986)

Evaluation standard was dissolved in 10 ml 90% ethanol and evaporated at 40 ºC under reduced pressure. The extracts were reconstituted in 2 ml dilute hydrochloric acid (0.05 M). Chlorophyll and wax were extracted with 5 ml ethyl-ether and the remaining aqueous layers

made basic by addition of about 0.2 ml 25 % ammonia solution. The alkaloids were extracted with 3 x 2 ml ethyl acetate, the ethyl acetate was evaporated and the alkaloids were reconstituted in 0.5 ml methanol and injected into the LC-MS/MS.

3.2.3.2 Solid phase extraction method (Mattocks 1986)

Evaluation standard was dissolved in 10 ml 90% ethanol. Dowex 50 (1 g) was added and the mixtures were left on a mechanical stirrer for 30 minutes. The mixtures were centrifuged and the liquid discarded. The alkaloids were eluted from the resin with 4 ml 10% ammonia in methanol. The eluates were evaporated and the alkaloids were reconstituted in 0.5 ml methanol and injected into the LC-MS/MS.

3.2.3.3 Liquid-liquid extraction method followed by solid phase extraction (Holstege et al. 1995)

Evaluation standard was dissolved in 10 ml 5% ethanol in ethyl acetate and the pH adjusted (pH>9) with 5 M sodium hydroxide. The mixtures were extracted twice with 2 ml hydrochloric acid (0.5 M) and the aqueous layers discarded. The organic layers were evaporated and reconstituted in 1 ml 0.5 M sodium hydroxide and passed through C_{18} Bond Elute solid phase columns. The alkaloids were eluted from the columns with ethyl acetate, the ethyl acetate was evaporated and the alkaloids were reconstituted in 0.5 ml methanol and injected into the LC-MS/MS.

3.2.3.4 Liquid-liquid extraction method (Ge Lin et al. 1998b)

Evaluation standard was dissolved in 10 ml 90% ethanol and evaporated at 40 ºC under reduced pressure. The extracts were reconstituted in 2 ml dilute sulphuric acid (0.5 M) and made basic by addition of about 0.5 ml 25% ammonia solution. The alkaloids were extracted with 2 x 10 ml dichloromethane, the dichloromethane was evaporated and the alkaloids were reconstituted in 0.5 ml methanol and injected into the LC-MS/MS.

3.2.3.5 Liquid-liquid extraction method followed by solid phase extraction (Mroczek et al. 2002)

Evaluation standard was dissolved in 10 ml methanol and evaporated at 40 ºC under reduced pressure. The extracts were reconstituted in 1.5 ml dilute hydrochloric acid (0.05 M) and passed through SCX (Phenomenex, Strata) solid phase columns. The alkaloids were eluted

from the columns with 2 ml 10% ammonia in methanol. The eluates were evaporated and the alkaloids were reconstituted in 0.5 ml methanol and injected on LC-MS/MS.

3.2.3.6 Solid phase extraction method (Franke and De Zeeuw, 1998)

Evaluation standard was diluted with 2 ml 0.1 M phosphate buffer (pH 6). The mixtures were passed through HCX (Isolute) solid phase columns. The alkaloids were eluted with 2 ml 10% acetone in ethyl acetate followed by 2 ml 3% ammonia in ethyl acetate. The eluates were evaporated and the alkaloids were reconstituted in 0.5 ml methanol and injected on LC-MS/MS.

3.2.3.7 Revised liquid-liquid extraction method

In spite of the excellent recoveries obtained with the first method (Table 3-2), the ethyl-ether cleanup step could not effectively remove the chlorophyll and it led to dirty extracts when *Crotalaria* plant material was extracted. Substituting the ether cleanup with 2 x 5 ml hexane cleanup steps led to clean extracts with recoveries similar to that of the first method and this was the final method used during the rest of the project.

3.2.4 Extraction of unsaturated pyrrolizidine alkaloids from plant material

The extraction procedure was adjusted according to the amount of sample extracted. Between 1.0 – 1.5 g sample was weighed and the volume of the extraction solutions adjusted accordingly. In general, 1 g milled sample was weighed, homogenized with 10 ml 90% ethanol and left on a mechanical shaker for about 4 hours. The solids were allowed to settle and the sample was centrifuged. The clear solution was divided into equal fractions A and B and evaporated at 40 °C under reduced pressure. The extracts were reconstituted in 2 ml dilute hydrochloric acid (0.1 M). Chlorophyll and waxes were extracted with 2 x 5 ml hexane and the hexane layer discarded. The *N*-oxides in fraction B were reduced by addition of about 0.5 g zinc powder and stirring overnight. Both fractions were then made basic (pH>9) by addition of about 0.5 ml 25% ammonia solution. The alkaloids were extracted with 3 x 5 ml ethyl acetate. The ethyl acetate was evaporated and the alkaloids stored dry at -20 °C until analysis. Samples were reconstituted in 1 ml methanol for both LC and GC analysis.

3.3 Results

3.3.1 Extraction optimization

The percentage recovery of each method was calculated to determine the extraction efficiency of each extraction procedure. The percentages recovered with triplicate extraction experiments were calculated as:

$$\frac{\text{Peak area in extracted standard} \quad x \ 100}{\text{Peak areas in non-extracted standard}}$$

The average recoveries of monocrotaline and retrorsine obtained with each extraction method are listed in Table 3-2. Possible reasons for the variations are discussed later in paragraph 3.4.1.

Table 3-2: Average recoveries obtained with the different extraction methods investigated

Method	Average % recovery of triplicate extractions	
	Monocrotaline	Retrorsine
1: Liquid-liquid extraction	97	98
2: Dowex resin extraction	54	47
3: LLE followed by C_{18} SPE	21	17
4: Liquid-liquid extraction	89	83
5: LLE followed by SCX SPE	103	92
6: LLE followed by HCX SPE	79	71
7: Revised LLE	105	98

3.3.2 Calibration curve and validation

Milled lucerne was extracted with ethanol as a blank medium and spiked with pure retrorsine and monocrotaline (1 $mg.ml^{-1}$ methanol) reference material. Serial dilutions with the blank matrix were used to prepare a standard curve consisting of eight different concentrations between 0.01 $\mu g.ml^{-1}$ and 100 $\mu g.ml^{-1}$. The standard solutions were evaporated, extracted with ethyl acetate and stored frozen until analysis.

Four aliquots of each standard concentration were extracted and injected using MRM experiments for the transitions *m/z* 326>120 for monocrotaline and *m/z* 352>120 for retrorsine.

The curves were linear, above the limit of detection to 100 µg.ml^{-1} for retrorsine [R^2=0.9979, y=11762x + 11794] and to 50 µg.ml^{-1} for monocrotaline [R^2=0.9861, y=17509x+13153].

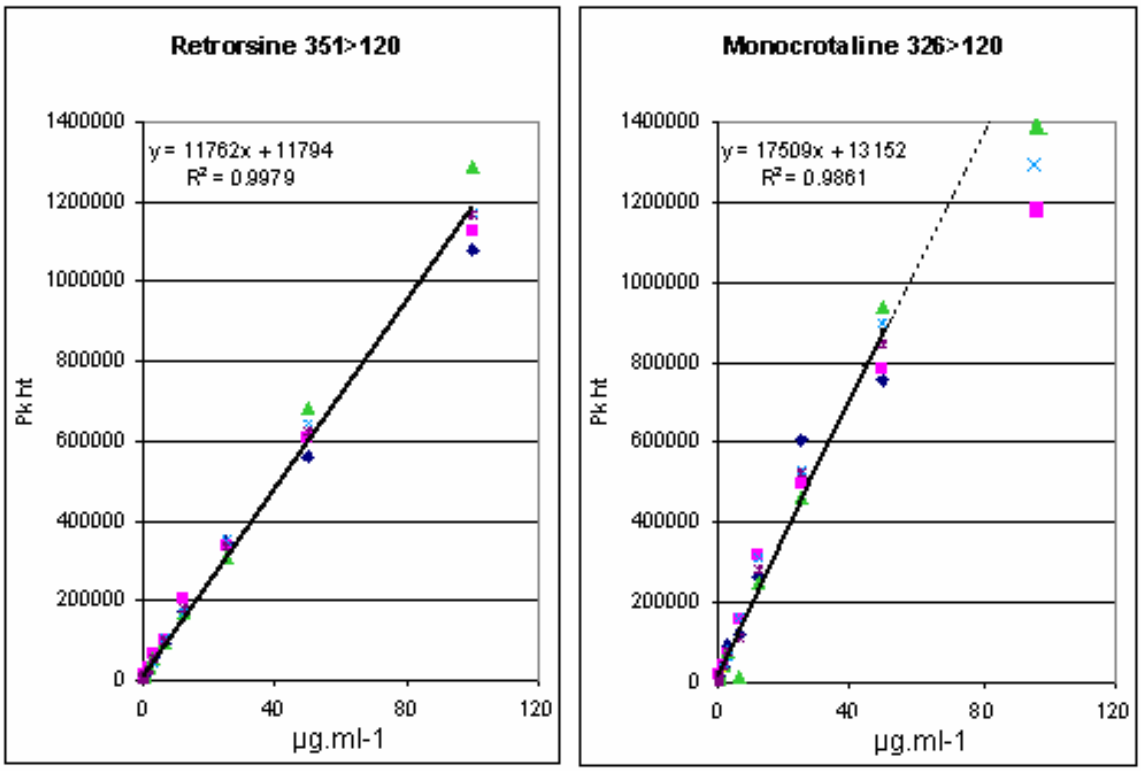


Figure 3-9: Calibration curves of retrorsine and monocrotaline spiked lucerne extracts

The recovery of monocrotaline, when calculated as retrorsine equivalents, ranged between 94% at 0.1 µg.ml^{-1} to 126% at 50 µg.ml^{-1}. The limit of quantification (signal > 10 noise) was 0.05 µg.ml^{-1} (0.05 ng "on column") when 10 µl of the extracted standards.

3.3.3 Moisture content

The moisture content of the green plants collected in February 2005 was determined on representative samples from the different plant parts. Samples were weighed and dried at 120°C for 24 hours. The moisture content was: roots 11%; stems 15%; secondary stems 51%; leaves, 53%; and green seeds 46%.

3.3.4 Experimental results

3.3.4.1 Precursor ion scans

Crotalaria sphaerocarpa samples were extracted as described, reconstituted in methanol, and injected. Precursor ion experiments were performed on all the samples for fragments *m/z* 120 and 138 for retronecine type PAs, and *m/z* 150 and 168 for otonecine type PAs. No otonecine type PA reference material was available, but it is known from experiments done by Ge Lin *et al.* (1998b) that otonecine type PAs will be detected under the same MS/MS conditions used for retronecine type PAs, if at all present. As no precursors of *m/z* 150 and 168 could be found, it was concluded that none of the samples contained otonecine type PAs.

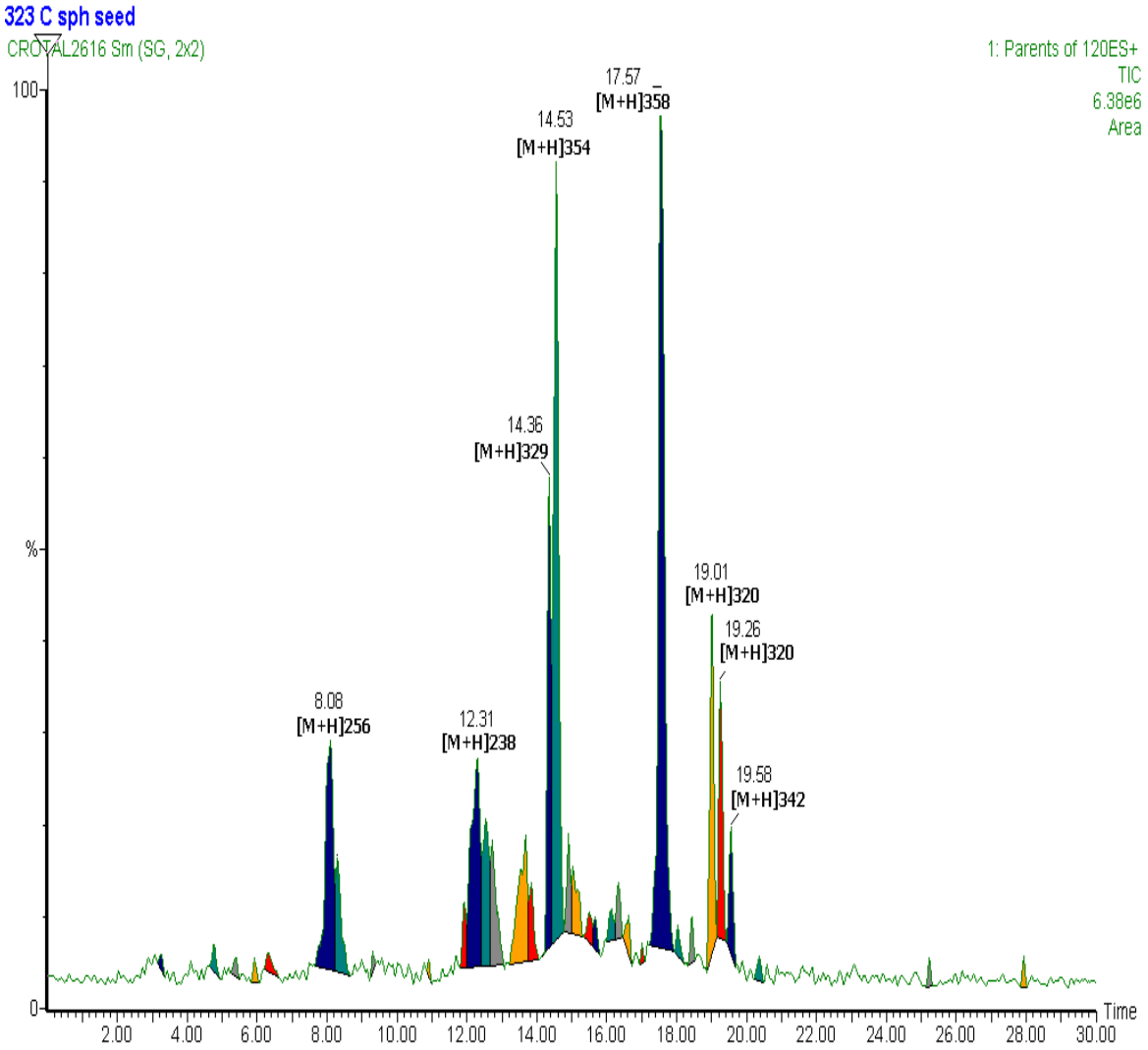


Figure 3-10: Example of a chromatogram obtained with the precursor scan (*C. sphaerocarpa* seed extract of sample A) indicating the $[M+H]^+$ mass of each precursor

Each peak found in the precursor chromatograms of the different samples was representative of a different precursor ion mass as can be seen in the example of the seed extract (Fig 3-10), where eight different unsaturated PAs were found.

A total of 11 different unsaturated PAs were found in the various parts of the *C. sphaerocarpa* plant. Using Masslynx software, the $[M+H]^+$ mass of the specific precursor ion was obtained by selecting the peaks of interest

The chromatogram can be reconstructed using the software, to show a trace for each of the different masses found (Fig 3-11).

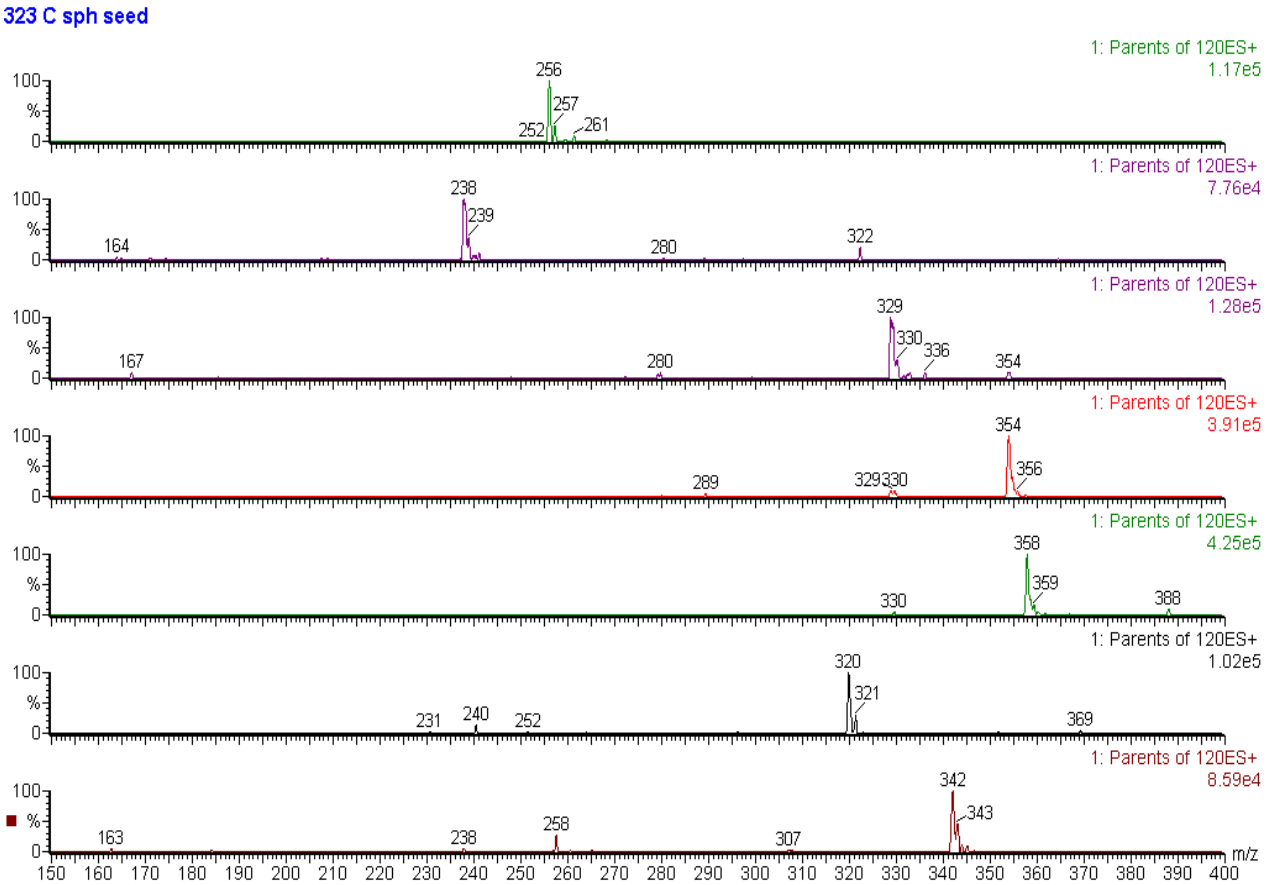


Figure 3-11: Reconstructed precursor ion $[M+H]^+$ masses of the fragment *m/z 120* obtained in Fig 3-10 at their corresponding retention times

3.3.4.2 Product ion scans

The *m/z* 120 fragments observed in the precursor ion scans could have been produced by compounds other than unsaturated PAs. The LC-MS/MS was programmed to record the product ion spectra of all the precursor ions at their respective retention times. The MS spectra obtained were again reconstructed by the software and plotted as individual traces for each specific mass. The spectra obtained with one of the extracts of sample E are shown in Figure 3-12 as an example. Each trace represents the mass fragments of a different pseudo-molecular ion that were fragmented at a specific retention time. These spectra were compared with the spectra of the pure PAs obtained during the infusion experiments. All the compounds that revealed the characteristic fragment *m/z* 120, 138 and 94 were assumed to be unsaturated PAs and quantified in subsequent MRM experiments.

