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:

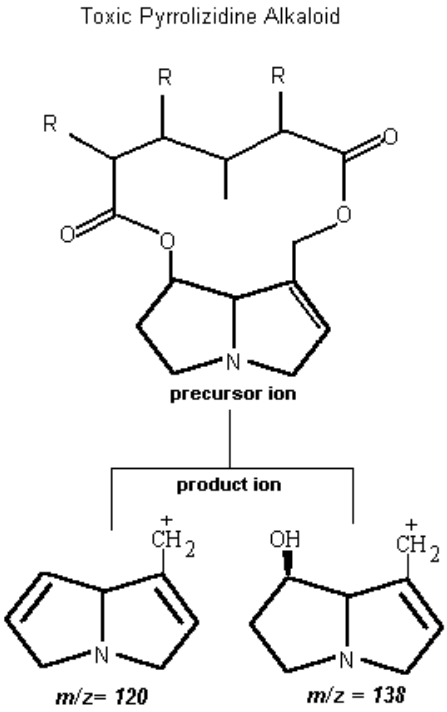

Figure 3-1: Generic structures of a retronecine type toxic PA with the characteristic product ions formed with CID in the collision cell

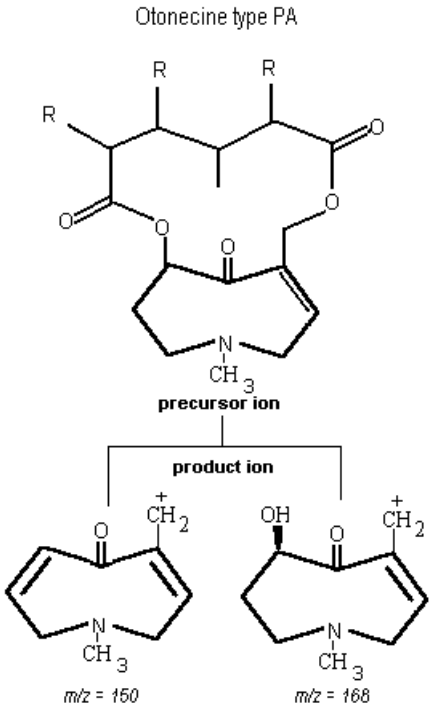

Figure 3-3: Generic structures of an otonecine type toxic PA with characteristic product ions formed with CID in the collision cell

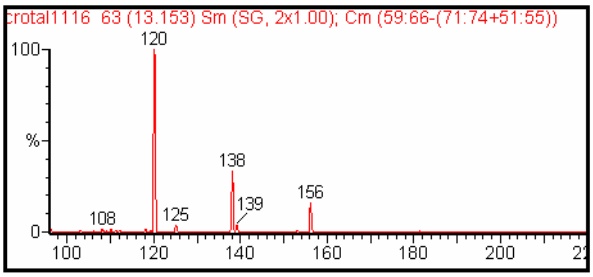

Figure 3-2: LC-MS/MS product ion spectrum of a 1,2-unsaturated necine base showing the characteristic *m/z* 120 and 138 fragments. Precursor ion scans of these fragments will reveal the $[M+H]^+$ mass of the retronecine type PA (Ge Lin 1998)

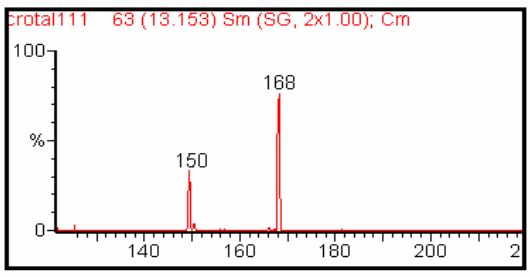

Figure 3-4: LC-MS/MS product ion spectrum of a 1,2-unsaturated otonecine base with characteristic fragments *m/z* 150 and 168. Precursor ion scans of these fragments will reveal the $[M+H]^+$ mass of the otonecine type PA (Ge Lin 1998)

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

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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.

