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.

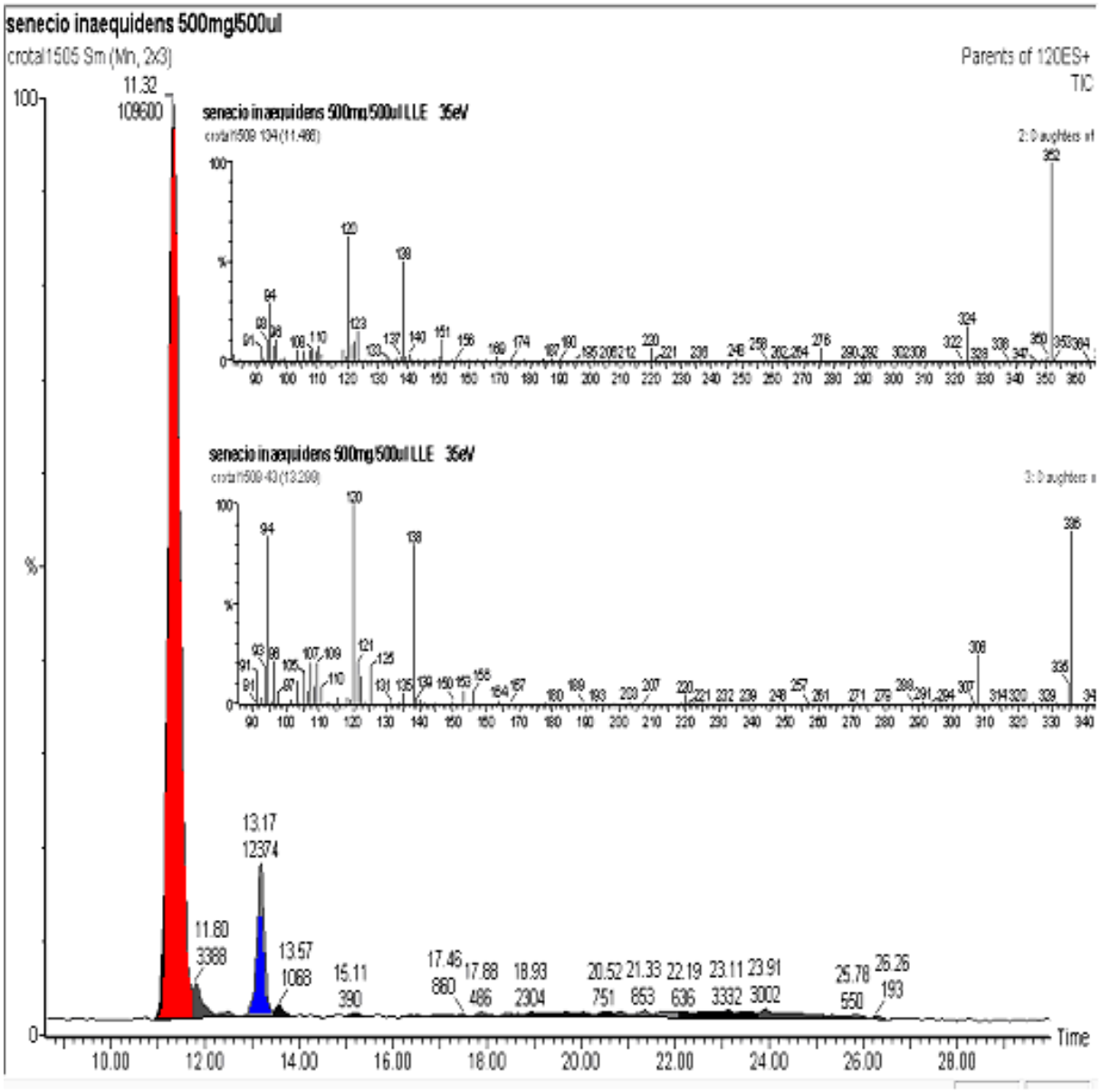

Figure 6-2: Total ion chromatogram [parent ions of *m/z* 120] of extracted *Senecio inaequidens* plant material. Inserted windows are the spectra of the two toxic PAs ($[M+H]^+$ 352 at 11 min and ($[M+H]^+$ 336 at 13 min)