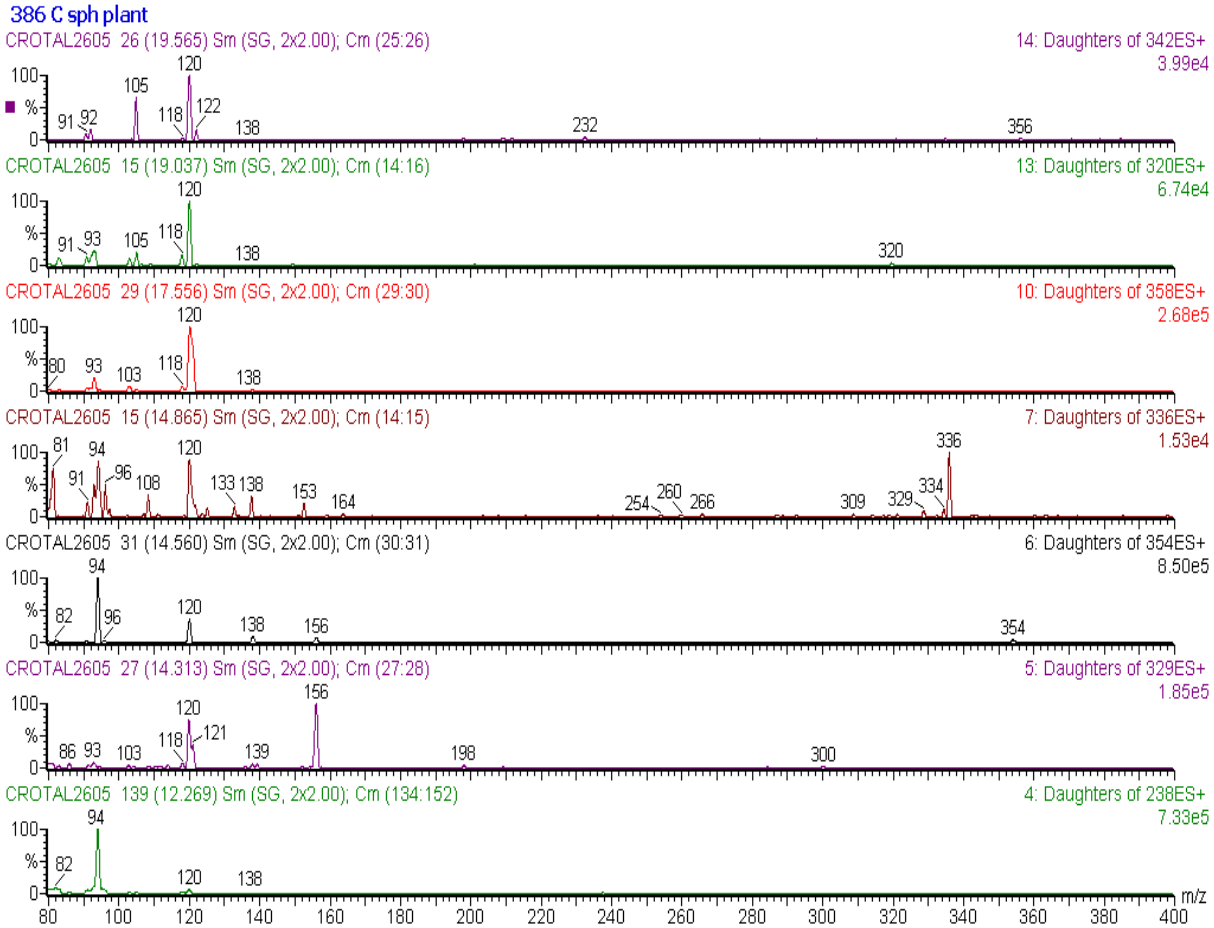


Figure 3-12: MS/MS spectra of the possible toxic PAs found in sample E. (Precursor ion mass listed in each trace as "Daughters of ...")

In the product ion experiments the relative abundance of the *m/z* 120 fragment was between 80 -100% for all the compounds except for the $[M+H]^+$ 238 ion, where the major fragment was at *m/z* 94. Due to the presence of the other characteristic fragments of unsaturated PAs this compound was assumed to be a possible toxic PA and was quantified as such in subsequent MRM experiments.

3.3.4.3 Multi reaction mode scans

MRM experiments are very specific and are used to quantify selected compounds of interest. The first quadrupole of the MS/MS detector is optimized to allow only a specific mass to pass through into the collision cell where it is fragmented. The second quadrupole is then optimized to allow only specific fragments to pass through to the detector where the abundance is then recorded. The sensitivity of MS/MS detectors is based on the selective filtering of specific precursor to product transitions. These transitions can be scanned sequentially in very short time cycles (0.16 ms) and the abundance recorded. The MRM transitions (precursor mass>product mass) for the unsaturated PAs were programmed in a MS method (see Table 3-3). Each transition measured can be reconstructed as a chromatogram of intensity vs. retention time. Concentration is a function of peak height or area and is calculated against an external calibration curve. The standards and samples were all injected and the toxic PAs quantified using these MRM experiments.

Table 3-3: MS method for the MRM transitions used for quantification

Transition	Dwell time (seconds)	Cone (kV)	Collision (kV)
238 > 120	0.16	20	40
256 > 120	0.16	20	40
320 > 120	0.16	20	40
326 > 120	0.16	20	40
329 > 120	0.16	20	40
336 > 120	0.16	20	40
338 > 120	0.16	20	40
342 > 120	0.16	20	40
352 > 120	0.16	20	40
354 > 120	0.16	20	40
356 > 120	0.16	20	40
358 > 120	0.16	20	40
396 > 120	0.16	20	40

The chromatograms were reconstructed using the MassLynx software to show the response found with different transitions, as can be seen in Figure 3-13.

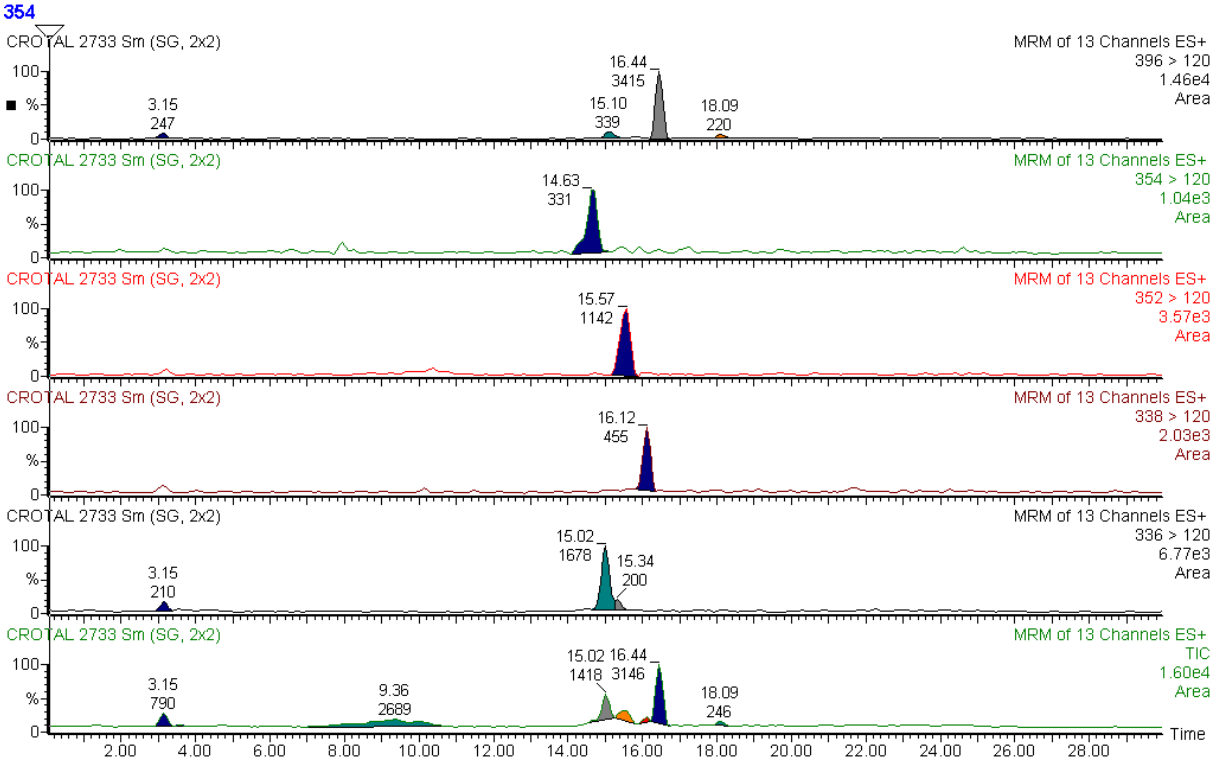


Figure 3-13: MRM chromatogram of the different transitions measured in sample E

The peak areas for each MRM transition were used to calculate the amount of the specific unsaturated PA from the retrorsine calibration curve.

3.3.5 Unsaturated pyrrolizidine alkaloids in *C. sphaerocarpa*

The quantitative results of the unsaturated PA found in the different seed samples are listed in Table 3-4. Two sets of results are listed for each sample: the basic unsaturated PA amount and the total PA amount (*N*-oxide + basic PA) found in the reduced fraction of each plant sample. Results for fresh green plants are based on the wet mass. Results are calculated using the retrorsine calibration curve and are expressed as µg retrorsine equivalents per gram sample

Table 3-4: Quantitative results of the possible toxic PAs present in *C. sphaerocarpa* seeds. Results are presented as the total PA fraction (*N*-oxide + basic) found in the reduced samples and as the basic PA fraction. (Sample A-E indicate the different plants).

* Results for green seeds are based on wet mass**.**

Plant extract	Unsaturated PA concentration as retrorsine equivalents (µg.g^{-1})											Total
Retention time (minutes)	8.2	12.3	14.4	14.6	14.7	15.6	16.2	16.4	17.7	19.5	19.7	
Pseudo-molecular ion mass	256	238	329	354	336	352	338	396	358	320	342	
Sample B green seeds (Feb) reduced				0.43				3.38				3.81
Sample B green seeds (Feb)	0.32			0.28				1.77				2.37
Sample B green seeds (March) reduced	0.29			0.30				0.82				1.41
Sample B green seeds (March)	0.30			0.26				0.39				0.95
Sample B dried seeds (May) reduced	24.76	7.50	1.86	23.35	0.52	0.14			12.92	1.99	1.51	74.55
Sample B dried seeds (May)	21.23	8.35	1.03	20.66	0.34				12.39	0.74	0.52	66.54
Sample A dried seeds reduced	25.17	7.90	20.23	60.13				2.02	30.50	2.79	1.62	150.36
Sample A dried seeds	22.09	11.82	16.68	54.24				11.07	22.31	1.24	0.69	140.26
Sample C dried seeds reduced	8.87	6.15	18.94	15.27	0.49		0.49	1.19	17.39	1.21	0.49	69.2
Sample C dried seeds	10.40	4.76	19.50	15.30	0.38			0.31	15.60	1.69	0.71	68.85
Sample D dried seeds reduced	22.89	15.3	31.37	25.64	0.83		0.15	6.05	16.46	5.0	1.7	125.39
Sample D dried seeds	24.95	13.92	33.97	19.70	0.70			0.49	15.38	5.85	2.14	117.10
Sample F dried seeds reduced	5.25	20.85	24.14	8.46				2.01	9.52	0.34	0.42	70.99
Sample F dried seeds	5.82	10.98	28.12	5.29				0.28	9.24	0.63		60.36

Table 3-5: Quantitative results of the possible toxic PAs present in *C. sphaerocarpa* roots and leaves. Results are presented as the total PA fraction (*N*-oxide + basic) found in the reduced samples and as the basic PA fraction. (Sample A-E indicate the different plants)

* Results for green roots are based on wet mass**.**

Plant extract	Unsaturated PA concentration as retrorsine equivalents (µg.g^{-1})											Total
Retention time (minutes)	8.2	12.3	14.4	14.6	14.7	15.6	16.2	16.4	17.7	19.5	19.7	
Pseudo-molecular ion mass	256	238	329	354	336	352	338	396	358	320	342	
Sample B green roots (Feb) reduced	22.57	10.34		10.97	167.1	10.17	4.46	216.6		0.47		442.7
Sample B green roots (Feb)	24.48	0.98		1.63	18.63	19.47	6.78	17.16		0.10		89.2
Sample B green roots (March) reduced	10.53	7.18		10.73	123.88	8.12	7.29	125.43				292.4
Sample B green roots (March)	9.19	0.54		0.57	20.04	3.12	1.80	24.17				59.4
Sample B dried roots (May) reduced		5.63		5.63	54.19	6.23	9.23	47.27	0.12			128.4
Sample B dried roots (May)		1.56		1.56	18.42	12.14	1.32	11.24	0.06			46.3
Sample D dried roots (May) reduced		6.85		6.85	103.3	9.56	13.68	97.65	0.14			238.0
Sample D dried roots (May)		1.98		1.98	43.81	16.58	2.70	21.39	0.07			88.5
Sample B green leaves (Feb) reduced	0.46				0.98	0.48		1.44				3.4
Sample B green leaves (Feb)												
Sample B green leaves (March) reduced								1.66				1.7
Sample B green leaves (March)								0.6				0.9
Sample B dried leaves May) reduced				1.04	8.41	1.33	0.77	6.50				18.1
Sample B dried leaves (May)				1.6	0.73	1.69		4.55				8.6
Sample A dried leaves reduced	1.86	0.71	3.39	9.46	10.55	0.73	0.71	16.26	1.08			44.8
Sample A dried leaves	2.07		3.86	7.30	4.22	1.47	0.27	4.47	0.79			24.5
Sample C dried leaves reduced	2.13	2.18	6.70	2.93	8.64	0.79	1.26	4.34	1.05			30.0
Sample C dried leaves	1.23		6.48	1.09	3.56	1.12	0.86	1.76	0.87			17.0

Table 3-6: Quantitative results of the possible toxic PAs present in *C. sphaerocarpa* stems and plant material. Results are presented as the total PA fraction (*N*-oxide + basic) found in the reduced samples and as the basic PA fraction.

* Results for green samples are based on wet mass

Plant extract	Unsaturated PA concentration as retrorsine equivalents (µg.g^{-1})											Total
Retention time (minutes)	8.2	12.3	14.4	14.6	14.7	15.6	16.2	16.4	17.7	19.5	19.7	
Pseudo-molecular ion mass	256	238	329	354	336	352	338	396	358	320	342	
Sample B green stems (Feb) reduced	0.67			6.34				64.81				71.8
Sample B green stems (Feb)	0.34			0.82				9.15				10.3
Sample B green stems (March) reduced	0.82			1.69	2.33	0.35	1.74	28.69				35.6
Sample B green stems (March)	1.0			1.15	0.90	0.33	0.89	11.64				15.9
Sample B dried stems (May) reduced	1.09		0.86	2.73	6.03	0.26	1.31	6.71	0.34			19.3
Sample B dried stems (May)	1.55		1.14	1.80	2.10	0.38	0.86	0.74				8.6
Sample C dried stems reduced			0.47	1.97	0.50			7.09				10.0
Sample C dried stems			0.92	1.34	0.49		0.49	1.58				4.8
Sample E dried plant material reduced	5.40	2.93	10.01	6.72	4.48	0.21	0.30	5.32	8.65	0.24		44.4
Sample E dried plant material	6.20	2.80	10.70	6.80	2.07	0.50		1.93	7.81	0.42		39.5

3.4 Discussion

3.4.1 Method and instrumentation

Retrorsine and monocrotaline were selected as reference standards as they were commercially available. The concentration of all the unsaturated PAs were calculated against a retrorsine calibration curve and reported as $\mu g.g^{-1}$ retrorsine equivalents. This is an estimation of the relative quantity of the specific PA present and not a reflection of the toxicity of the compounds.

The optimization conditions were compared between two different MS detectors. Standard solutions were infused in a Micromass Quatro Micro detector and a Micromass Quatro Ultima detector. The optimum conditions were nearly identical in both instruments, with the capillary voltage and cone settings slightly lower (2.5 V and 15 V) in the Ultima when compared to the Micro (3.2 V and 20 V). The same precursor ions were identified in extracted samples when these were injected on both instruments. It is clear from the comparison that the screening method was not instrument dependent and that the same precursors would be found on different detectors, once the instrument conditions has been optimized with a reference standard solution.

The HPLC mobile phase was chosen to be compatible with the LC-MS systems. Ammonium acetate is very volatile and promotes desolvation of the mobile phase. The nitrogen atoms of the necine bases are ionized at pH 3.84 which enhances the formation of the $[M+H]^+$ pseudo-molecular ions in the source. The gradient system ranging from 10 % to 90 % organic content can easily be adjusted to achieve separation of most co-eluting compounds. This specific HPLC mobile phase is also often used in systematic toxicological analysis in which the elution order of many toxic compounds is already known (Fitzgerald *et al*. 1999). Analysis of PAs on this system will therefore add useful information to existing toxicology screening procedures.

It is clear from the results in Table 3-2 that poor recoveries were achieved when PAs were extracted from acidic solutions using liquid-liquid extraction (method 3). Higher recoveries were achieved with liquid-liquid extraction from basic (pH>9) solutions (method 1, 4 and 7). Extracts need to be clean as dirty samples may cause matrix interferences which may lead to ion suppression in the source. Extractions with hexane (method 7) removed unwanted waxes and chlorophyll more effectively than ethyl ether (method 1) and led to cleaner extracts.

Extraction of the PAs with ethyl acetate was less problematic than dichloromethane (method 4), which tended to form emulsions. Solid phase extraction with cation exchange (SCX, method 5) and mixed mode columns (HCX, method 6) yield reasonable to high recoveries, but some of the plant extracts caused column blockages (insoluble waxes), limiting this application to clean samples, which are completely soluble in aqueous solutions.

3.4.2 Quantification

The lack of commercially available reference materials is the major limitation in PA analysis and results were therefore calculated as $\mu g.g^{-1}$ retrorsine equivalents. The method was developed as a screening for unsaturated PA molecules, and normalizing results to retrorsine equivalents allows quantitative comparisons between plants. This approach has been used in other studies e.g. expressing results as heliotridine equivalents in the ANZFA report (2000).

Variation in response of early eluting compounds may overestimate the calculated amount, e.g. 126 % recovery of monocrotaline at 50 $ug.ml^{-1}$. Peak broadening and tailing of higher concentrations may also invalidate results. Dilution of these samples before quantification will, however, eliminate the problem.

3.4.3 Possible toxic PAs in *C. sphaerocarpa*

Crotalaria spp. are reported to contain mainly basic PAs and little *N*-oxides. Except for the roots, this was also true for *C. sphaerocarpa*. Marais (1944) found that the PA crude extract made up about 0.05 % of the *Crotalaria* plant mass. In this study the total unsaturated PA content in the various plant parts ranged between 0.001 – 0.04 % mass per mass.