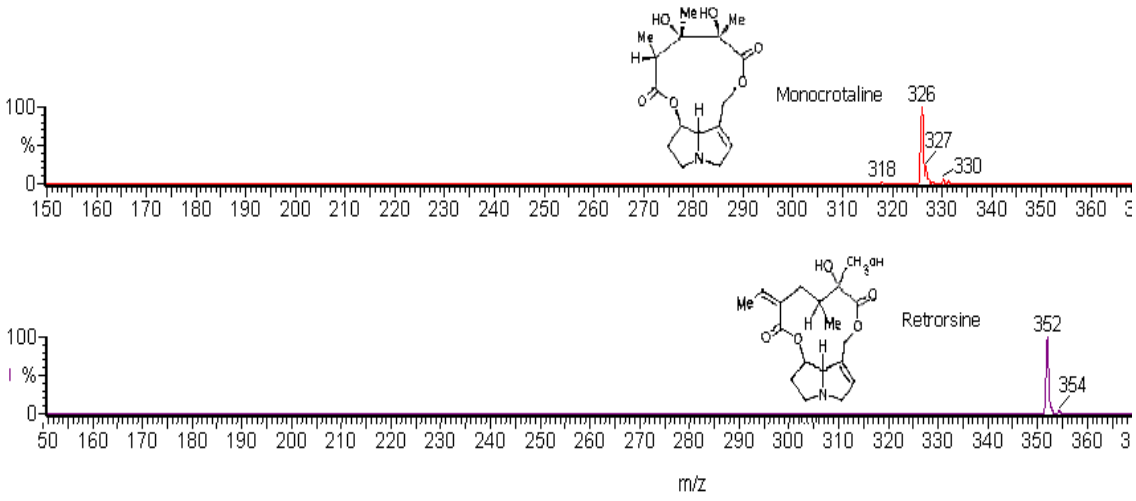

Figure 3-6: ESI$^+$ mass spectra of monocrotaline and retrorsine showing the $[M+H]^+$ ions obtained with the infusion experiments

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.

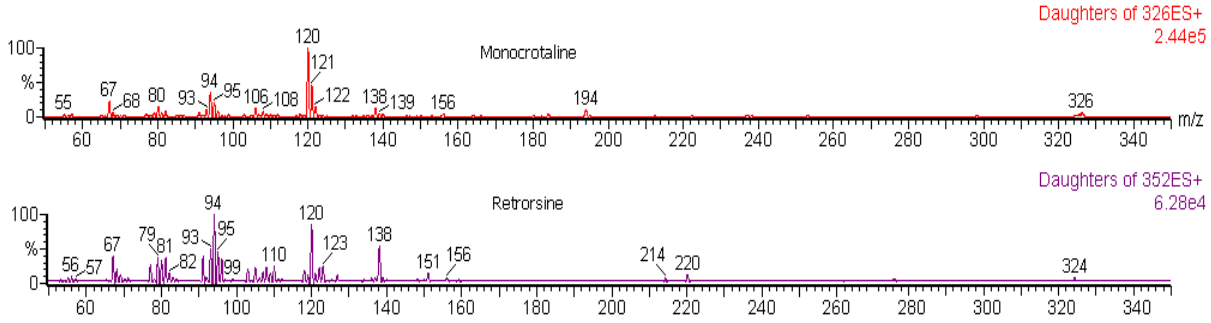

Figure 3-7: MS/MS spectra of monocrotaline and retrorsine with collision induced fragmentation in the collision cell (collision gas = 29) showing the fragments characteristic of unsaturated PAs

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.