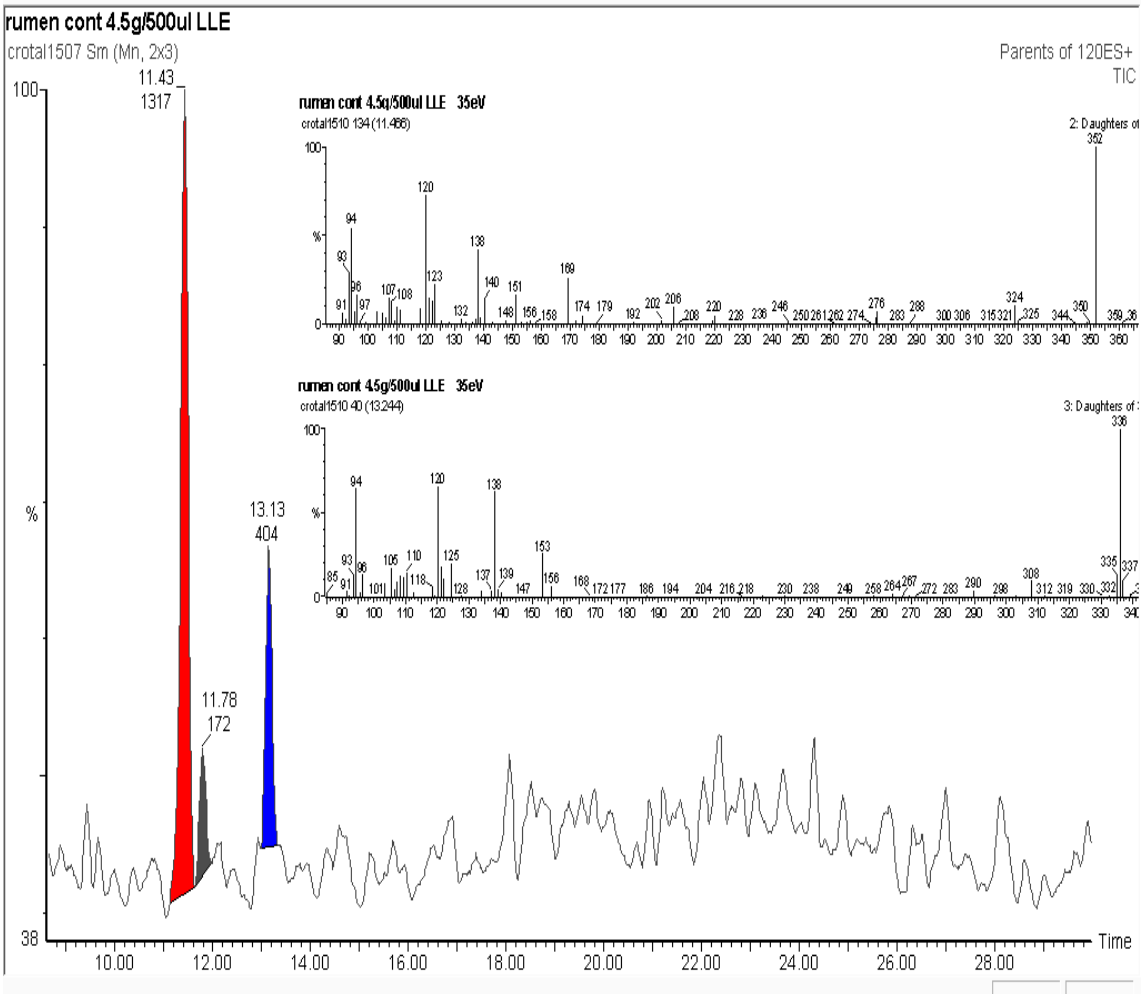

Figure 6-3: Total ion chromatogram [parent ions of *m/z* 120] of extracted rumen content. The spectra of the toxic PAs ($[M+H]^+$ 352 at 11 min and $[M+H]^+$ 336 at 13 min) are identical to the plant extracts.

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).