Very low unsaturated PA concentrations were found in green seeds. The concentrations of unsaturated PAs in the dried seed samples ranged between 60 and 150 $\mu g.g^{-1}$. These PAs were present as basic alkaloids with molecular masses 255, 237, 328, 353 and 357 present in significant quantities. These PAs could, however, not be identified. Sample A, which had the highest PA concentration in the seeds, was obtained from ARC where it was grown under optimum conditions, to test the efficacy of certain herbicides on these plants in field trials. These seeds were generally heavier (14.2 mg ± 2.8) and intact and showed few signs of insect damage. The other seed samples weighed slightly less (11.1 mg ± 2.2 mg sample B; 12.2 mg ± 4.1 mg sample C) and many of these seeds showed signs of insect infestations, with many of

the seed pods empty or occupied by larvae, which may explain the lower PA per gram concentrations. The unsaturated PA content of the empty pods were investigated and found to be below detectable levels. The ratio of pod mass to seed mass in the samples may add to the difference in the final concentration of the seed samples.

The highest concentration of unsaturated PAs in the different plant parts was found in the roots, mainly as the *N*-oxides (443 $\mu g.g^{-1}$ in green roots collected in February 2005). These results correlate with data of Ober and Hartmann (1999), who proposed that PAs are produced in the roots of plants as *N*-oxides and then transported to other parts. The roots contained mainly two PAs with molecular masses 395 and 335. For some compounds found in the roots, e.g. $[M]^+$ 352, the ratio of basic alkaloid : total PA is inverted which is possibly due to interfering substances present in the samples that were not reduced.

The concentrations in other parts of the plant were much lower (approximately 1 - 70 $\mu g.g^{-1}$). Green stems contained about 10-15 $\mu g.g^{-1}$ basic alkaloid and 35-70 $\mu g.g^{-1}$ total PA. When the moisture content (51%) is taken into account the concentration in the green stems is 20-30 $\mu g.g^{-1}$ basic alkaloid and 70-140 $\mu g.g^{-1}$ total PA. The concentration decreased with age to 5-10 $\mu g.g^{-1}$ basic and 10-20 $\mu g.g^{-1}$ total PA in the mature, senescent stage, respectively. This also conforms to the notion that the alkaloids are produced and transported as the *N*-oxides, which are then converted to the basic alkaloids as the plant matures. The concentration of PAs in the green leaves was below 4 $\mu g.g^{-1}$ and increased with age to 45 $\mu g.g^{-1}$ with the ratio of 1:2 for basic alkaloid to total PA.

In general, when comparing the various dried plant samples, sample A contained the highest concentration of unsaturated PAs in the plant parts analyzed, followed by sample D, while the concentrations in samples B, F and C were very similar (see page 44 for origin of samples). There was no correlation in the results of the genetically related samples (A, grown at Potchefstroom and B, grown in Centurion). Correlating results were found in sample B and F, both grown in the Centurion area. This indicates that habitat and climatic factors may have an effect on the levels of PAs. Samples C and D were both grown in the Bothaville district but the PA levels differed. This may have been due to a residual effect of herbicide treatment of only sample C. These tentative conclusions will have to be confirmed by further analysis.

Crotalaria species are generally expected to contain high concentrations of dicrotaline and related compounds. In this study the retention behavior of unsaturated PAs present in *C.*

sphaerocarpa were retained longer on the C_{18} column (retention time between 8 – 20 minutes), indicate that the compounds are less polar than monocrotaline (retention time 4 minutes), and similar to retrorsine (retention time 15 minutes). It was concluded from the results obtained with this screening method that *C. sphaerocarpa* contained various unsaturated PAs, which are structurally closely related to retrorsine. The results were used to calculate the allowable level of contamination of grain with *C. sphaerocarpa* seeds (see paragraph 7.3)

With the low levels of these unsaturated PAs present in seeds, it would be a challenge to isolate sufficient material for structural elucidation. Isolation of the two *N*-oxides found in high concentrations in green roots may be a practical approach to follow.

CHAPTER 4: STABILITY OF PYRROLIZIDINE ALKALOIDS DURING FOOD PREPARATION

4.1 Introduction

One of the objectives of the study was to determine the stability of unsaturated PAs during food preparation. The potential toxicity and hazard to human health will be considerably lower if the necine bases become degraded at high temperatures during food preparation. The stability of unsaturated PAs at high temperatures during maize porridge cooking and herbal tea preparation was investigated. Control samples which were not exposed to high temperatures were prepared together with the cooked samples. The control samples were used to determine whether loss of response was due to PA degradation or due to matrix effect during extraction and analysis.

4.2 Experimental procedure

A standard solution containing retrorsine (50 µg.ml^{-1}) in diluted hydrochloric acid (0.1 M) was prepared. The standard solution (1 ml) was diluted in 50 ml water and injected as a reference sample.

Two maize meal samples were prepared; a cooked sample and a raw control sample. Maize meal (2 g) was weighed into two glass beakers. Standard solution (1 ml) and water (50 ml) was added to each sample.

Dried, milled lucerne (4 g) was used to simulate herbal tea and two samples (cooked and raw) were again prepared. Standard solution (1 ml) and water (50 ml) were added to each sample.

The cooked samples were heated in a boiling water bath for three hours, while the raw samples were left at room temperature. The diluted standard solution and prepared samples were made basic (pH>9) with ammonia solution and extracted with ethyl acetate. The ethyl acetate was evaporated under reduced pressure and the samples reconstituted in 2 ml methanol and analyzed on LC-MS in MRM mode as described in Chapter 3. The peak areas

of retrorsine in the reference sample were compared with the retrorsine peak areas obtained with each sample.

4.2.1 Results

Table 4-1: Stability of retrorsine in different matrices after cooking

Samples	Peak area	% of reference peak
Reference sample	43498	
Raw herbal tea sample	41450	95.3
Cooked herbal tea sample	40947	94.1
Raw maize sample	17591	40.4
Cooked maize sample	10624	24.4

4.2.2 Discussion

The small difference in the results between the reference and the tea samples was within the analytical variation of the method and the retrorsine concentration in this sample was not affected by the cooking process.

Severe problems with emulsion formation were experienced during the extraction of the maize samples, and the low concentration of retrorsine in both the raw and cooked samples can be ascribed to extraction inefficiency, rather than instability of the PAs during cooking. One of the objectives of the study was to determine the LLOD for toxic PAs in maize. After these experiments it became clear that this would not be possible, due, rather to extraction problems, than to the analytical method.

It is concluded from the results obtained with the tea samples that toxic retronecine type PAs are not affected by high temperatures during normal cooking procedures. This is consistent with an outbreak of “Bread poisoning” in South Africa where contaminated wheat flour was used to bake bread (Willmot and Robertson 1920).

CHAPTER 5: OTHER ANALYTICAL METHODS

5.1 Background

Some of the analytical methods discussed in Chapter 2 were investigated in an effort to evaluate the sensitivity and specificity of the LC-MS/MS screening method. The results of these experiments are discussed in this chapter.

5.2 Ehrlich's screening methods

5.2.1 Introduction

The method described by Mattocks and Jukes (1987) uses Ehrlich's reagent to determine whether 1,2-unsaturated PAs and PA *N*-oxides are present in plant samples. When the analysis of *C. sphaerocarpa*, *C. laburnifolia, C dura* and *S. inaequidens* samples were first attempted using this method both the sample and the blank gave a positive colour reaction, possibly due to other unknown indoles present in the plants. It was therefore decided to evaluate the method on extracted plant samples.

5.2.2 Materials and method

Ascorbic acid, glacial acetic acid, perchloric acid 70%, ortho-chloranil, sodium nitroprusside and 4-diaminebenzaldehyde were obtained from Merck (SA)

The same liquid-liquid extraction method was used to extract the unsaturated PAs from the different plant samples. The samples were divided into reduced and unreduced fractions and Ehrlich's screening tests were performed on both fractions of all the plant samples.

To test for the presence of *N*-oxides, extracted plant material was reconstituted in methanol and aliquots (0.1 ml) of each extract were transferred to separate test tubes and labeled as "sample" and "blank". Aqueous ascorbic acid (5%, 1 ml) was added to each tube. An aqueous solution (0.1 ml) of sodium prusside (5% in 1mM sodium hydroxide) was added to the test

sample. Both samples were heated for 1 minute at 75 ºC. Ehrlich's reagent (0.2 ml) [containing 4-dimethylaminobenzaldehyde (5 g) dissolved in water (30 ml), acetic acid (60 ml) and perchloric acid (10 ml)] was added to both samples and heating continued for an additional minute. Any magenta colour in the test sample was due to the presence of unsaturated *N*-oxides.

To test for unsaturated necine bases the procedure was changed slightly: extracted plant material was reconstituted in methanol and aliquots (0.1 ml) of each extract were again used as "sample" and "blank". Chloroform (1.0 ml) was added to each tube. The solution was heated slightly with a solution (0.1 ml) of ortho-chloranil (0.5% in acetonitrile) and then with Ehrlich reagent. A magenta colour in this test sample was due to the presence of unsaturated necine bases.

5.2.3 Results

Table 5-1: Results of the screening test with Ehrlich's reagent. (- no colour, + slight colour, ++ moderate colour, +++ intense colour)

Plant extract	Mass (g)	Fraction	PA conc. µg.g^{-1}	*N*-oxide screen		Basic alkaloid screen	
				blank	test	blank	test
C. sphaerocarpa seeds from sample A	0.5	basic	140	-	-	-	+
		reduced	150	-	-	-	+
C. sphaerocarpa powdered plant from sample E	0.5	basic	39	-	-	-	-
		reduced	44	-	-	-	-
C. laburnifolia powdered plant material *	2.5	reduced	20	+	+	+	+
C. dura powdered plant material *	2.5	reduced	589	++	++	+++	+++
S. inaequidens powdered plant material	0.12	basic	250	-	+++	-	++
		reduced	12000	-	+	-	+++

* Results for *C laburnifolia* and *C dura* from Chapter 6

5.2.4 Discussion

The slight positive result with the *C. sphaerocarpa* seed sample is in accordance with the low unsaturated PA concentration found with LC-MS/MS. The concentration of unsaturated PAs in the powdered *C. sphaerocarpa* plant sample was below the visible detection limit of the method. The positive results with the blank samples of both *C. laburnifolia* and *C. dura* were

most likely due to the presence of other pyrroles and indoles which were co-extracted. *Senecio inaequidens* contained high concentrations of unsaturated PAs, mainly as the *N*-oxides. The positive result obtained with the *N*-oxide screen indicates that some of the *N*-oxides were co-extracted together with the basic unsaturated PAs, mainly due to the high concentration of *N*-oxides present.

All the blank samples gave positive colour reactions when the plant samples were initially investigated with this screening method. This was most likely due to other unsaturated pyrroles and indoles, which were removed, except from *C. laburnifolia* and *C. dura,* by first extracting the unsaturated PAs. The results found in the extracted samples agreed with the unsaturated PA concentrations found with the LC-MS/MS method.

This screening method with Ehrlich's reagent may be useful for the detection of unsaturated PAs in the absence of more sophisticated equipment, e.g. at the silo where grain is received, provided that the suspected contaminant seeds are first isolated and extracted according to the method described here.

5.3 GC-MS methods

5.3.1 Introduction

Various unsaturated PAs were detected by the LC-MS/MS method. The molecular masses of these were obtained with the precursor ion experiments and the unsaturated necine structures were confirmed with product ion scans. Many unsaturated PAs exist as isomers, or share the same molecular mass and the information obtained with the LC-MS/MS method could not positively identify these toxic PAs. All the extracts prepared for LC analysis were analyzed on GC-MS in an attempt to identify the toxic PAs with MS library matching. The more meaningful results are discussed in this section

5.3.2 Gas Chromatography with EI

5.3.2.1 Instrument and method

The GC used was a Hewlett Packard HP5973 (Agilent Technologies, SA) GC-MS instrument. The column was a CPsil 5CB 25 m x 0.32 mm x 0.25 µm (CROMPAK). The MS detector was set at 230 ºC with the auxillary line at 280 ºC. Injector was at 240 ºC. Oven temperature

program was 50 ºC at 0-0.5 min; increased 10 ºC.min^{-1} to 200 ºC; increased 30 º C.min^{-1} to 290 ºC and held 6 minutes (total runtime was 25 min). The column flow was 2.6 ml.min^{-1}. The library used was Wiley Version 4.

5.3.2.2 Results and discussion

Unsaturated PAs all revealed intense fragments at *m/z* 94, 120 and 138 when using EI. A spiked lucerne standard (20 ug.ml^{-1} retrorsine and monocrotaline) was injected into the GC. The ion, *m/z* 120 was used to extract the unsaturated PAs from the total ion chromatograms (Fig 5.1). The mass spectrum of each of the peaks was obtained and a spectra library search was done on each spectrum against the library.

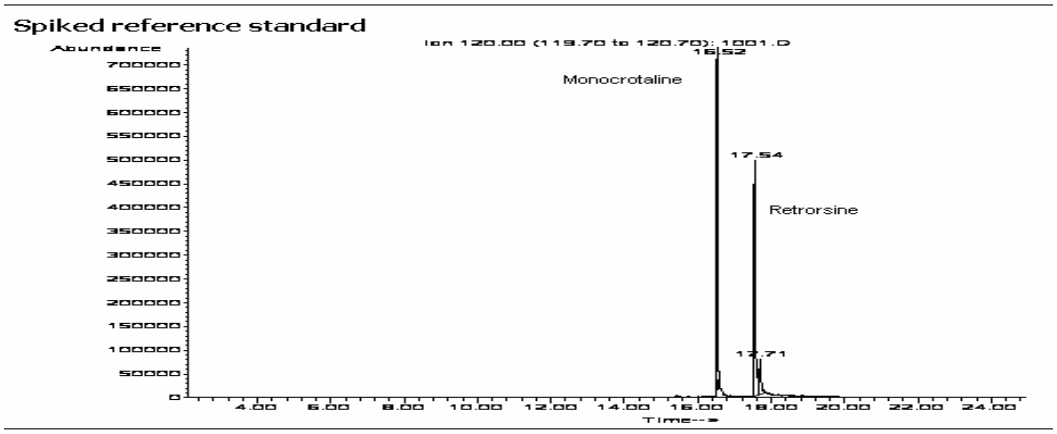


Figure 5-1: Reconstructed EI-MS chromatogram of the extracted ion *m/z* 120, of a reference standard containing monocrotaline and retrorsine in lucerne extract

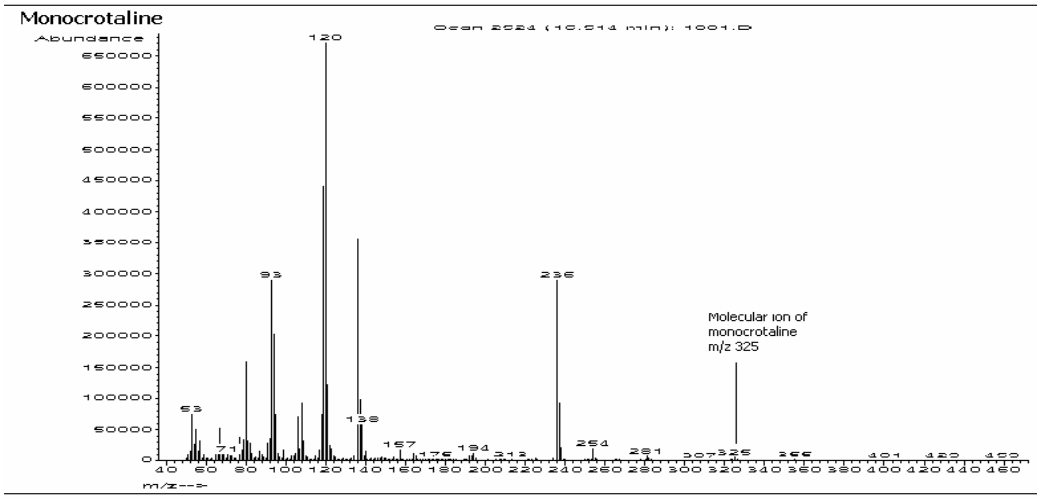


Figure 5-2: EI-MS spectrum of monocrotaline

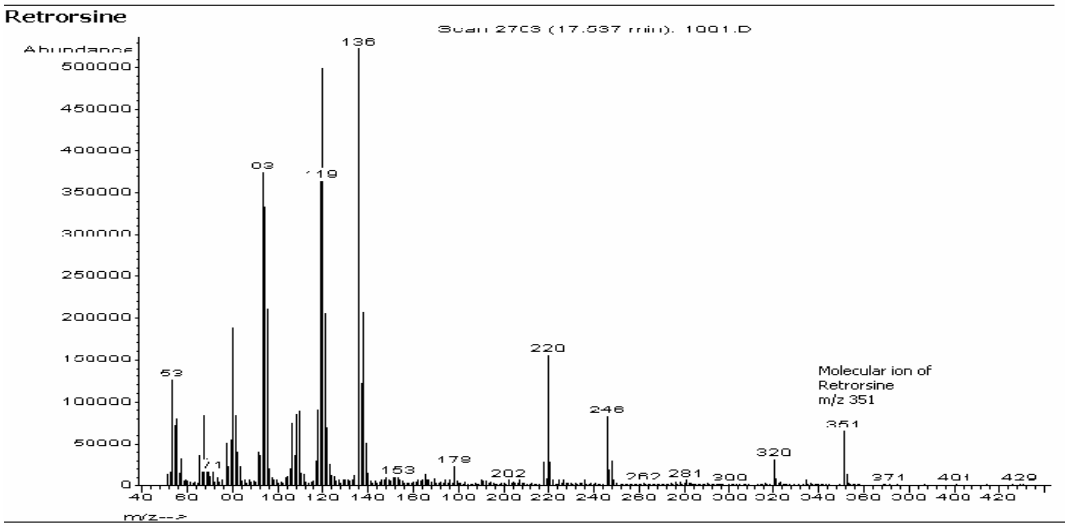


Figure 5-3: EI-MS spectrum of retrorsine

The characteristic fragments of unsaturated PAs (*m/z* 80, 94 and 111), as discussed in Chapter 2, are all present in the EI spectra of the reference compounds together with prominent fragments at *m/z* 120 and 136, confirming the diester structures of monocrotaline and retrorsine (Fig 5-2 and Fig 5.3). These fragments appeared in different ratios in the spectra of the two standard compounds, but were all products of the unsaturated retronecine base, while little information could be gained about the acid moiety. Monocrotaline was fragmented to such an extent that the molecular ion $[M]^+$ 325 was difficult to identify, serving as a good example of the limitations when trying to identify unknown compounds by their GC/MS spectra. The abundance of the molecular ion $[M]^+$ 351 in the retrorsine spectrum was much higher and it was therefore possible to identify retrorsine in the chromatogram. Monocrotaline was, however, positively identified when using the library search options. Retrorsine, on the other hand, could only be matched with usaramine in the library search. Usaramine has the same molecular mass as retrorsine, but the acid moiety has a different configuration and the ratios of the fragments are also slightly different.

A reduced extract of the powdered plant specimen (sample E) was injected into the GC (Fig 5-4). Three unsaturated PAs were found by extracting the ion *m/z* 120. The spectrum search for the peak $[M]^+$ 335 at 16.89 minutes gave a 97% library match with integerrimine (Fig 5-5). This was also found with the LC-MS/MS method (pseudo-molecular mass 336 at a concentration of 4.5 $\mu g.g^{-1}$). The other peak $[M]^+$ 335 at 17.72 minutes could not be identified (Fig 5-7). The $[M]^+$ 351 compound at 17.56 minutes was probably a retrorsine isomer (Fig 5-6) as the retention time was identical to retrorsine in the standard injection, but the ratios of

the fragments did not correspond. The fragments derived from the acid moiety in this compound was also different when compared to that obtained with the retrorsine peak in the standard chromatogram.