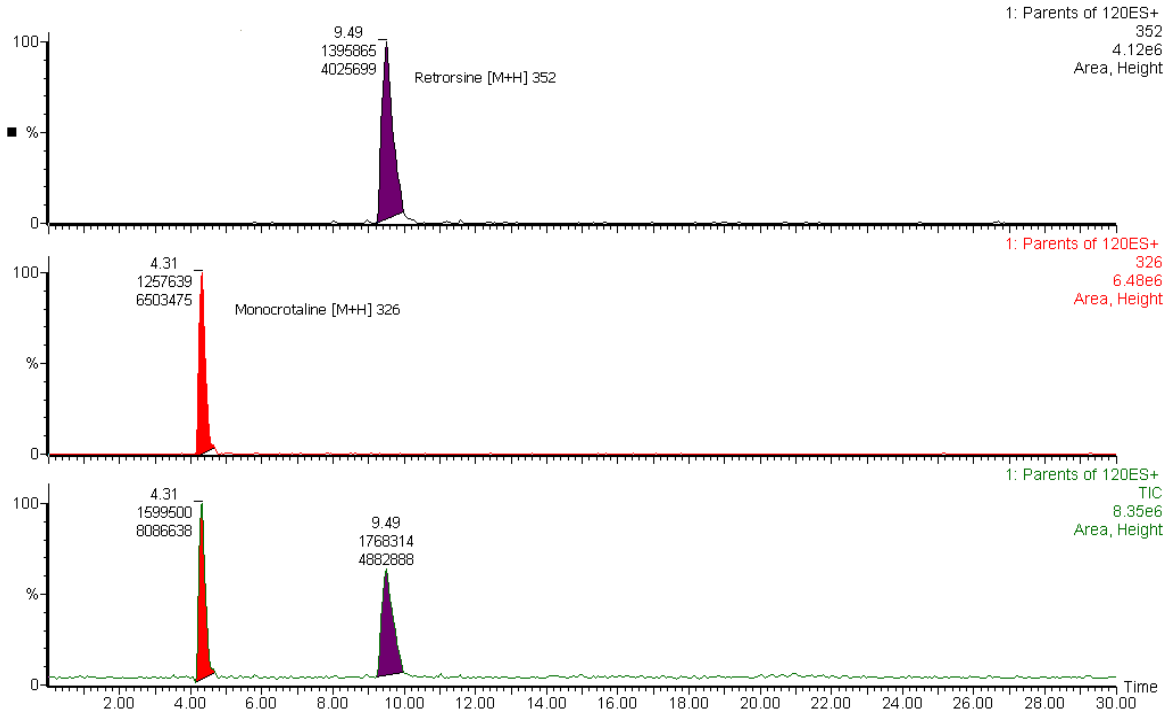

Figure 3-8: The LC-MS/MS total ion chromatogram (TIC) of a mixture of monocrotaline and retrorsine solution and the reconstructed chromatograms of the $[M+H]^+$ precursor ions 326 (monocrotaline) and 352 (retrorsine) which gave rise to the *m/z* 120 fragments

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].

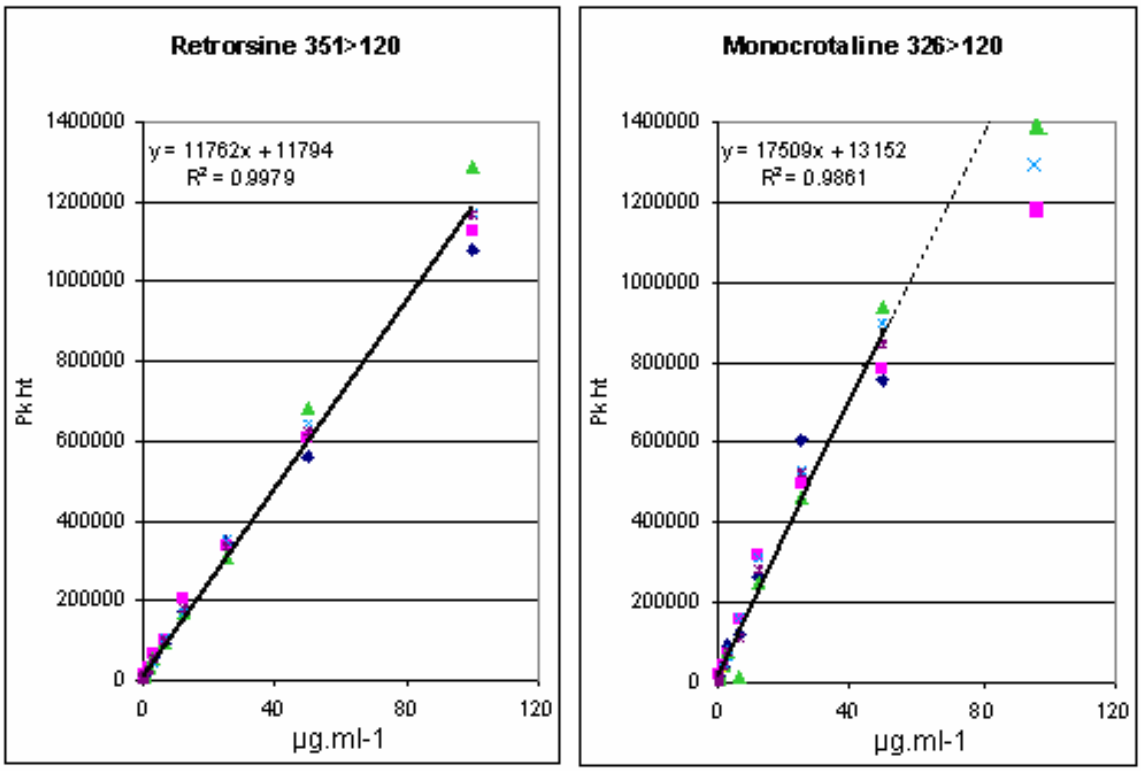

Figure 3-9: Calibration curves of retrorsine and monocrotaline spiked lucerne extracts

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.