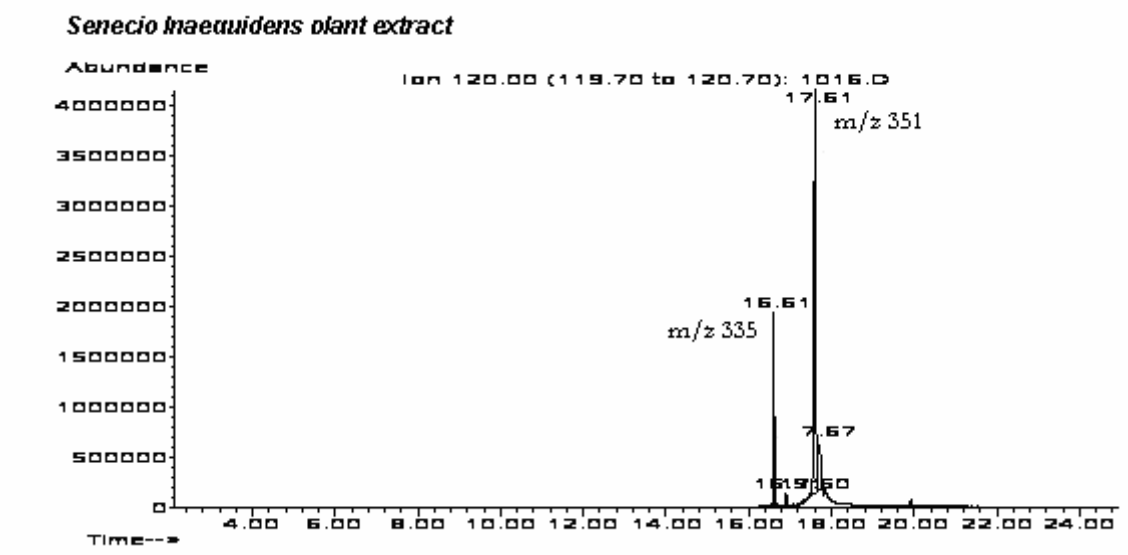

Figure 6-4: Reconstructed chromatogram of the extracted ion *m/z* 120 of a reduced *S. inaequidens* plant extract