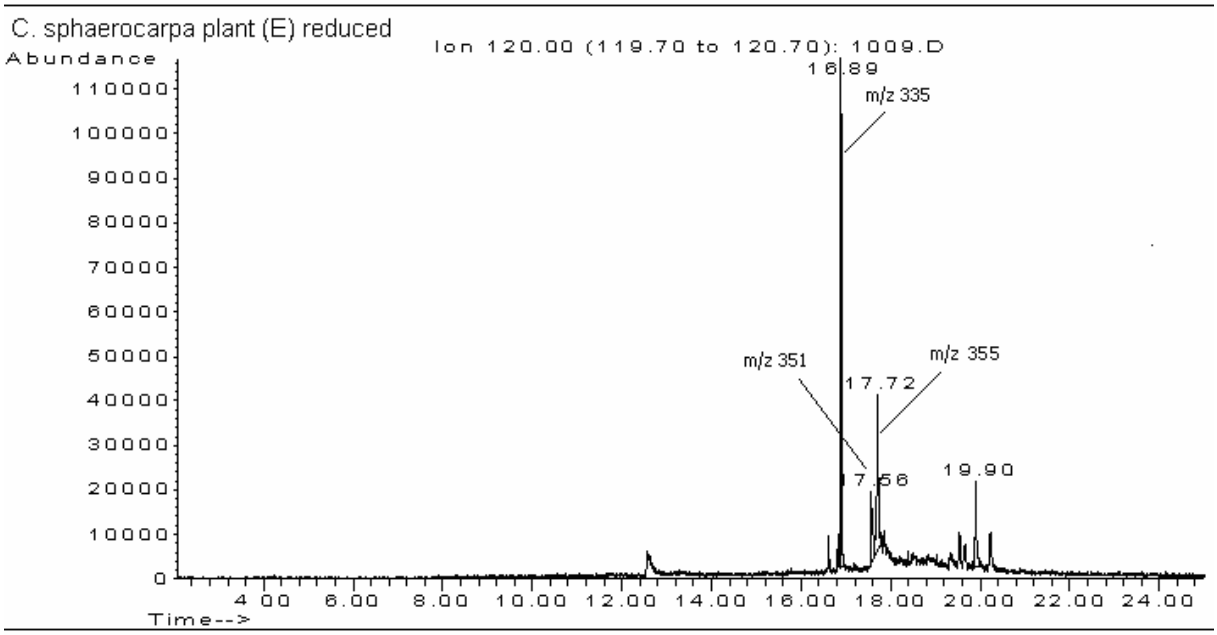


Figure 5-4: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of sample E

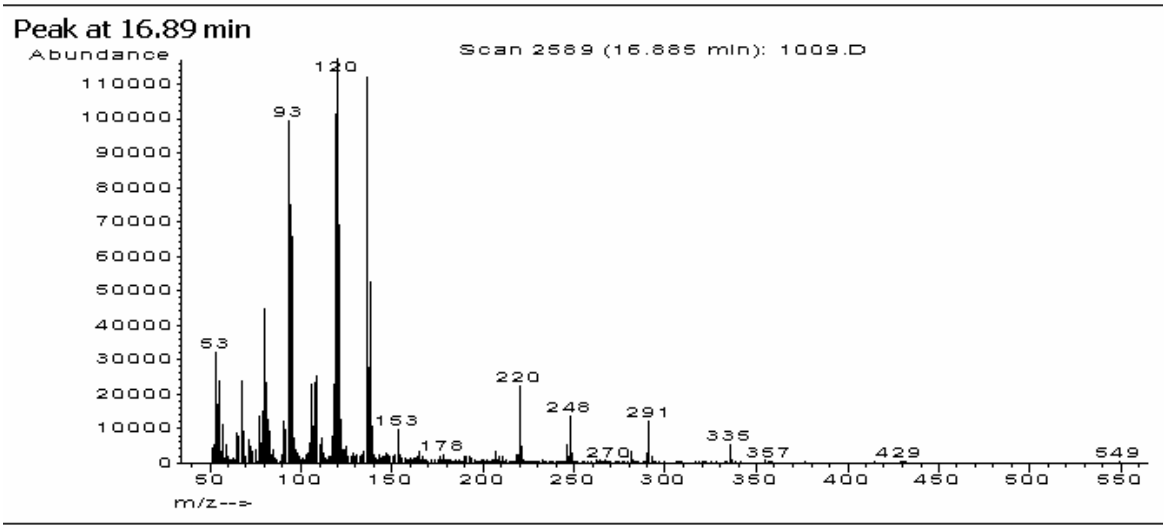


Figure 5-5: EI-MS spectrum of the peak $[M]^+$ 335 at 16.9 minutes, identified as integerrimine

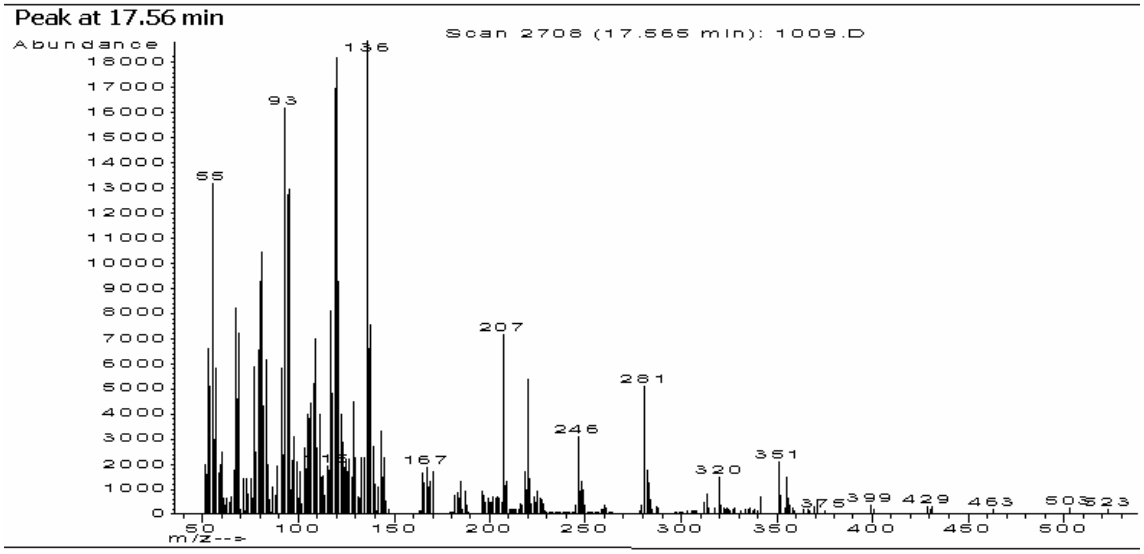


Figure 5-6: EI-MS spectrum of peak $[M]^+$ 351 at 17.56 minutes

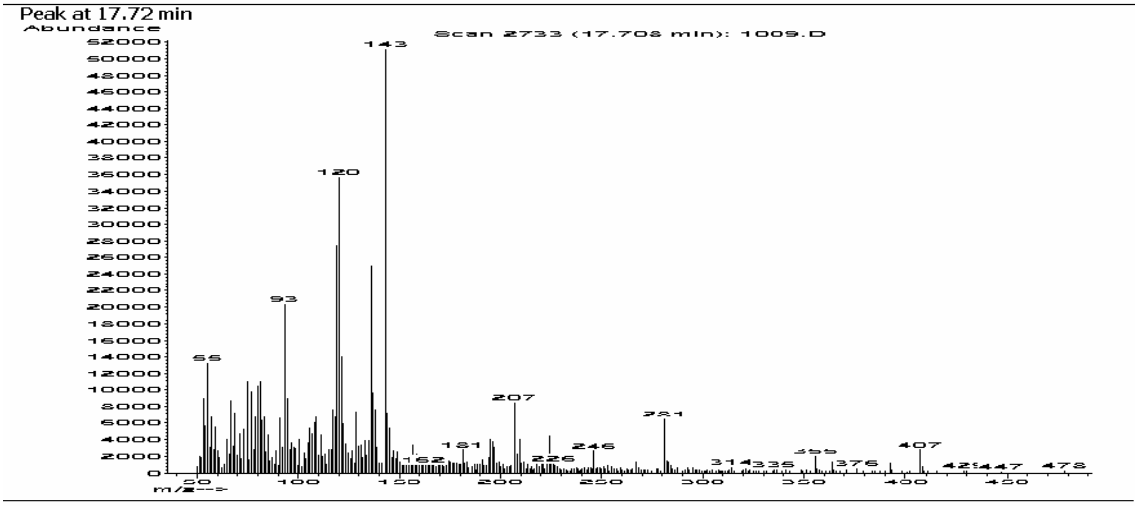


Figure 5-7: EI-MS spectrum of peak $[M]^+$ 355 at 17.72 minutes

A reduced seed sample (A) was also injected in the GC and the ion *m/z* 120 was extracted from the chromatogram (Fig 5-8). The overall abundance of the extracted peaks was much lower than sample E, indicating very low concentrations of unsaturated PAs found with GC. Two unsaturated PAs were however detected. The retention time of the $[M]^+$ 355 compound at 17.71 minutes (Fig 5-9) was identical to the compound $[M]^+$ 355 found at 17.72 minutes in the chromatogram of sample E, but the spectrum revealed different fragments, which could once again not be identified by library matching. The second compound at 18.45 minutes (Fig 5-10) also revealed a molecular ion $[M]^+$ 355. The spectrum of this compound revealed different fragments to that found in the peak at 17.72 minutes and no

library match was found for this spectrum either. The spectrum of peak at 12.5 minutes did not match that of unsaturated PAs.

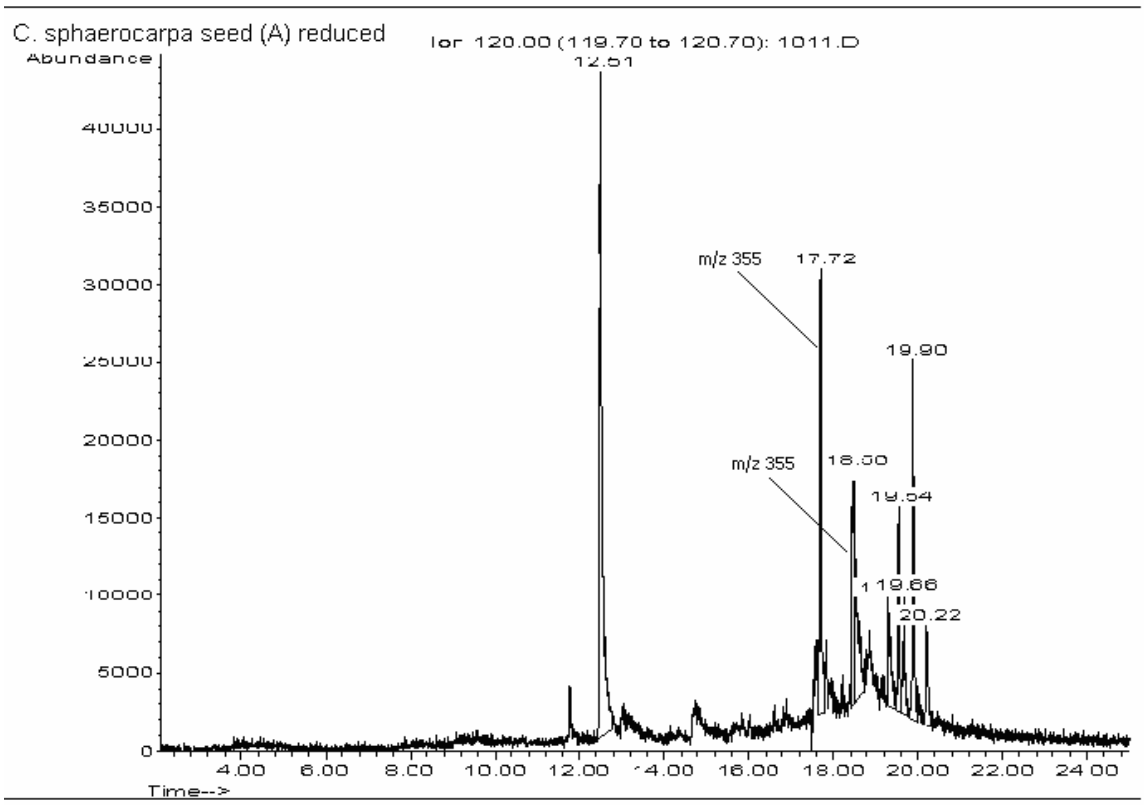


Figure 5-8: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced sample A in EI mode

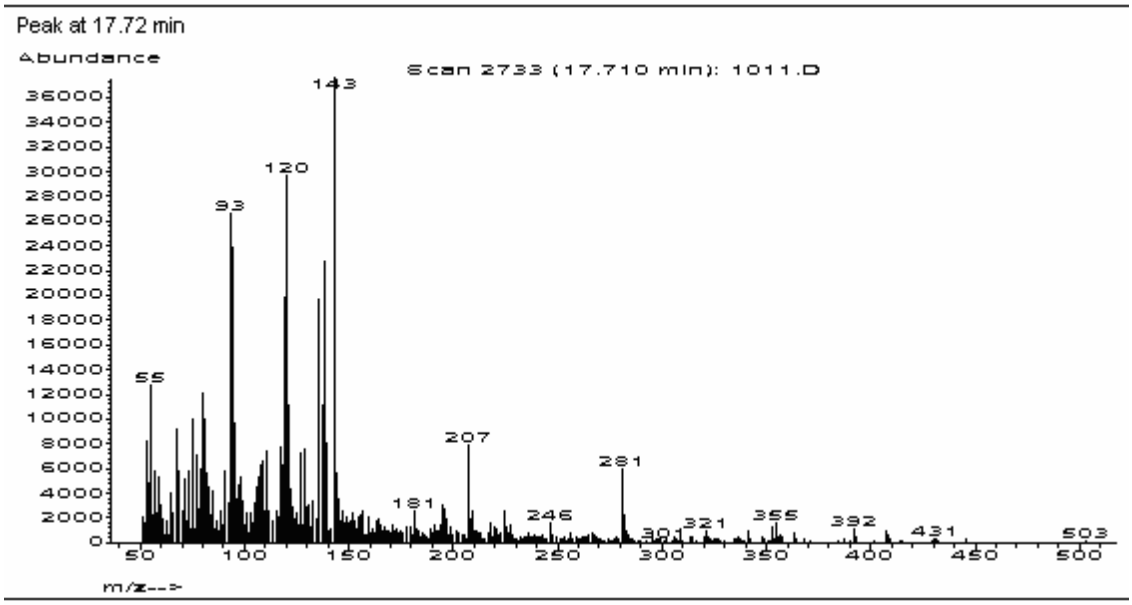


Figure 5-9: EI spectrum of peak $[M]^+$ 355 at 17.71 minutes

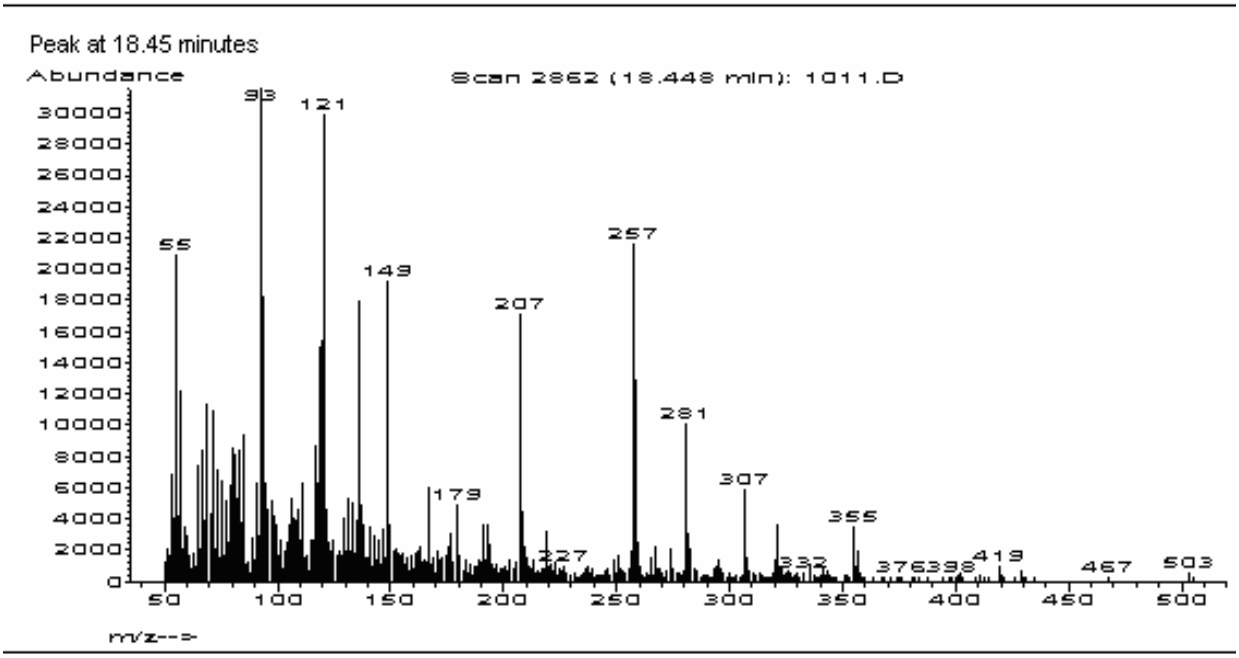


Figure 5-10: EI spectrum of peak $[M]^+$ 355 at 17.71 minutes

5.3.3 Gas Chromatography with CI

Positive chemical ionization is not generally used for identification purposes, as the ionization energy used is not universal, but needs to be set to produce the fragments of interest. It is however a soft ionization technique which yields high abundances of the pseudo-molecular ions $[M+H]^+$ in the case of positive CI, and can be used to confirm the molecular mass of the compounds of interest. The extracted samples prepared for the LC-MS/MS screening method were also injected into GC-MS with PCI in an effort to confirm the unsaturated PA structure of the components found with the LC-MS/MS screening method.

5.3.3.1 Instrument and method

The GC used was a Thermo MDQ with PCI (Thermo, SA) GC-MS instrument. The column was Rtx-1, 30 m x 0.25 mm x 0.1 µm id. (Thermo). The MS detector was set at 180 ºC with the reaction gas (methane) set at 1.6 $ml.min^{-1}$. Injector was at 220 ºC. Oven temperature program was 80 ºC at 0-4 min; increased 10 $ºC.min^{-1}$ to 290 ºC; and held 5 minutes (total runtime was 30 min). The column flow was 1 $ml.min^{-1}$.

5.3.3.2 Results and discussion

With PCI the pseudo-molecular ion $[M+H]^+$ is more stable and can easily be identified as seen in the spectra of monocrotaline (Fig 5-11) and retrorsine (Fig 5-12). Using methane as reaction gas, two other major fragments were detected at *m/z* 120 and 138 derived from the necine base and a third fragment [M+29] (possibly due to addition of two methyl groups to the di-ester groups).

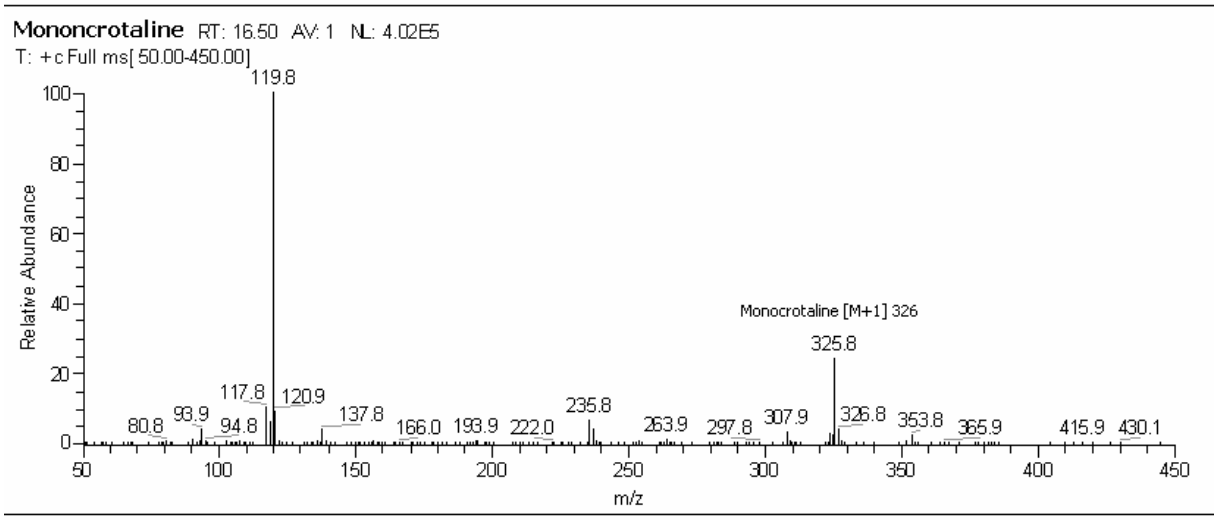


Figure 5-11: MS spectrum of monocrotaline obtained with PCI

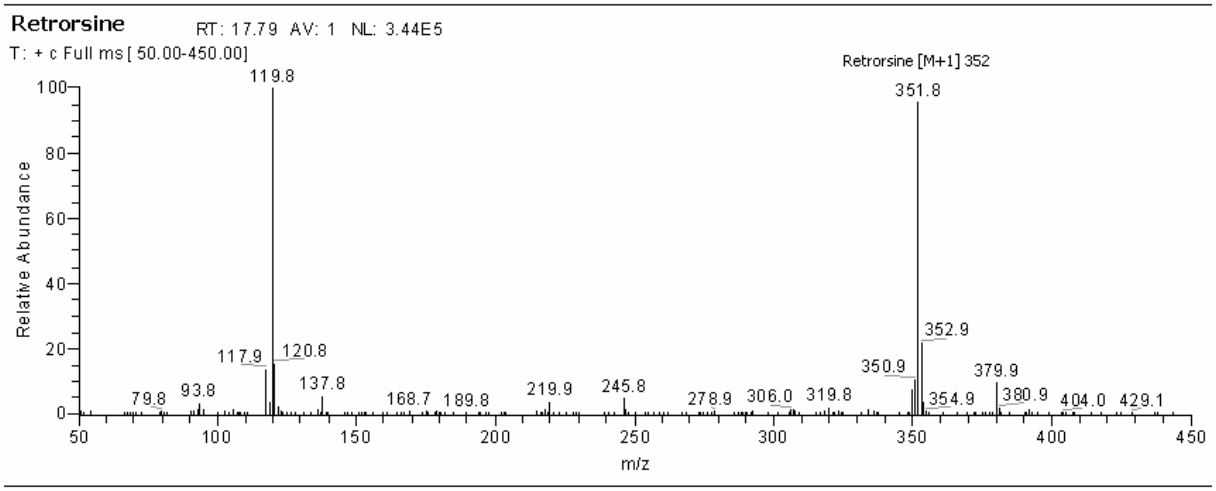


Figure 5-12: CI-MS spectrum of retrorsine

A reduced extract of the powdered plant specimen (sample E) was injected (Fig 5-13). The *m/z* 120 ion was used to extract possible unsaturated PAs. More unsaturated PAs were found with PCI, which is generally known to be more sensitive than EI. The retention times of the compounds were slightly longer than with GC-EI, due to the different column dimensions and gas flow settings. The $[M]^+$ 335 mass, identified with EI as integerrimine, was present at 19.06 minutes as $[M+H]^+$ 336. The other compounds did not correspond to those found with EI and could not be identified. In the spectra of all the peaks the abundance of the $[M+H]^+$ ion was the highest, with the *m/z* 120 fragment present between 60 – 100 % relative abundance. The fragments *m/z* 138 and the [M+29] fragments found with retrorsine and monocrotaline were also present in the spectra of other possible unsaturated PAs.

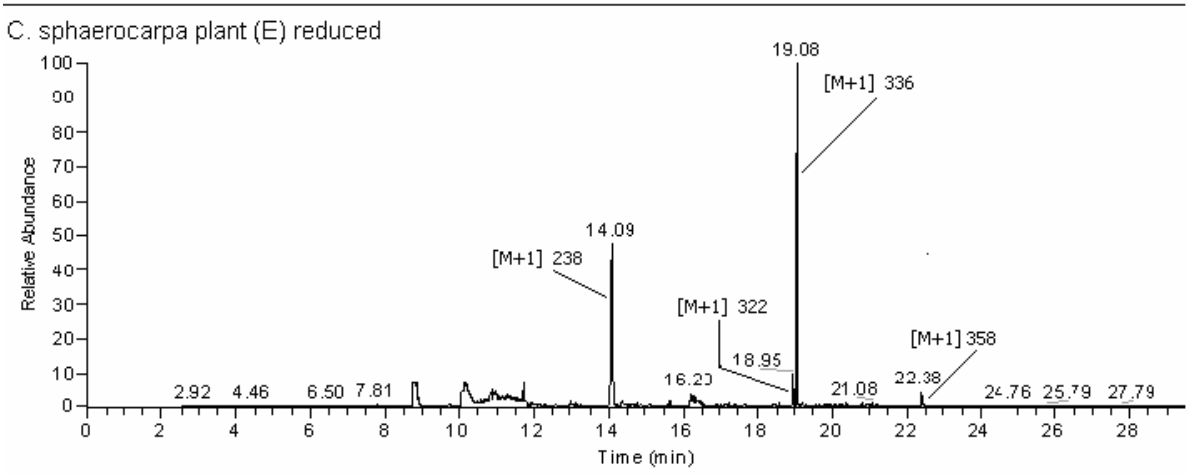


Figure 5-13: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of sample E in PCI mode

A reduced seed sample (A) was injected into the GC and the *m/z* 120 ion was extracted from the chromatograms. Ten of the unsaturated PAs found with the LC-MS/MS were detected. The spectra of these compounds also possessed fragments corresponding to *m/z* 120, 138, $[M+H]^+$ and [M+29]. Although the compounds could not be positively identified, the spectra obtained with PCI were accepted as confirmation that the compounds contained unsaturated necine basis.

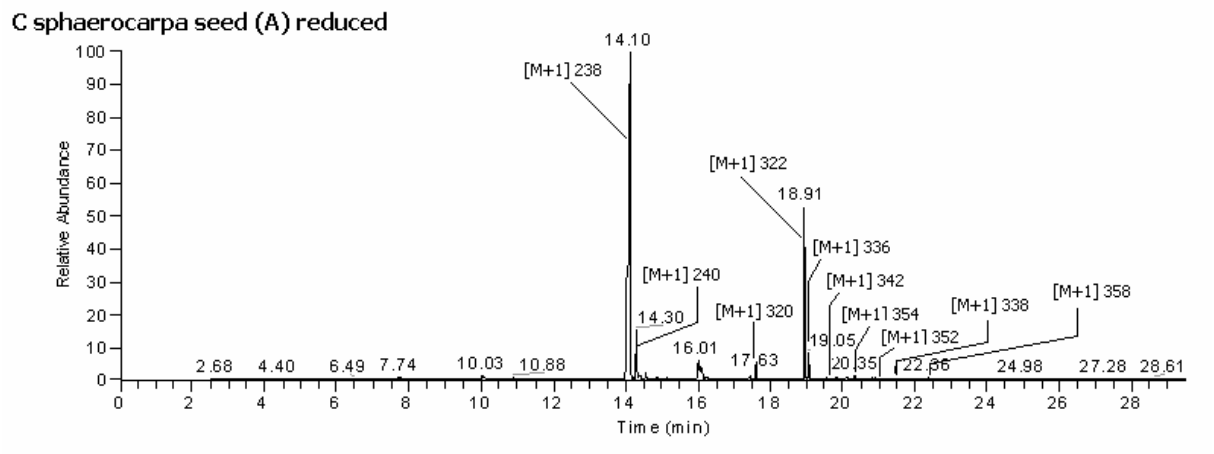


Figure 5-14: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of a seed sample (A) in PCI mode

5.3.4 Discussion of GC results

The elution order of the different PAs roughly followed molecular weight in agreement with previous reports (Stelljes *et al*, 1992). In general, GC-MS with EI was less sensitive than LC-MS/MS and was not very useful when attempts were made to identify the unknown PAs with commercially available library matching. Due to the lack of reference standards, appropriate RI data was also not available, making identification impossible in most cases. The intensity of the molecular ion for many of the PAs was often too low to be detectable, and this made it virtually impossible to relate the characteristic fragments to the original molecule.

GC-MS with PCI, on the other hand, gave fewer fragments and the molecular mass of the compound could easily be related back to the $[M+H]^+$ masses. Based on the findings with PCI most of the molecular masses of the unsaturated PAs detected with the LC-MS/MS method could be confirmed.

In general GC-MS with PCI is more sensitive and can also be used as a screening method for unsaturated PAs in plants by using the *m/z* 120 fragment to extract the possible unsaturated PAs from the total ion chromatogram. This fragment is, however, not unique to unsaturated PAs and confirmation on GC-MS with EI will be essential before any quantitative results can be accepted.

CHAPTER 6: ANALYSIS OF OTHER PLANTS CONTAINING PYRROLIZIDINE ALKALOIDS

6.1 Introduction

Another objective of the project was to determine if the technique could be applied to other plant species. *Senecio inaequidens* milled plant material was provided by Prof. Botha, Faculty of Veterinary Science, Onderstepoort. *Crotalaria dura* and *C. laburnifolia* milled plant material was supplied by Prof. Naudé, Faculty of Veterinary Science, Onderstepoort. Analysis of these samples served to test the ability of the LC-MS/MS method developed during this study, to detect toxic PAs in plants.

6.2 Senecio inaequidens

6.2.1 Background

It is well known that *Senecio* spp. contain toxic PAs. From the 1968/69 Annual Report, Onderstepoort Research Institute it appeared that *Senecio* spp. were responsible for the heaviest stock losses in South Africa during that period. Livestock may be either acutely or chronically poisoned, depending on the amount ingested and duration of exposure. Acutely intoxicated animals usually start dying within a day or two.

In October 2004 nine out of 29 adult cows died near Frankfort in the Free State Province, Republic of South Africa after grazing on plants suspected of containing PAs. Some of the plants growing there were collected and submitted for identification. The plants were identified by the National Herbarium of the South African National Biodiversity Institute as the potentially toxic *Senecio inaequidens* (DC) (Fig 6.1).

Although all *Senecio* spp. must be regarded as potentially toxic, no previous reports of poisoning by *S. inaequidens* in South Africa could be found. To confirm the circumstantial evidence linking the toxicity to this species, plant specimens as well as some of the rumen content were extracted and analyzed for toxic PAs using the LC-MS/MS method described.

Figure 6-1: *Senecio inaequidens*

A sample of the rumen content was collected during necropsy and stored frozen until analysis. Two plant samples were supplied: fresh plant material, which was collected and dried in the shade and milled, and plant material which had been stored in plastic bags, which became slightly mouldy before it was dried and milled.

6.2.2 Results

The samples were extracted as before and precursor ion scans were performed on the extracts. Very high concentrations of two possible hepatotoxic PAs were found in the plant samples (Fig 6-2). These components were also present in lower concentrations in the rumen sample (Fig 6-3). The peak at 11.3 minutes had a mass $[M+H]^+$ 352 and the one at 13.7 minutes a mass $[M+H]^+$ 336. The daughter ion spectra of these compounds (inserts) revealed the characteristic fragments *m/z* 120 and *m/z* 138 associated with unsaturated toxic PAs.

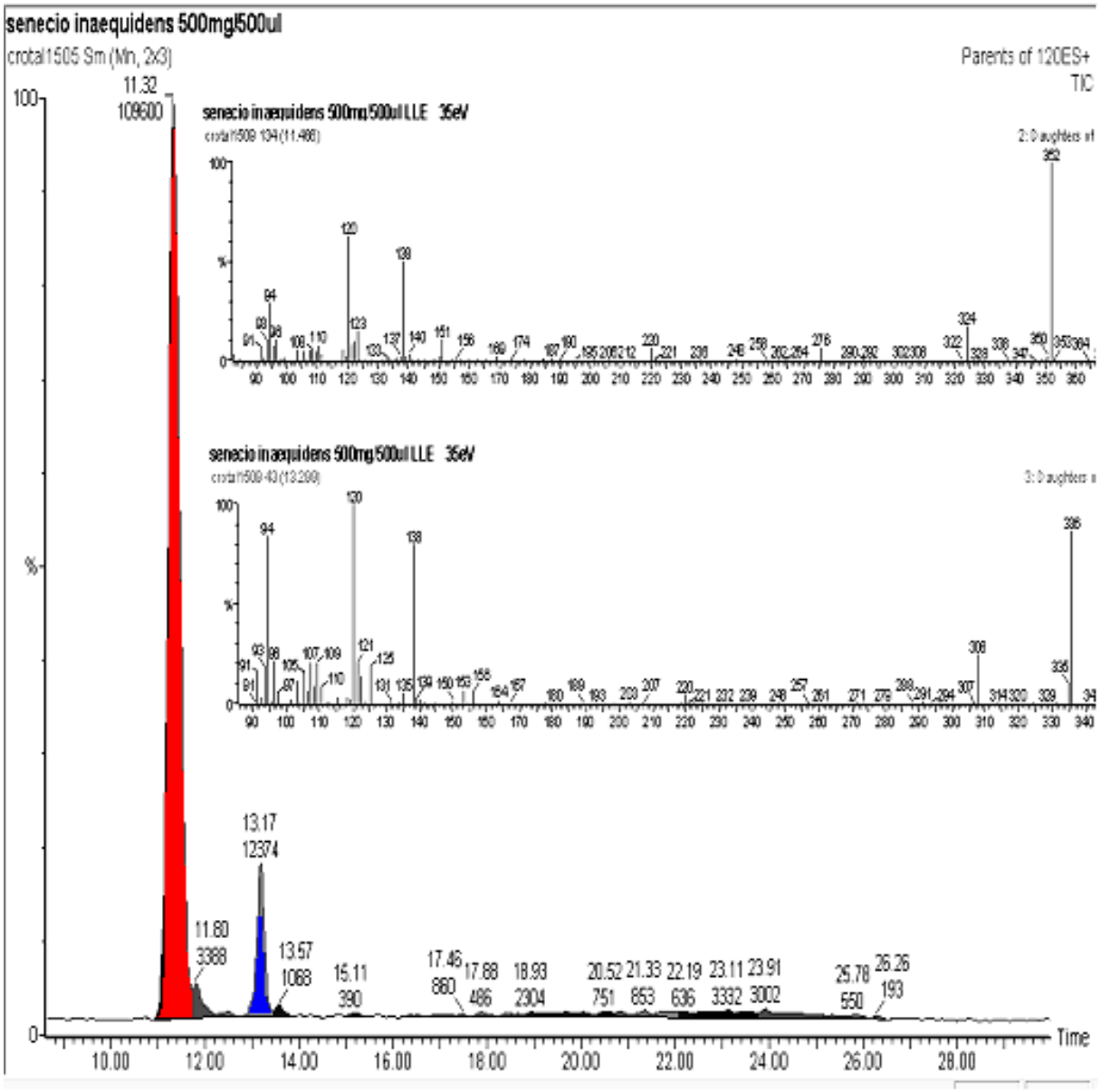


Figure 6-2: Total ion chromatogram [parent ions of *m/z* 120] of extracted *Senecio inaequidens* plant material. Inserted windows are the spectra of the two toxic PAs ($[M+H]^+$ 352 at 11 min and ($[M+H]^+$ 336 at 13 min)

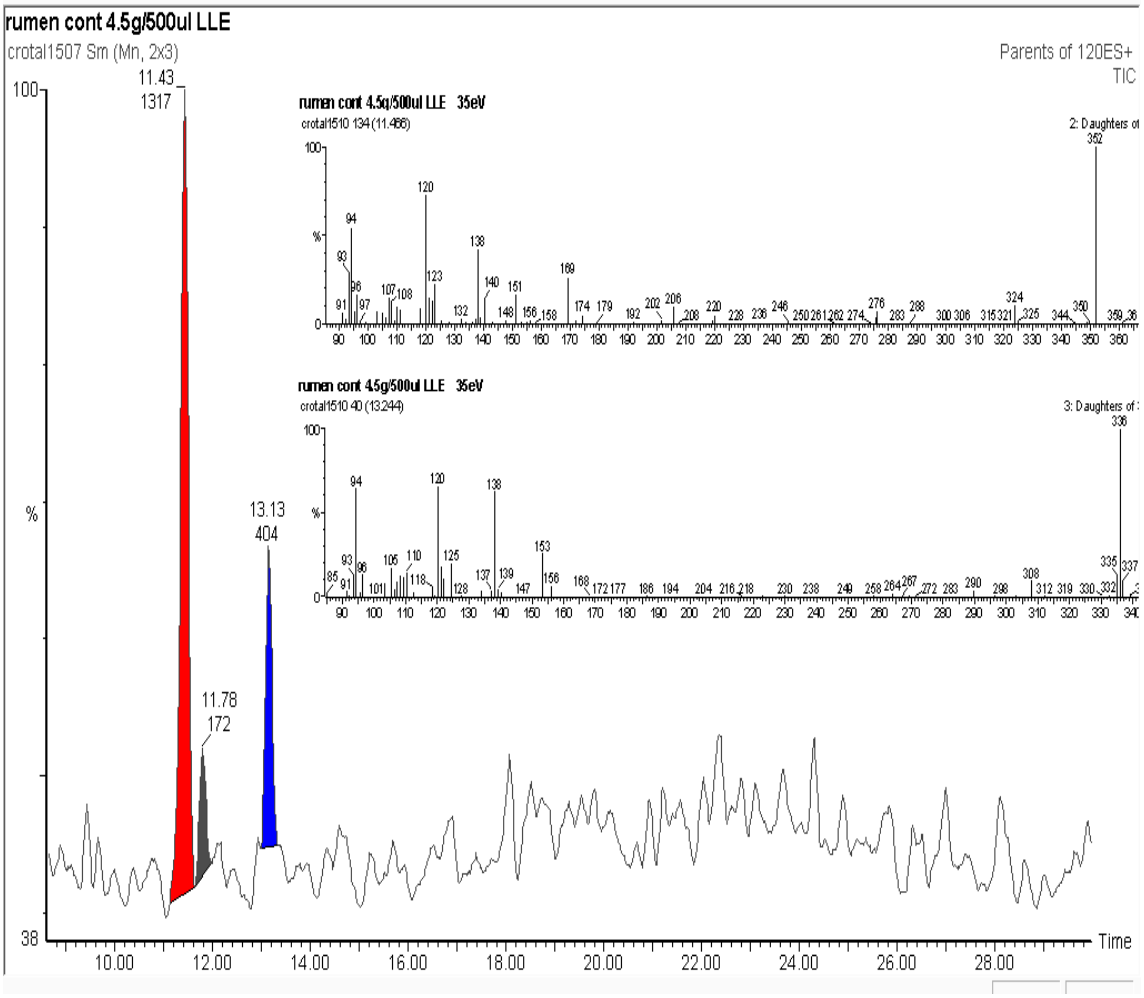


Figure 6-3: Total ion chromatogram [parent ions of *m/z* 120] of extracted rumen content. The spectra of the toxic PAs ($[M+H]^+$ 352 at 11 min and $[M+H]^+$ 336 at 13 min) are identical to the plant extracts.