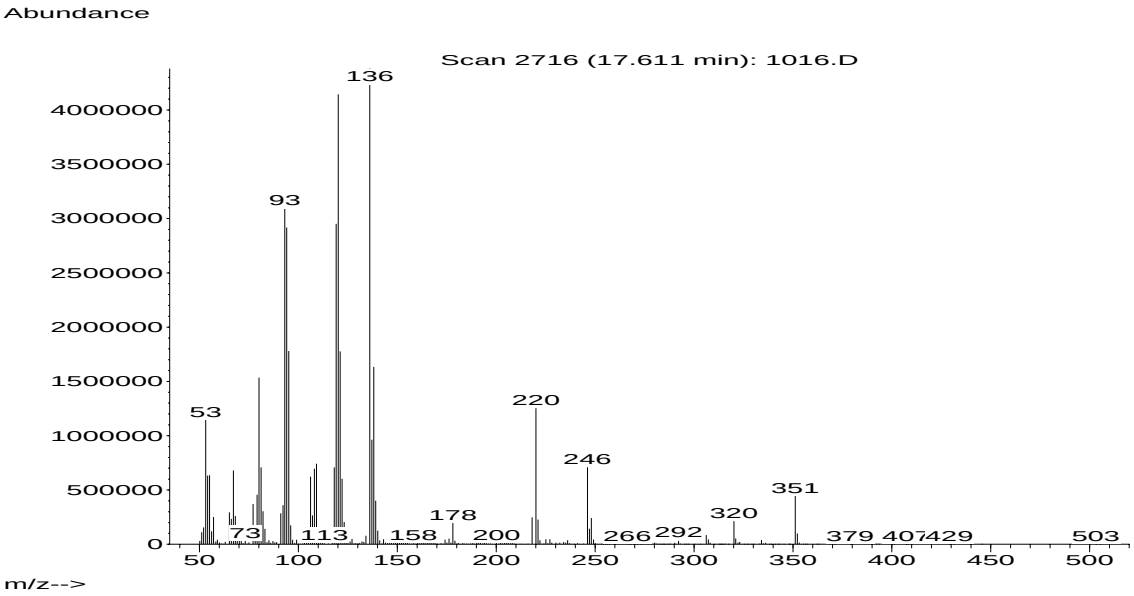

Figure 6-5: GC-MS spectrum of retrorsine in *S. inaequidens*

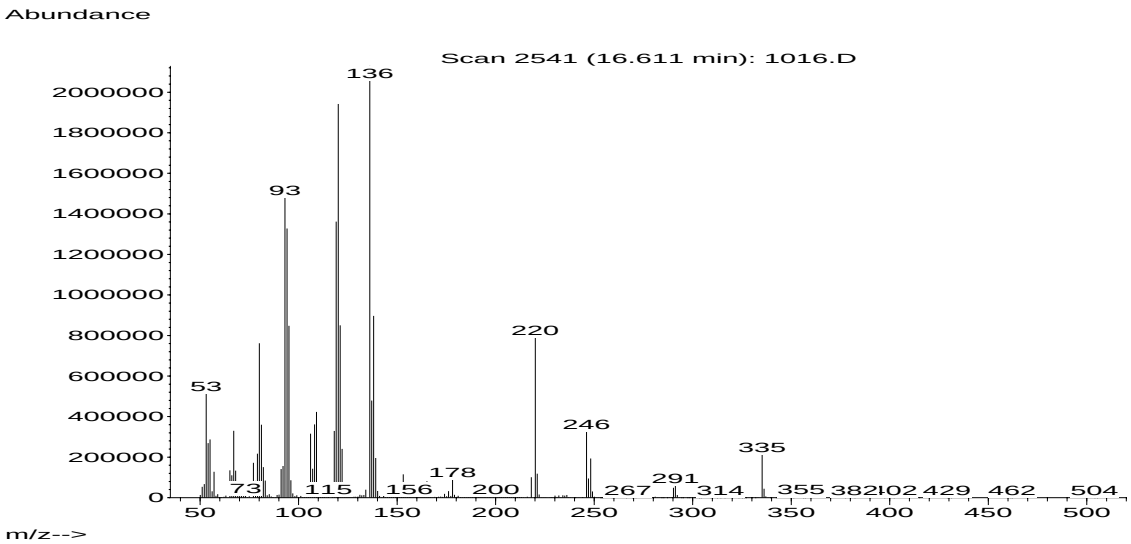

Figure 6-6: GC-MS spectrum of senecionine in *S. inaequidens*

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.

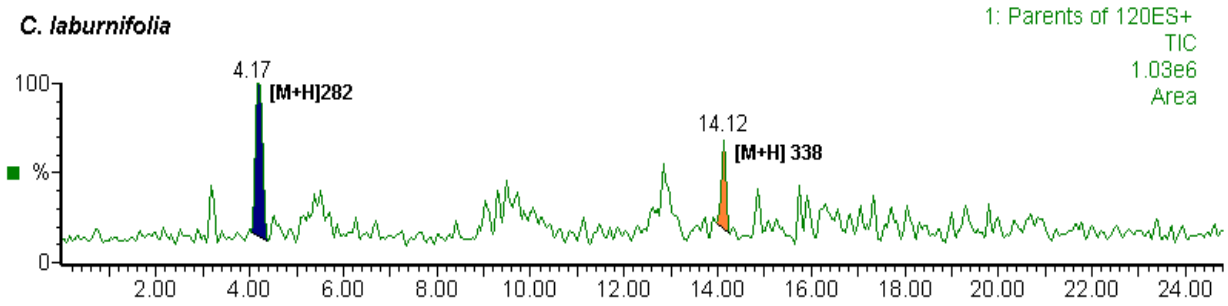

Figure 6-9: Precursor scan of a reduced *C. laburnifolia* plant extract

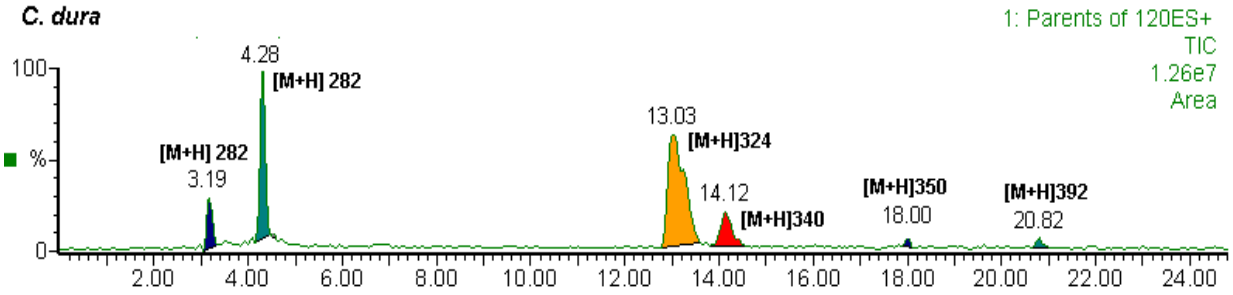

Figure 6-10: Precursor scan of a reduced *C. dura* plant extract

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).