MRM experiments were performed for the transitions 352>120 and 336>120 and the compounds were quantified against a retrorsine calibration curve. The quantitative results are expressed as µg retrorsine equivalents per gram sample (Table 6-1).

Table 6-1: Concentration (µg.g^{-1}) of unsaturated PAs in *Senecio inaequidens*

Extract	Toxic PA (µg.g^{-1})	
	Retrorsine	Senecionine
Plant material	234	19
Reduced plant material	11 446	550
Mouldy material	160	15
Reduced mouldy material	3697	427
Rumen content	0.52	0.04

The sample extracts were also injected on GC-MS with EI and library search matching was used to identify the peaks. Retrorsine was identified by retention time and spectra matching with the reference standard (Fig 6.5). The other peak was identified with library matching (99% match) as senecionine (Fig 6-6).

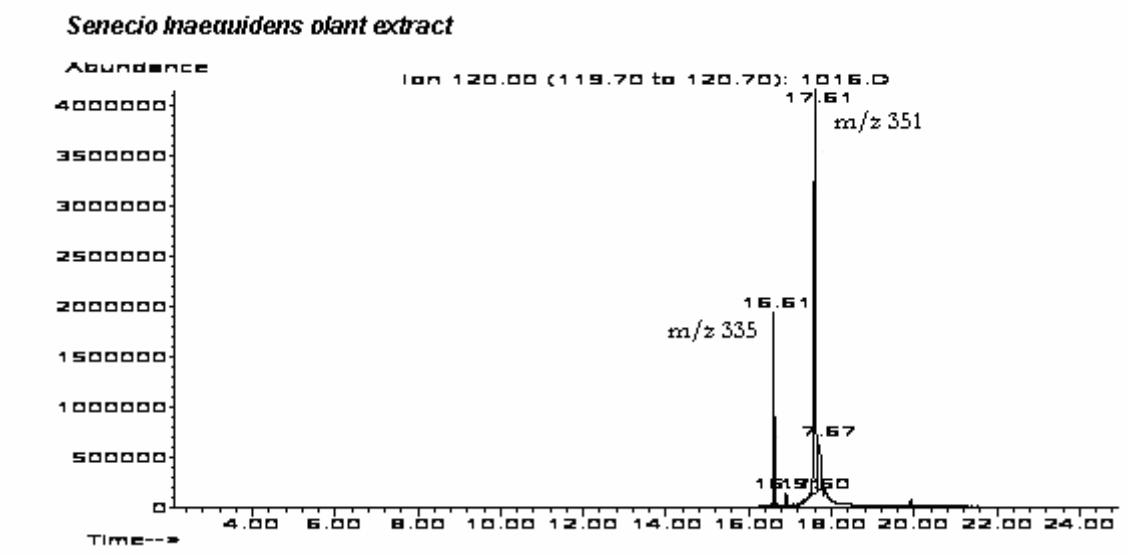


Figure 6-4: Reconstructed chromatogram of the extracted ion *m/z* 120 of a reduced *S. inaequidens* plant extract

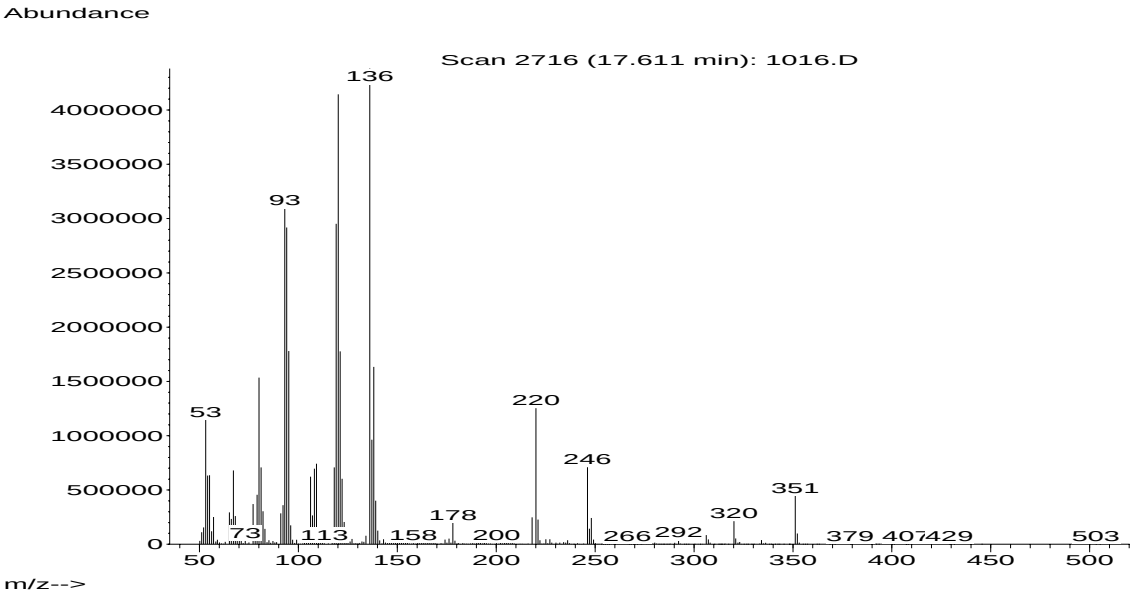


Figure 6-5: GC-MS spectrum of retrorsine in *S. inaequidens*

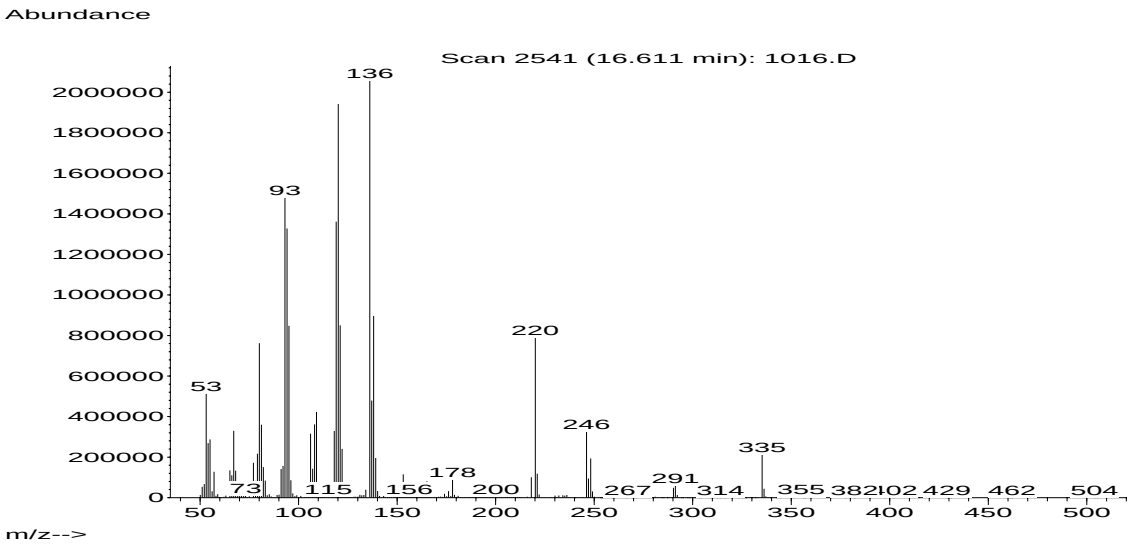


Figure 6-6: GC-MS spectrum of senecionine in *S. inaequidens*

6.2.3 Discussion

The plants contained high concentrations of retrorsine and roughly 10 times less senecionine. The oral LD_{50} for retrorsine in male rats is 38 $mg.kg^{-1}$ for and 85 $mg.kg^{-1}$ for senecionine (Cheeke 1989). The unsaturated PAs were mostly present as the *N*-oxides, as can be seen in the concentrations of the basic fraction compared with the reduced fraction.

The total concentration of toxic PAs decreased from about 12 $mg.g^{-1}$ to 4 $mg.g^{-1}$, in the slightly mouldy sample. This raises questions about the stability of unsaturated PAs during organic degradation and indicate that fungi may be able to metabolize PAs.

Finding the toxic PAs in the rumen content confirmed that the cows died from PA poisoning. This was also corroborated by histopathological examinations where liver samples revealed diffuse centrilobular to massive necrosis and haemorrhage (Prof Botha, Faculty of Veterinary Science, Onderstepoort, Personal communication 2004).

The analytical screening method developed during this study was successfully used to determine the concentration of unsaturated PAs in *S. inaequidens* plant material and confirmed the presence of these PAs in the rumen content collected during necropsy. The method could prove the suspected but unconfirmed toxicity of *S inaequidens* at a much lower cost than carrying out animal experiments.

6.3 *Crotalaria* species

6.3.1 Background

Crotalaria laburnifolia (Fig 6-7) and *Crotalaria dura* (Fig 6-8) specimens were submitted by Prof. Naudé, Faculty of Veterinary Science, Onderstepoort. *C. dura* is known to be toxic and is associated with "jaagsiekte" in horses, as mentioned in Chapter 1. The analytical method developed in this study was used to compare the unsaturated PA content in these two plants.

Figure 6-7: *Crotalaria laburnifolia* from KwaZulu-Natal. Photographs by Lyndy McGaw

Figure 6-8*: Crotalaria dura* from KwaZulu-Natal. Photographs by Lyndy McGaw

6.3.2 Results

The samples were extracted as described before. Using precursor ion scans two unsaturated PAs were detected in *C. laburnifolia* (Fig 6-9) and six in *C. dura* (Fig 6-10). The pseudo-molecular ion $[M+H]^+$ masses found are shown in the respective chromatograms.

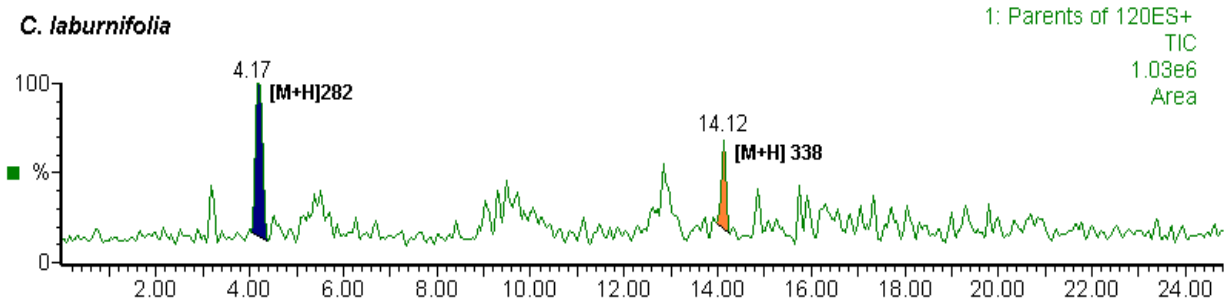


Figure 6-9: Precursor scan of a reduced *C. laburnifolia* plant extract

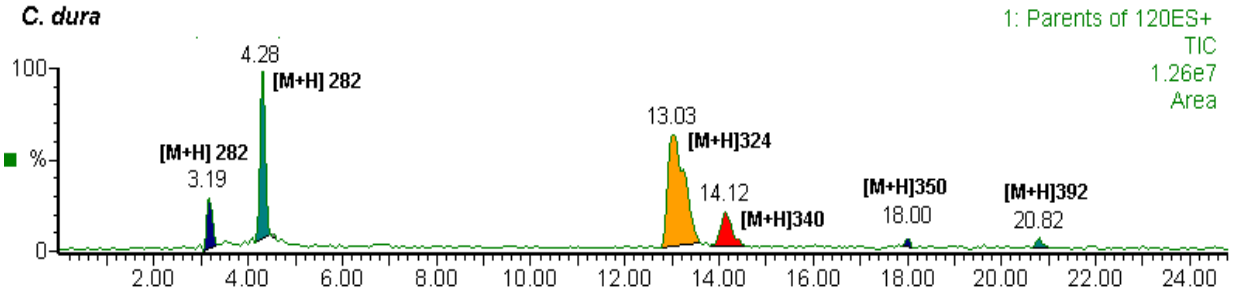


Figure 6-10: Precursor scan of a reduced *C. dura* plant extract

MRM transitions of pseudo-molecular ions and the fragment *m/z* 120 were used to quantify the unsaturated PAs. The results are listed in Table 6-2.

Table 6-2: Unsaturated PA concentrations in *C. laburnifolia* and *C. dura*

C. laburnifolia reduced						
Retention time (min)	4.17	14.2				
Pseudo-molecular ion	282	338				
PA concentration (µg.g^{-1})	13.6	5.7				
C. dura reduced						
Retention time (min)	3.19	4.28	13.03	14.12	18.0	20.8
Pseudo-molecular ion	282	282	324	340	350	392
PA concentration (µg.g^{-1})	41.0	152.8	305.2	66.1	12.6	11.6

The extracts were analyzed on GC-MS with EI. The software was used to extract the relative abundance of the *m/z* 120 ion and the spectra of the peaks found were investigated (Fig 6.11 and Fig 6-12).

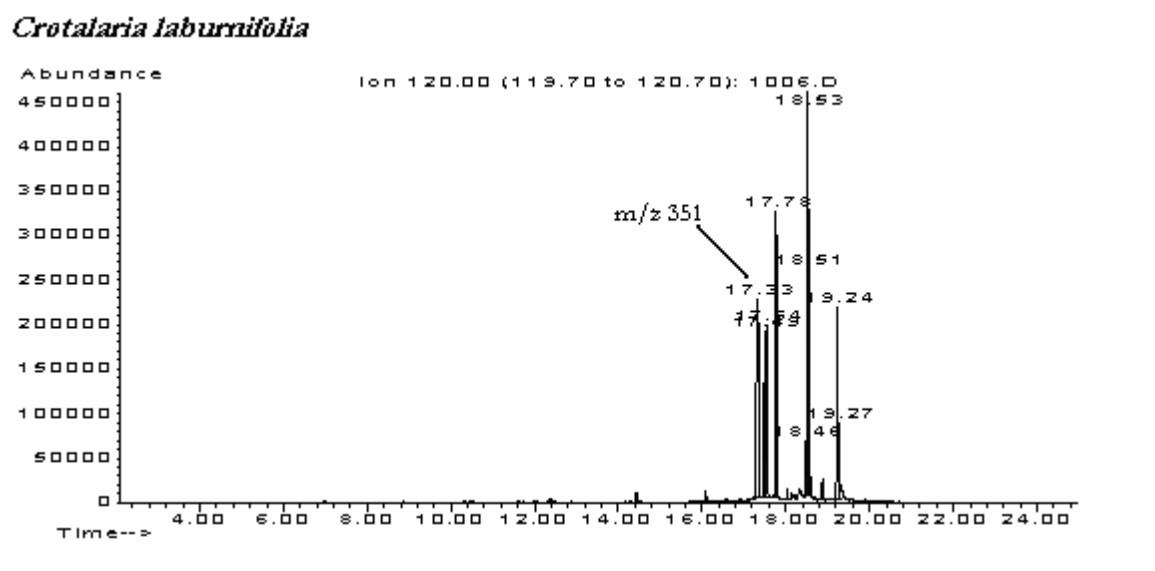


Figure 6-11: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of *C. laburnifolia*

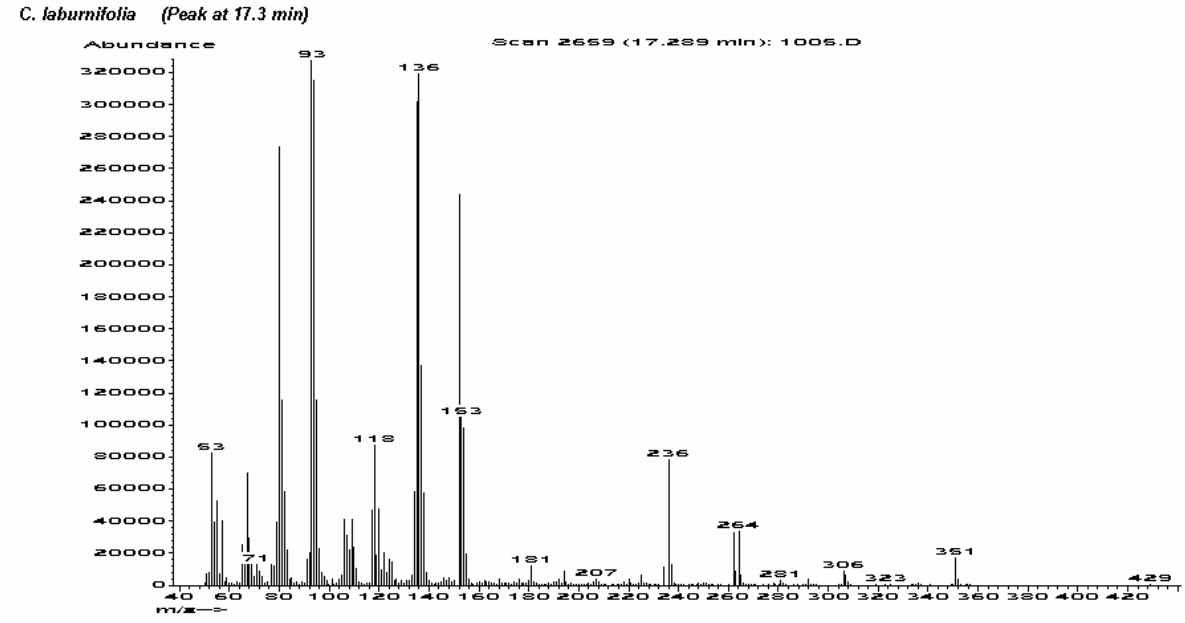


Figure 6-12: GC-MS spectrum of peak at 17.31 minutes

The characteristic fragments of unsaturated PA structures were only seen in the spectrum of the peak at 17.33 minutes, with an unexplained low abundance of the *m/z* 120 fragment. The

molecular ion has the same mass as retrorsine, but both the spectrum and the retention time were different.

Two unsaturated PAs were found in *C. dura* on GC-MS (Fig 6-13) and the spectra of both compounds were characteristic of unsaturated PAs (Fig 6-14 and Fig 6-15).

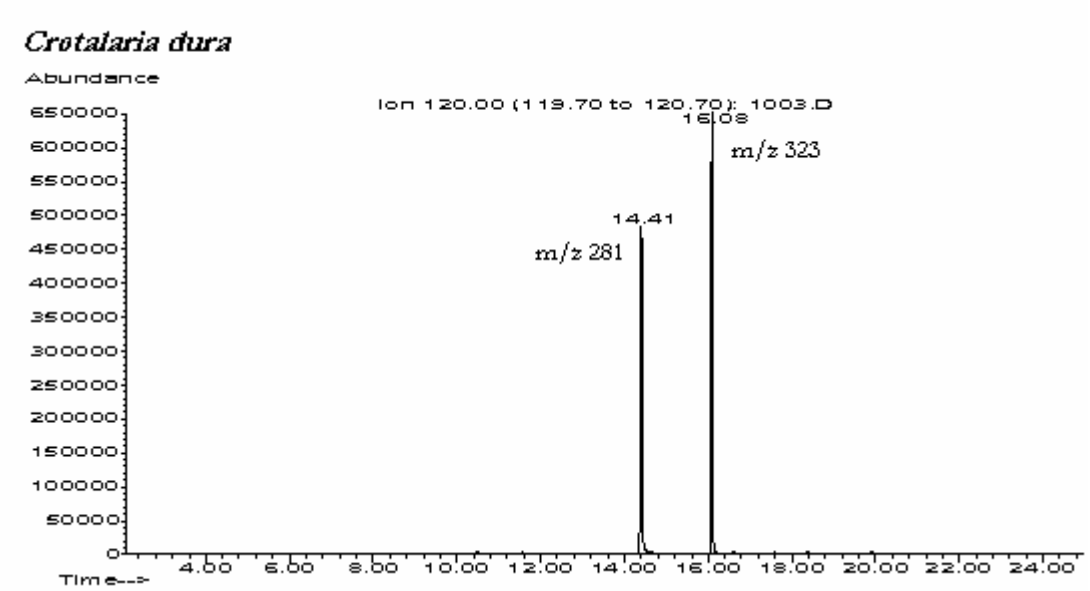


Figure 6-13: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of *C. dura*

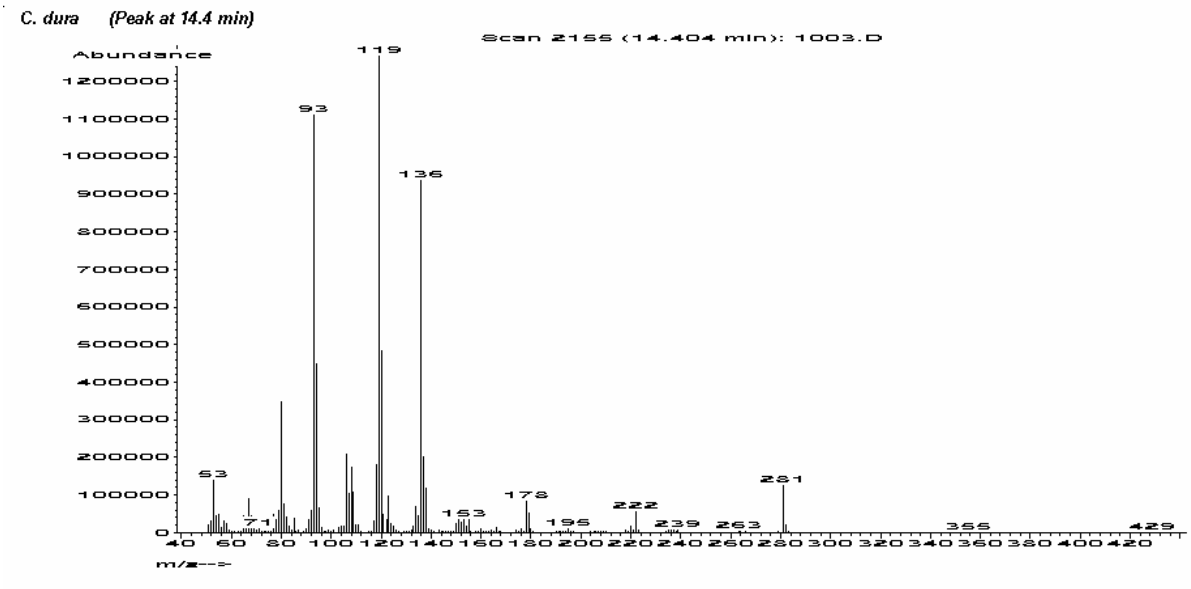


Figure 6-14: CG-MS spectrum of peak $[M]^+$ 281 at 14.4 minutes

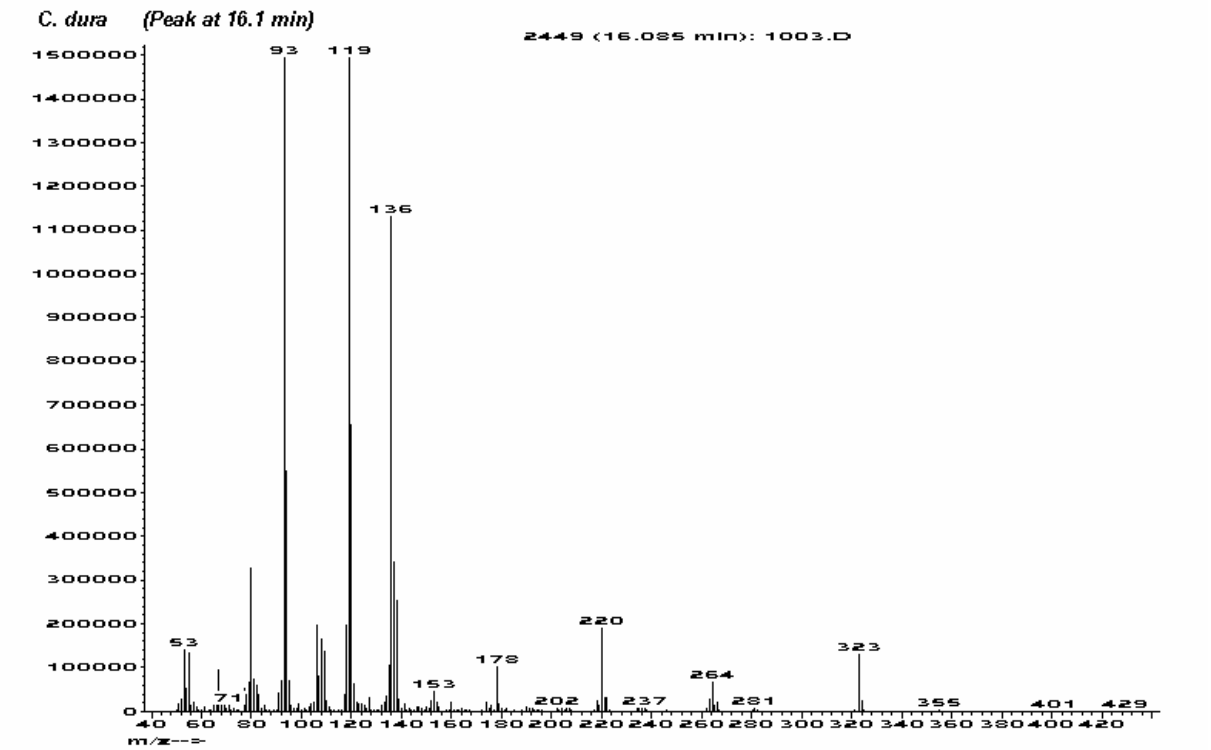


Figure 6-15: GC-MS spectrum of peak $[M]^+$ 323 at 16.1 minutes

6.3.3 Discussion

Two unsaturated PAs were found in the reduced fraction of *C. laburnifolia,* with molecular masses 281 and 337 respectively. These compounds were, however, not present in the GC-MS chromatogram. The total unsaturated PA content in this plant was less than 20 $\mu g.g^{-1}$ and it is unlikely that this would be toxic to any animals.

Crotalaria dura, on the other hand, is known to be pneumotoxic, and has been shown to cause liver damage in some instances (Kellerman *et al*. 1988). Marais (1944) extracted large amounts (0.27%) of dicrotaline from *C. dura.* A total of six unsaturated PAs were found with the LC-MS method with molecular masses 281, 281, 323, 339, 349 and 391and the total unsaturated PA concentration was 590 $\mu g.g^{-1}$ (0.06%). One of the 281 PAs and the PA with mass 323 could be confirmed with GC-MS. The molecular mass of dicrotaline is 281 found at 14.4 minutes in the GC chromatogram (Fig 6-13) and was most likely the compound found at 4.28 minutes in the LC chromatogram (Fig 6-10) and. The retention times of the other unsaturated PAs found with the LC-MS method indicate that the structures are more closely related to retrorsine. The presence of these PAs may be the cause of liver damage, sometimes seen together with lung damage, after ingestion of *C. dura*.

6.4 Conclusion

Other *Crotalaria* spp. were analyzed using the LC-MS/MS method developed during this study. *Crotalaria laburnifolia*, which is generally considered as being non-toxic, contained very low concentrations (<20 µg.g^{-1}) of unsaturated PAs. The known toxic *Crotalaria dura* contained 585 µg unsaturated PAs per gram of plant material. The presence of dicrotaline in *C. dura*, found by Marias (1944) was confirmed. Other unsaturated PAs were also found in *C. dura*, which are structurally more related to retrorsine, which might explain why this plant can cause both lung and liver damage when ingested.

CHAPTER 7: GENERAL DISCUSSION AND RECOMMENDATIONS

7.1 Analytical method

A screening method for the detection of unsaturated PAs in natural products was developed. The method specifically targets the 1,2-unsaturated necine bases to discriminate between toxic and non-toxic PAs. The pseudo-molecular ions of each of the unsaturated PAs were established. The concentrations of each of the individual unsaturated PAs could also be determined.

The method was partly validated using monocrotaline and retrorsine reference materials. The linearity, limit of quantification and recovery from spiked extracts were determined for these compounds. The method was, however, developed as a screening method for unknown unsaturated PAs and the validation was therefore limited.

Various extraction methods found in the literature were investigated. The final method consisted of a robust liquid-liquid extraction of unsaturated PAs from small amounts (1 g) of milled sample. The extraction method is suitable for a wide variety of matrices, e.g. plant materials, biological specimens, foods and even rumen content.

The use of retrorsine equivalents to express the amount of unsaturated PAs allows quantitative comparisons between compounds and plants and allows estimations of toxicity in the absence of authentic reference materials. It is, however, important to keep in mind that the amount of unsaturated PAs present in any sample is only an approximation of possible toxicity and the toxicity of each compound should still be determined in isolation.

Limited attempts were made to determine unsaturated PAs in maize meal, but severe problems were encountered with emulsions and co-extraction of other contaminants during sample preparation. In the case of *C. sphaerocarpa*, even allowing 50 seeds per 10 kg of maize, the total unsaturated PA concentration will only be about 0.01 $\mu g.g^{-1}$, which is well below the limit of quantification of the LC-MS/MS method (0.05 $\mu g.ml^{-1}$ for extracted samples).

It is therefore advisable to determine the level of contamination before milling, when the seeds can physically be removed and analyzed, or by improving the de-fatting step during the extraction procedure to overcome problems with emulsions and contaminants.

The stability of PAs at high temperatures was investigated to determine the effect of cooking on PAs present in foods. PAs were stable at high temperatures and it was concluded that the possible toxic effect will not be reduced by cooking.

Other analytical methods were also investigated in an attempt to confirm the LC-MS/MS results. The screening method with Ehrlich's reagent, described by Mattocks and Jukes (1987), proved useful for some of the plants investigated, only after interfering substances were removed.

Extracts were also analyzed on GC-MS with both electron impact and chemical impact ionization. Fewer unsaturated PAs were found with GC-MS possibly due to co-eluting PAs on the GC column and variations in concentration where low concentrations were masked by more abundant compounds. Attempts to identify the PAs with spectral matching were not successful, mainly due to instability of the molecular ions and also due to a limited number of PA spectra available on the library used.

7.2 Further recommendations

NMR is still essential for positive identification of PAs and should be considered for the major compounds found in *C. sphaerocarpa* seeds (PAs with molecular masses 238, 237, 328, 353 and 357). Cytotoxic studies should also still be performed on *C. sphaerocarpa* seeds to exclude the possibility that some of the PAs may be highly toxic, or that other toxins may be present in the seeds.

The variation in unsaturated PA content between *C. sphaerocarpa* plants could not be explained. Results of genetically related plants and plants growing in specific areas need to be investigated further. Statistical sampling from more grain producing regions in South Africa should also be considered before the allowable level of noxious seeds can be finalized.

When all the LC-MS/MS results were evaluated a certain correlation between structure, toxicity and elution order on reverse phase was noted. All the compounds that could be identified

contained unsaturated necine bases with cyclic diester necic acids. Compounds like monocrotaline and dicrotaline have three carbon atoms between the two ester groups in their necic acid moiety. Both compounds are known to be pneumotoxic and both eluted early with the LC-MS/MS method (more polar). Retrorsine, senecionine and integerrimine all contain four carbon atoms between the two esters in the acid moiety, eluted much later (less polar) and are known to be hepatotoxic. More authentic pyrrolizidine alkaloids should be analyzed with this method to test the relationship between polarity, structure and toxicity as this might predict the eventual toxic effect.

Herbal preparations and traditional medicines containing hepatotoxic PAs may pose a real threat as large quantities are normally consumed and children are often the ones who are treated in this manner. The LC-MS/MS screening method developed in this study may be of value for the determination of toxic PAs in these samples.

In S. *inaequidens* the concentration of unsaturated PAs in fresh, dried plants were about three times higher than in the samples that were slightly mouldy before they were dried and milled. This may have been due to bacterial/fungal degradation of the PAs during the storage period. It may also be that the plants were collected a few days earlier than the fresh samples and that the concentration changed drastically in the maturing plants. These findings merit further investigation.

Careful consideration is needed when identification of PAs are attempted with spectral EI libraries. It is difficult to identify unknown PAs, due to the similar fragments which are derived from the necine base, and the low abundance of the molecular ions in many instances. Good library matches are often difficult to achieve and positive matches may in some instances be achieved with many of the PA spectra in the library. PCI is recommended for the confirmation of unsaturated structures and the determination of molecular masses. A considerable amount of information accumulated during the development of this project and the GC-MS spectral data is available for further evaluation. Other research groups interested in pyrrolizidine alkaloids may be able to identify some of these alkaloids by spectral matching with their own private spectral libraries.

7.3 Allowable number of *C. sphaerocarpa* seeds in grain

The quantitative results obtained with the LC-MS/MS method were used to calculate the allowable seed level in maize, using the same approach as Eloff *et al*. (2003). Contamination problems arise when the whole seed pod (3-4 x 4-6 mm) is harvested together with grain. The highest PA content (*N*-oxide + basic alkaloid) found in the seed samples was 150 $\mu g.g^{-1}$ in sample A (2.1 µg per seed for seed mass 14.2 mg). To reach the suggested NOAEL of 5 $\mu g.kg^{-1}$ per day, a 70 kg person would have to consume 164 seeds per day, which relates to 3280 seeds in 10 kg maize. To limit the daily intake to 1 $\mu g.kg^{-1}day^{-1}$, as suggested by the Australian criteria for chronic exposure safety, 656 seeds per 10 kg of maize should be the maximum allowed. The newest guideline of 10 seeds per 10 kg can therefore be applied with safety. If the results found here are confirmed with *C. sphaerocarpa* from different production areas the current allowable level could be increased up to 50-fold. This would lead to a substantial benefit to producers of maize or soybean in South Africa.

7.4 Final conclusion

The aim of this study was to develop an analytical method that could selectively detect unsaturated PAs in *C. sphaerocarpa* seed. This was achieved by the development of the LC-MS/MS screening method described, that selectively targets the fragments produced by unsaturated PAs in the precursor ion mode, to identify the toxic compounds.

One of the objectives was to identify and quantify the toxic PAs present. Although the identity of most of the PAs could not be finalized, the molecular masses of the compounds were obtained, which already narrows down the range of possible structures. The compounds of interest were quantified against a retrorsine calibration curve as $\mu g.g^{-1}$ retrorsine equivalents. This allows comparisons and estimations of the amount of PAs present in the absence of authenticated reference standards. The method was used to determine the unsaturated PA content of the seed samples and the allowable level of *C. sphaerocarpa* seed in grain was calculated accordingly.

Another objective was to compare the PA content in *C. sphaerocarpa* plants from different locations and during different stages of maturation. Some correlation was observed in PA levels in certain plants but careful statistical data still need to be collected before these relationships can be confirmed. It was found that unsaturated PAs were present in the roots of

young plants mainly as the *N*-oxides and were present in the mature aerial parts as the basic alkaloids.

The stability of PAs during cooking was determined as this may affect the possible threat to human health. Unsaturated PAs were found to be stable at high temperatures and toxicity is therefore not reduced by cooking procedures.

The last objective of the study was to demonstrate the effectiveness of the analytical method by analyzing other PA containing plants. The unsaturated PA content *of C. laburnifolia, C. dura* and *Senecio inaequidens* were successfully determined. The method was also used to confirm the presence of unsaturated PAs in rumen content.

BIBLIOGRAPHY

1. Altee MY, Mahmood MH. (1998). An outbreak of veno-occlusive disease of the liver in northern Iraq. http://www.emro.who.int/Publications
2. Ames MM, Powis G. (1978). Determination of indicine N-oxide and indicine in plasma and urine by electron-capture gas-liquid chromatography. *Journal of Chromatography.* **166**, 519-526.
3. Anon. (1969) Onderstepoort; Annual report 1968/9. http://www.nda.agric.za/vetweb
4. Anon. (1987). Regulations governing the tolerances for certain seeds in certain agricultural products, R1466, in terms of the Foodstuffs, Cosmetics and Disinfectants Act, 1972. Department of Health, Pretoria.
5. Anon. (1995). Application of food analysis to food standards issues. Report of the joint FAO/WHO expert consultation. WHO: Geneva, Switzerland.
6. Anon. (1997). Poisonous plants- Pyrrolizidine alkaloids. http://www.ansi.cornell.edu/courses/as625/1997/term/Sopheak/pyrol625.htm
7. Anon. VRI (1999). Hepatotoxicity associated with pyrrolizidine alkaloid (*Crotalaria* spp.) ingestion in a horse on Easter Island. http://www.veterinaryreliefinternational.com/hepatotoxicity
8. Anon. (2002). Regulations governing the tolerances for certain seeds in certain agricultural products. Department of Health, Pretoria.
9. Anon. (2003a). U.S. Food and Drug Administration/Centre for Food Safety and Applied Nutrition, Office of Plants and Dairy Foods. FDA Poisonous Plant Database. http://www.cfsan.fda.gov/
10. Anon. (2003b). NTP Report. Toxicology and carcinogenesis studies of riddelliine in F344/N rats and B6C3F_1 mice. National Toxicology Program, US Department of Health and Human Services.
11. ANZFA. (2001). Australia New Zealand Food Authority. Pyrrolizidine alkaloids in food: A toxicological review and risk assessment. Technical Report No 2. http://www.anzfa.gov.au
12. AOAC (1990) Official Methods of Analysis, Section 960.07. 15 th Ed. Arlington, VA
13. Arnold TH, de Wet BC. (1993). Plants of Southern Africa: Names and distribution. *Memoirs Botanical Survey South Africa.* **62**. National Botanical Institute, Pretoria.