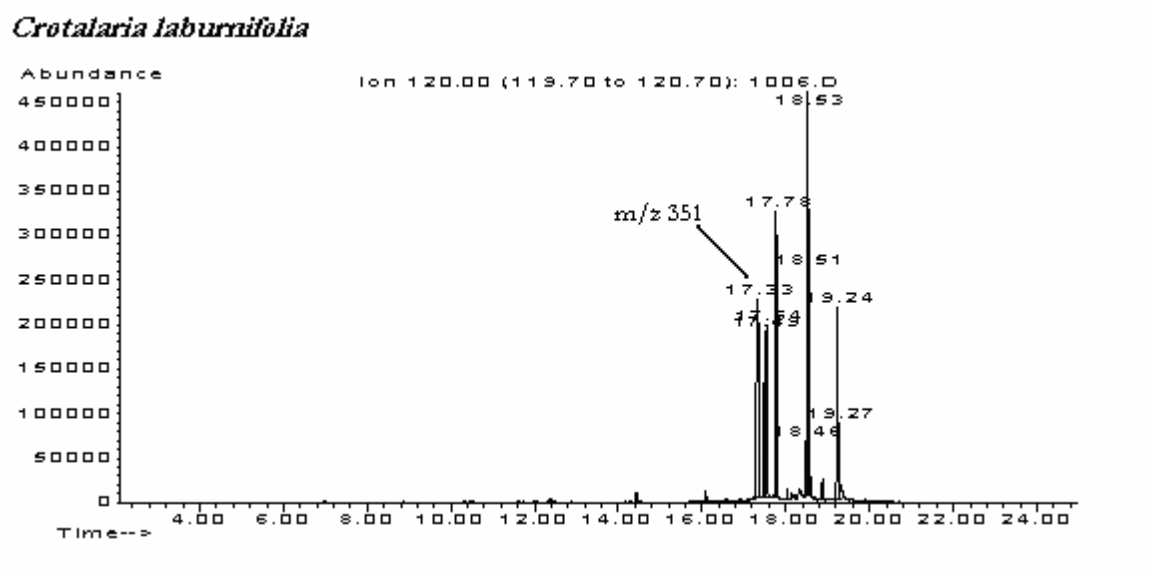

Figure 6-11: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of *C. laburnifolia*