14. Azadbakht M, Talavaki M. (2003). Qualitative and quantitative determination of pyrrolizidine alkaloids of wheat and flour contaminated with *Senecio* in Mazandaran Province. *Iranian Journal of Pharmaceutical Research.* **2**, 179-183.
15. Beasley V. (1999) Poisonous plants that affect the liver. In: Veterinary Toxicology, Beasely V Ed. International Veterinary information service. http://www.ivis.org/advances/Beasley
16. Boppre M. (1990). Lepidoptera and pyrrolizidine alkaloids: exemplification of complexity in chemical ecology. *Journal of Chemical Ecology.* **16**, 165-185.
17. Bras G, Brooks SHE, Walter DC. (1961). Cirrhosis of liver in Jamaica. *Journal of Pathological Bacteriology.* **82**, 503-512.
18. Bromilow C. (1996). Problem plants of South Africa. Bayer SA.
19. Bull LB, Culvenor CCJ, Dick AT. (1968) The pyrrolizidine alkaloids; Their chemistry, pathogenicity and other biological properties. North Holland Publishing Company, Amsterdam. 115-132.
20. Castagnoli N, Rimoldi JM, Bloomquist J, Castagnoli KP. (1997). Potential metabolic activation pathways involving cyclic tertiary amines and azoarenes. *Chemical Research in Toxicology.* **10**, 924-940.
21. Chauvin P, Dillon JC, Moren A. (1994). An outbreak of *Heliotrope* food poisoning, Tadjikista, Nov 1992 – Mar 1993. *Cahiers Sante.* **4**, 263-268.
22. Cheeke PR. (1989) Toxicants of plant origin; Volume I Alkaloids. CRC Press, Florida. 2-21.
23. Chojkier M. (2003). Hepatic sinusoidal-obstruction syndrome: toxicity of pyrrolizidine alkaloids. *Journal of Hepatology.* **39**, 437-446.
24. Clark AM. (1959). Mutagenic activity of the alkaloid heliotrine in *Drosophila. Nature.* **183**, 731-732.
25. Craig A M. (2002). Pyrrolizidine alkaloids: Health problem in South Africa. Research Proposal (unpublished).
26. Craig A M. (1995). Detoxification of plant and fungal toxins by ruminant bacteria. In: *Ruminant Physiology: Digestion, metabolism, growth and production*. Ferdinand Enke Verslag, Stuttgart. 271-288.
27. Crews C, Startin JR, Clarke (1997). Determination of pyrrolizidine alkaloids in honey from selected sites by solid phase extraction and HPLC-MS. *Food Addition Contamination.* **14**(5), 419-428.

28. Culvenor CCJ, Edgar JA, Jago MV, Outteridge A, Peterson JE, Smith LW. (1976). Hepato-and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. *Chemical Biological Interacta.* **12**, 299-324.
29. Culvenor CCJ, Edgar JA, Smith LW. (1981). Pyrrolizidine alkoloids in honey from *Echium plantagineum* . *Journal of Agriculture and Food Chemistry.* **29**, 958-960.
30. Damron BL, Jacob JP. (1998). Toxicity of weed seeds common to the South Eastern US. *Journal of Applied Poultry Research.* **10**, 104-110.
31. Damron BL. (2001). Toxicity to poultry of common weed seeds. hppt://edis.ifas.ufl.edu/
32. Deinzer ML, Thomason PA, Burgett DM, Isaacson DL. (1977). Pyrrolizidine alkaloids: Their occurrence in honey from Tansy Ragwort (*S. jacobaea* L) *Science.* **195**, 497-499.
33. De Medeiros RMT, Gorniak SL, Guerra JL. (1999). Effects of milk from goat fed *Crotalaria spectabilis* seeds on growing rats. *Brazilian Journal of Veterinary Research and Animal Science.* **36**, (2)
34. Deuker SR, Lame MW, Morin D, Wilson DW, Segall HJ. (1992). Guinea pig and rat hepatic microsomal metabolism of monocrotaline. *Drug Metabolism and Disposition* **20**, 275-280
35. Dharmananda S. (2002). Safety issues affecting herbs: pyrrolizidine alkaloids. http://www.itmonline.org/arts/pas.htm
36. Dickinson JO, Cooke MP, King RR Mohamed PA. (1976). Milk transfer of pyrrolizidine alkaloids in cattle. *Journal of American Veterinary. Medical Association*. **169**, 1192-1196.
37. Edgar JA, Smith WS. (2000). Transfer of pyrrolizidine alkaloids into eggs: Food safety implications. In: *Natural and selected synthetic toxins. Biological implications*. ACS Symposium series 745. American Chemical Society, Washington, DC. 118-128.
38. Eloff JN, Jansen van Rijssen FW, Naudé TW, Joubert JPJ. (2003). What levels of potentially toxic seed should be allowed in grains in South Africa? I. Background and pyrrolizidine alkaloid-containing plants. *South African Journal of Science*. **99**, 337-344.
39. El-Shazly A. (2002). Pyrrolizidine Alkaloid profiles of some *Senecio* species from Egypt. *Verlag der Zeitschrift fur Naturforschung*. **57c**, 429-433.
40. Estep JE, Lame MW, Morin D, Segall HJ, Wilson DW. (1992). ^{14}C-Monocrotaline tissue distribution, covalent binding and kinetics in the rat. In: *Poisonous Plants; Proceedings of the Third International Symposium*. Iowa State University Press. 584-588.

41. Evans JV, Daley SK, McClusky GA, Nielsen CJ. (1980). Direct quantitative analysis of incidine *N*-oxide in cancer patient samples by gas chromatography using the internal standard heliotrine *N*-oxide including a mass spectral comparison of their trimethylsilyl derivatives. *Biomedical Mass Spectrometry*. **7**, 65-73.
42. Faftine O, Alage A, Muir,JP. (2000). Characterization of forage selected by cattle on communal range in Manhica, Mozambique. hppt://Stephenville.tamu.edu/
43. Fitzgerald RL, Rivera JD, Herold DA. (1999). Broad spectrum drug identification directly from urine using liquid chromatography tandem mass spectrometry. *Clinical Chemistry.* **45**, 8, 1224-1234.
44. Franke JP, De Zeeuw RA. (1998). Solid phase extraction procedures in systematic toxicological analysis. *Journal of Chromatography B.* **713**, 51-59.
45. Fu PP, Xia Q, Lin G,Chou MW. (2002a). Genotoxic pyrrolizidine alkaloids – mechanism leading to DNA adduct formation and tumorigenicity. *International Journal of Molecular Sciences.* **3**, 948-964.
46. Fu PP, Yang Y, Xia Q, Chou MW,Cui YY Lin G. (2002b). Pyrrolizidine alkaloids – Tumorigenic components in Chinese herbal medicines and dietary supplements. *Journal of Food and Drug Analysis.* **10**(4), 198-211.
47. Ge Lin, Yan-Yan Cui, Hawes EM. (1998a). Microsomal formation of a pyrrolic alcohol glutathion conjugate of Clivorine. *Drug Metabolism and Disposition.* **26**(2), 181-184.
48. Ge Lin, Ke-Yu Zhou, Xian-Guo Zhao, Zhen-Tao Wang, Paul Pui Hay But. (1998b). Determination of hepatotoxic pyrrolizidine alkaloids by on-line high performance liquid chromatography mass spectrometry with electrospray interface. *Rapid Communications in Mass Spectrometry.* **12**, 1445-1456.
49. Harris PN, Reddy J, Chiga M, Svoboda D. (1969). Polysomal disaggregation by lasiocarpine. *Biophysica Acta* **180**, 587-589.
50. Hartmann T, Sander H, Adolph RD, Toppel G. (1988). Metabolic links between the biosynthesis of pyrrolizidine alkaloids and polyamines in root cultures of *Senecio vulgaris. Planta*. **175**, 80-92.
51. Hartmann T, (1997) C-10-2 The mechanistic basis of generating chemical diversity: exemplified by Senecionine type pyrrolizidine alkaloids. http://www.chemecol.org/meetings/97/C-10-2.html.
52. Hincks JR, Kim HY, Segall HJ, Molyneux RJ, Strmitz FR, Coulombe RA. (1991). DNA cross linking in mammalian cells by pyrrolizidine alkaloids: structure-activity relationship. *Toxicology and Applied Pharmacology.* **111**(1), 90-98.

53. Hol WGH, Vrieling K, van Veen JA. (2003). Nutrients decrease pyrrolizidine alkaloid concentrations in *Senecio jacobaea*. *New Phytologist.* **158**(1), 175-179.
54. Holstege DM, Seiber JN Galey FD. (1995). Rapid multiresidue screen for alkaloids in plant material and biological samples. *Journal of Agricultural and Food Chemistry.* **43**, 691-699.
55. Hooper PT, Scalan WA. (1977). *Crotalaria retusa* poisoning in pigs and poultry. *Australian Veterinary Journal.* **53**, 109-114.
56. Hough JM, Haney CA, Voyksner RD, Bereman RD. (2000). Evaluation of electrospray transport CID for the generation of searchable libraries. *Analytical Chemistry* **72**, 2265-2270.
57. Huxtable RJ. (1989). Human health implications of pyrrolizidine alkaloids and herbs containing them. In: *Toxicants of plant origin*; Volume I Alkaloids. Cheeke PR Ed. CRC Press, Florida. 42-86.
58. IARC. (1976) Pyrrolizidine alkaloids. In: *Some naturally occurring substances*. Lyons, International Agency for Research on Cancer. (IARC Monograph on the evaluation of carcinogenic risks of chemicals to man). **10**, 265-342.
59. IPCS. (1989). International Programme on Chemical Safety; Pyrrolizidine Alkaloid Health and Safety Guide 26. WHO:Geneva. http://www.inchem.org/documents/hsg/hsg/hsg026.htm
60. Jago MV, Lanigan GW, Bingley JB, Piercy DWT, Whittem JH, Titchen DA. (1969). Excretion of the pyrrolizidine alkaloid heliotrine in the urine and bile of sheep. *Journal of Pathology.* **98**, 115-128.
61. Kedzierski B, Buhler DR. (1986). The formation of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizidine, a metabolite of pyrrolizidine alkaloids. *Chemical Biological Interacta.* **57**, 217-222.
62. Kellerman TS, Coetzer JAW, Naudé TW. (1988). Plant poisonings and mycotoxicoses of livestock in Southern Africa. Oxford University Press, Cape Town.
63. Kellerman TS, Fourie N, Naudé TW. (1996). The distribution, diagnosis and estimated economic impact of plant poisonings and mycotoxicosis in South Africa. *Onderstepoort Journal of Veterinary Research.* **63,** 65-90.
64. Lafranconi WM, Ohuma S, Huxtable RS. (1985). Biliary excretion of novel pneumotoxic metabolites of the pyrrolizidine alkaloid monocrotaline. *Toxicon*. **23**, 903-992.
65. Lindigkeit R, Biller A, Buch M, Schiebel HM, Boppre M, Hartmann T. (1997). The two facies of pyrrolizidine alkaloid: the role of the tertiary amine and its *N*-oxide in chemical

defence of insects with acquired plant alkaloids. *European Journal of Biochemistry.* **245**, 626-636.

66. Lips AGAM, Lameijer W, Fokkens RH, Nibbering NMM. (2001). Methodology for the development of a drug library based upon collision-induced fragmentation for the identification of toxicologically relevant drugs in plasma samples. *Journal of Chromatography B.* **759**, 191-207.
67. Logie CG, Grue R, Liddell JR. (1994). Proton NMR spectroscopy of pyrrolizidine alkaloids. Review article No. 93. *Phytochemistry.* **37**(1), 43-109.
68. Marais JSC. (1944) Dicrotaline: The toxic alkaloid from *Crotalaria dura* and C*rotalaria globifera. Onderstepoort Journal of Veterinary Science and Animal Industry* **20**(1), 61-65.
69. Mattocks AR. (1967). Detection of pyrrolizidine alkaloids on thin layer chromatography. *Journal of Chromatography.* **27**, 505-508.
70. Mattocks AR. (1971). The occurrence and analysis of pyrrolizidine alkaloid *N*-oxides. *Xenobiotica.* **1**, 451-453.
71. Mattocks AR. (1977). Tissue distribution of radioactivity in rats given tritiated analogues of hepatotoxic pyrrolizidine alkaloids. *Xenobiotica*. **7**, 665-670.
72. Mattocks AR. (1986). Chemistry and toxicology of pyrrolizidine alkaloids. Academic Press, Orlando, Florida.
73. Mattocks AR, Jukes R. (1987). The simple detection of potentially toxic PAs under field conditions. In: *Poisonous plants: Proceedings of the Third International Symposium*. Eds. James, Keeler Iowa State University Press. 553-555.
74. Mattocks AR, Culvenor CCJ, Tandon HJ, Prost A. (1988). IPCS:Criteria 80. Environmental health criteria for pyrrolizidine alkaloids, World Health Organization, Geneva. http://www.inchem.org/documents/ehc/ehc/ehc080.htm
75. Mattocks AR (1992) Pyrrolizidine Alkaloids: What metabolites are responsible for extrahepatic tissue damage in animals. In *Poisonous Plants; Proceedings of the Third International Symposium*. Iowa State University Press. 192-197.
76. Mohabbat O, Svrivastava RN, Younos MS, Sediq GG, Mendaz AA, Aram GN. (1976). An outbreak of veno-occlusive disease in North-western Afghanistan. *Lancet* **7**, 269-271.
77. Mroczek T, Glowniak K, Wlaszczyk A. (2002). Simultaneous determination of *N*-oxides and free bases of pyrrolizidine alkaloids by cation-exchange solid-phase

extraction and ion-pair high-performance liquid chromatography. *Journal of Chromatography A.* **949**(1-2), 249-262.

78. Naudé TW, Coetzer JAW, Kellerman TS. (1992). Variation in animal species' response to plant poisonings and mycotoxicoses in Southern Africa. In: *Poisonous Plants; Proceedings of the Third International Symposium*. Iowa State University Press.
79. Ober D, Hartmann T. (1999). Homospermidine synthase, the first pathway-specific enzyme of pyrrolizidine alkaloid biosynthesis, evolved from deoxyhypusine synthase. *Procedures in Natural Academic Science*. **96**(26), 14777-14782. www.pnas.org
80. Prakash AS, Perira TN, Reilly PEB, Seawright AA. (1999). Pyrrolizidine alkaloids in human diet. *Mutation Research.* **443**, 53-67.
81. Qualls CW, Segall HJ. (1978). Rapid isolation and identification of pyrrolizidine alkaloids (*Senecio vulgaris*) by high performance liquid chromatography. *Journal of Chromatography.* **50**, 202-205.
82. Ramsdell AS, Buhler DR. (1981). High performance liquid chromatographic analysis of pyrrolizidine (*Senecio*) alkaloids using a reverse phase styrene-divinylbenzene resin column. *Journal of Chromatography*. **210**,154-158.
83. Ridker PM, Ohkuma S, McDermott WV, Trey C, Huxtable RJ. (1985). Hepatic veno-occlusive disease associated with consumption of pyrrolizidine alkaloid containing dietary supplement. *Gastroenterology.* **88**, 1050-1054.
84. Röder E. (1990). Carbon-13 NMR spectrometry of pyrrolizidine alkaloids. *Phytochemistry.* **29**, 11-29.
85. Röder E. (1995). Medicinal plants in Europe containing pyrrolizidine alkaloids. *Pharmazie.* **50**, 83-98.
86. Schoental R, Head MA. (1955). Pathological changes in rats as result of treatment with monocrotaline. *British Journal of Cancer*. **9**, 229-237.
87. Schoental R, Magee PN. (1959). Further observations on the subacute and chronic liver changes in rats after a single dose of various pyrrolizidine alkaloids. *Journal of Pathological Bacteriology.* **78**, 471-482.
88. Schoental R, Bensted JPM. (1963). Effects of whole body irradiation of portal hepatectomy on the liver lesions induced in rats by a single dose of retrorsine, a pyrrolizidine (*Senecio*) alkaloid. *British Journal of Cancer*. **17**, 242-251.
89. Segall HJ. (1979). Preparative isolation of pyrrolizidine alkaloids derived from *Senecio vulgaris. Liquid Chromatography.* **2**, 1319-1323.

90. Segall HJ, Wilson DW, Dallas JL, Haddon WJ. (1985). Trans-4-hydroxy-2-hexanol: a reactive metabolite from the macrocyclic pyrrolizidine alkaloid senecionine. *Science.* **229**, 472-475.
91. Sippel W L. (1964). Crotalaria poisoning in livestock and poultry. *Annals of the New York Academy of Science*. **111**, 562-570
92. Shubat PJ, Huxtable RJ. (1992). The threshold for toxicity of the pyrrolizidine alkaloid, monocrotaline. In: *Poisonous Plants; Proceedings of the Third International Symposium*. Iowa State University Press. 186-191.
93. Shumaker RC, Robertson KA, Hsu IC, Allen JR. (1976). Noeplastic transformation in tissues of rats exposed to monocrotaline or dehydroretronecine. *Journal of National Cancer Institute.* **56**, 787-789.
94. Srungbomee S, Makasame C. (1981). A preliminary study on the toxicity of *Crotalaria juncea* to cattle. *Sattawaphaet San.* **32**, 91-107.
95. Steenkamp V, Stewart MJ, Zuckerman M. (2000). Clinical and analytical aspects of pyrrolizidine poisoning caused by South African traditional medicines. *Therapeutic Drug Monitoring.* **22**, 302-306.
96. Stegelmeier BL, Edgar JA, Colegate SM, Gardner DR, Scoch TK, Coulombe RA, Moleneux RJ. (1999). Pyrrolizidine alkaloid plants, metabolism and toxicology. *Journal of Natural Toxins.* **8**, 95-116.
97. Stelljes ME, Kelley RB, Seiber JN. (1992). Chromatographic techniques for the identification of pyrrolizidine alkaloids. In *Poisonous Plants; Proceedings of the third International Symposium*. Iowa State University Press. 548-552.
98. Steyn DG, De Kock G. (1932). Crotalariosis in sheep. *18th Report of the Directorate of Veterinary Service and Animal Industry*. 947-953.
99. Stuart KL, Bras G. (1957). Veno-occlusive disease of the liver. *Quebec Journal of Medicine.* 291-315.
100. Swick RA, Cheeke PR, Patton NM, Buhler DR. (1982). Absorption and excretion of pyrrolizidine (senecio) alkaloids and their effects on mineral metabolism in rabbits. *Journal of Animal Science*. **55**, 1417-1424.
101. Tandon RK, Tandon BN, Tandon HB, Bhatia ML, Bhargava S, Arora RR. (1976). Study of an epidemic of veno-occlusive disease in India. *Gut.* **17**, 849-855.
102. Thomas HC, Lame MW, Morin D, Wilson DW, Segall HJ. (1998). Prolonged cell-cycle arrest associated with altered cdc2 kinase in monocrotaline pyrrole-treated pulmonary artery endothelial cells. *American Journal of Respiratory Cell Molecular Biology.* **19**(1), 129-142.

103. Tittel G, Hinz H, Wagner H. (1979). Quantitative determination of pyrrolizidine alkaloids in *Symphyti radix*. *Planta Medica.* **37,** 1-8.

104. Venisse N, Marquet P, Duchoslav E, Dupuy JL, Lachatre G. (2003). A general unknown screening procedure for drugs and toxic compounds in serum using liquid chromatography-electrospray single quadrupole mass spectrometry. *Journal of Analytical Toxicology.* **27,** 7-14.

105. Wagner H, Neidhardt U, Tittel G. (1981). TLC and HPLC analysis of pyrrolizidine *N*-oxide alkaloids of *Symphyti radix*. *Planta Medica.* **41**, 232-239.

106. Willmot FC, Robertson AW. (1920). *Senecio* disease or cirrhosis of the liver due to senecio poisoning. *Lancet.* **2**, 848-849.

107. Winter CK, Segall HJ. (1989). Metabolism of pyrrolizidine alkaloids. In: *Toxicants of plant origin*; Volume I Alkaloids. Cheeke PR Ed. CRC Press, Florida 23-38.

108. Witte L, Rubiolo P, Bicchi C, Hartmann T. (1993). Comparative analysis of pyrrolizidine alkaloids from natural sources by gas chromatography-mass spectrometry. *Phytochemistry*. **32**(1), 187-196

109. Yan CC, Huxtable RJ. (1995). Relationship between glutathione concentration and metabolism of the pyrrolizidine, monocrotaline, in isolated, perfused liver. *Toxicology and Applied Pharmacology* **130**, 13-17.