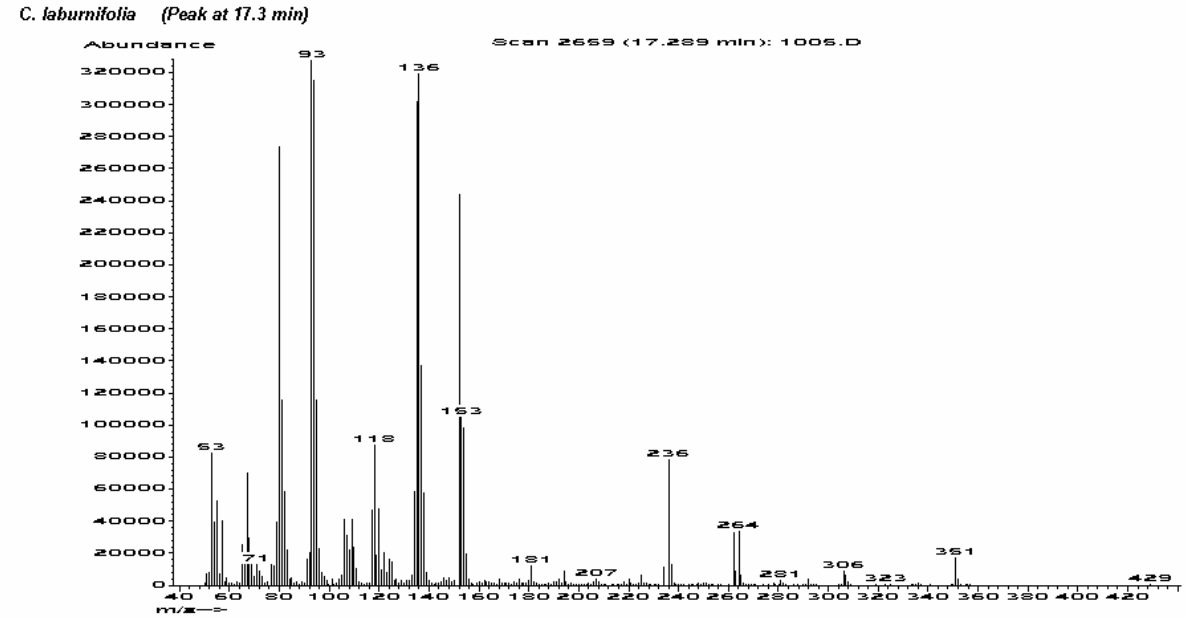

Figure 6-12: GC-MS spectrum of peak at 17.31 minutes

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).

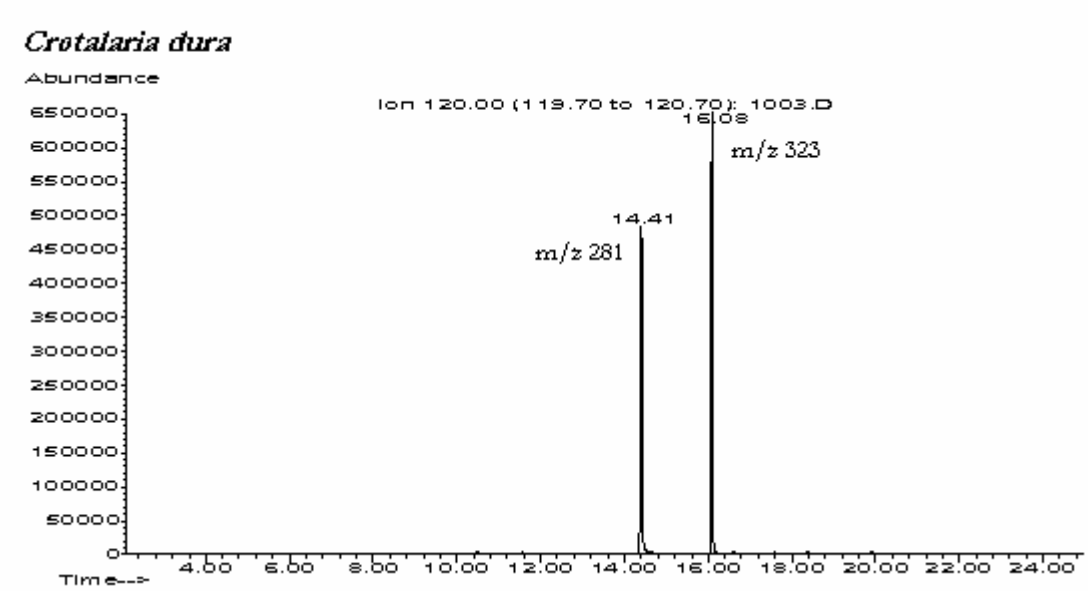

Figure 6-13: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of *C. dura*

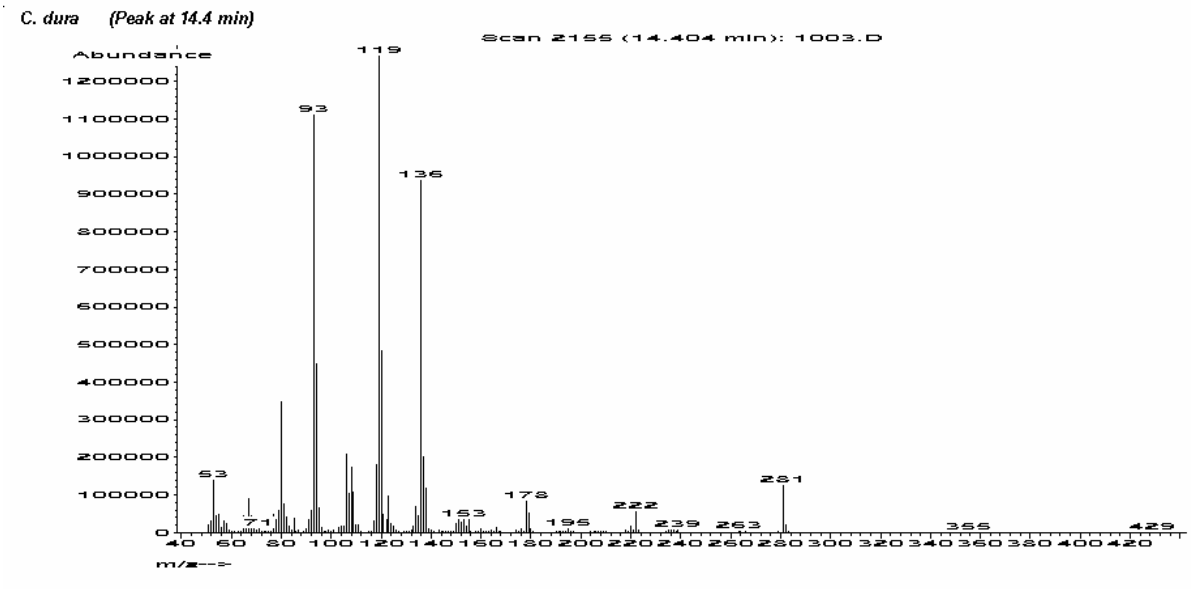

Figure 6-14: CG-MS spectrum of peak $[M]^+$ 281 at 14.4 minutes

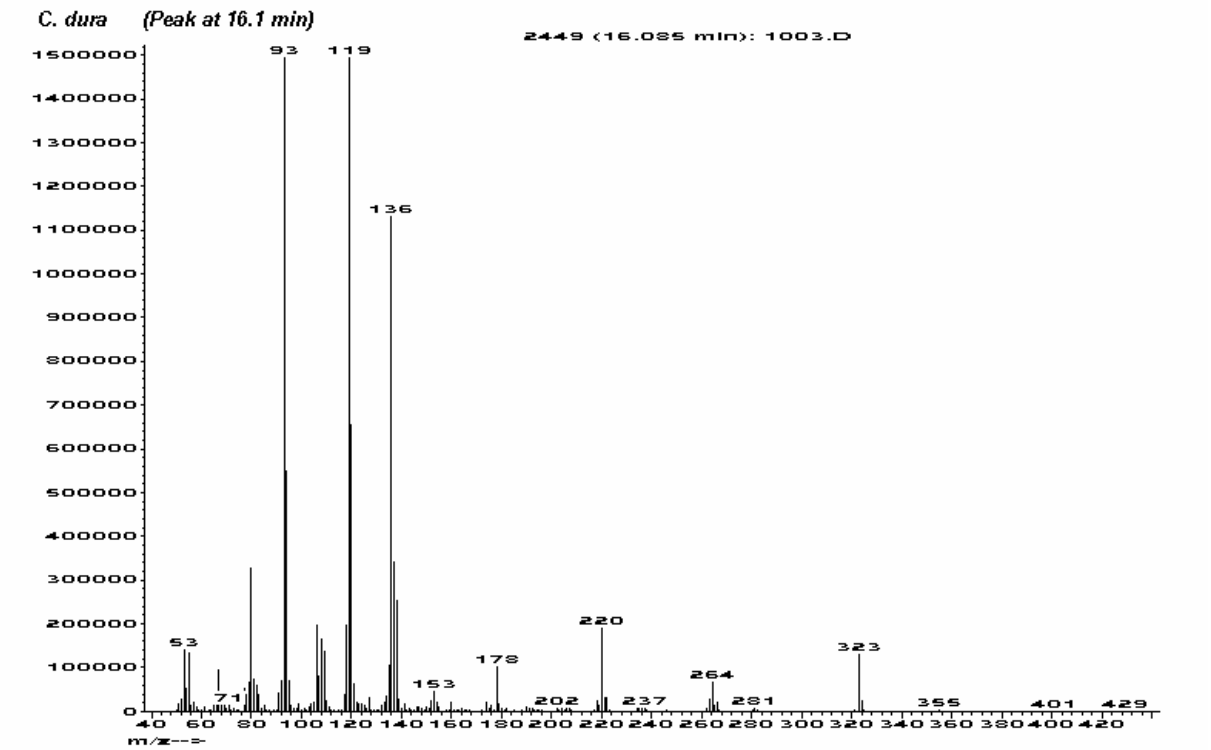

Figure 6-15: GC-MS spectrum of peak $[M]^+$ 323 at 16.1 minutes

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.