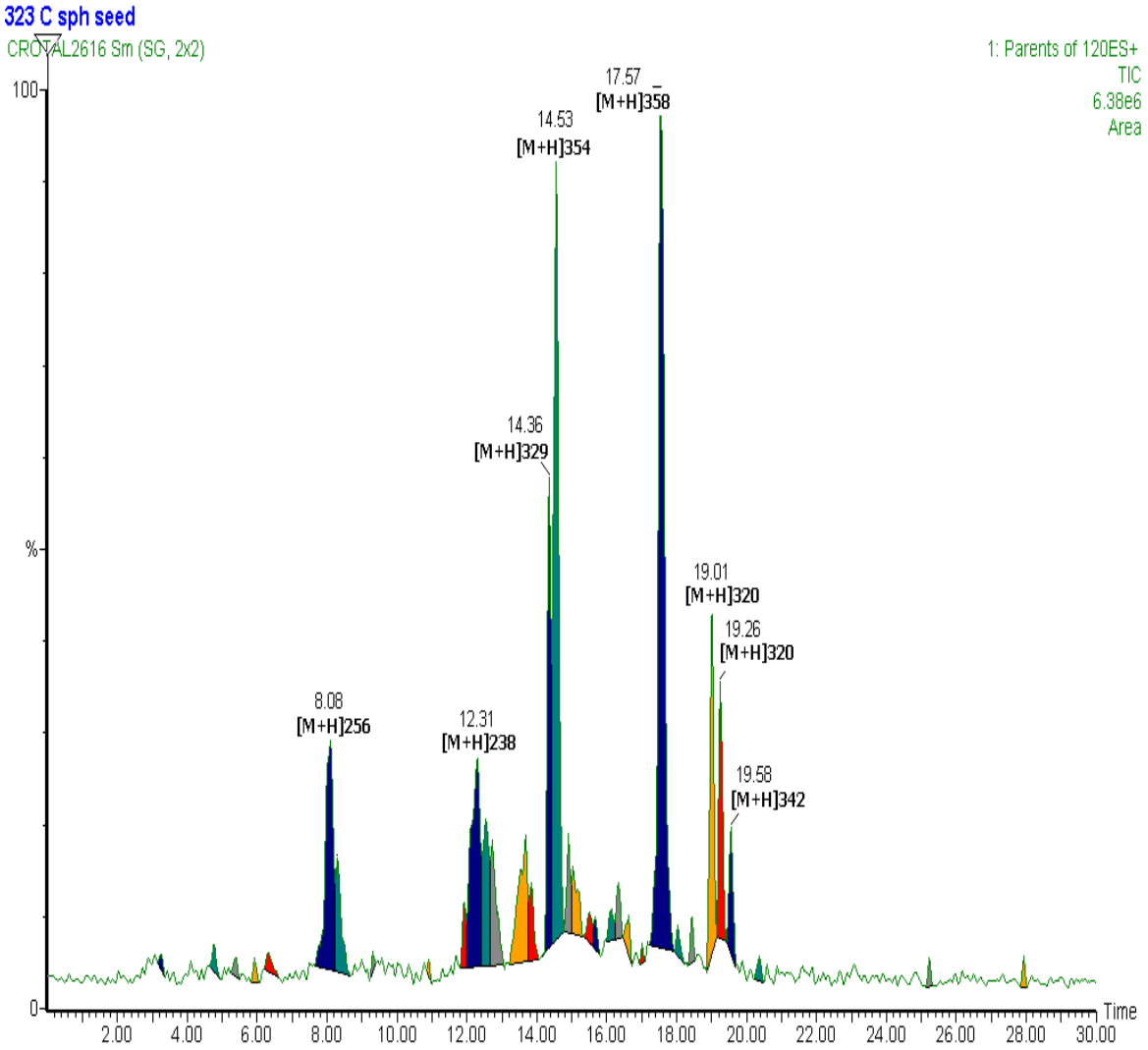

Figure 3-10: Example of a chromatogram obtained with the precursor scan (*C. sphaerocarpa* seed extract of sample A) indicating the $[M+H]^+$ mass of each precursor

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).

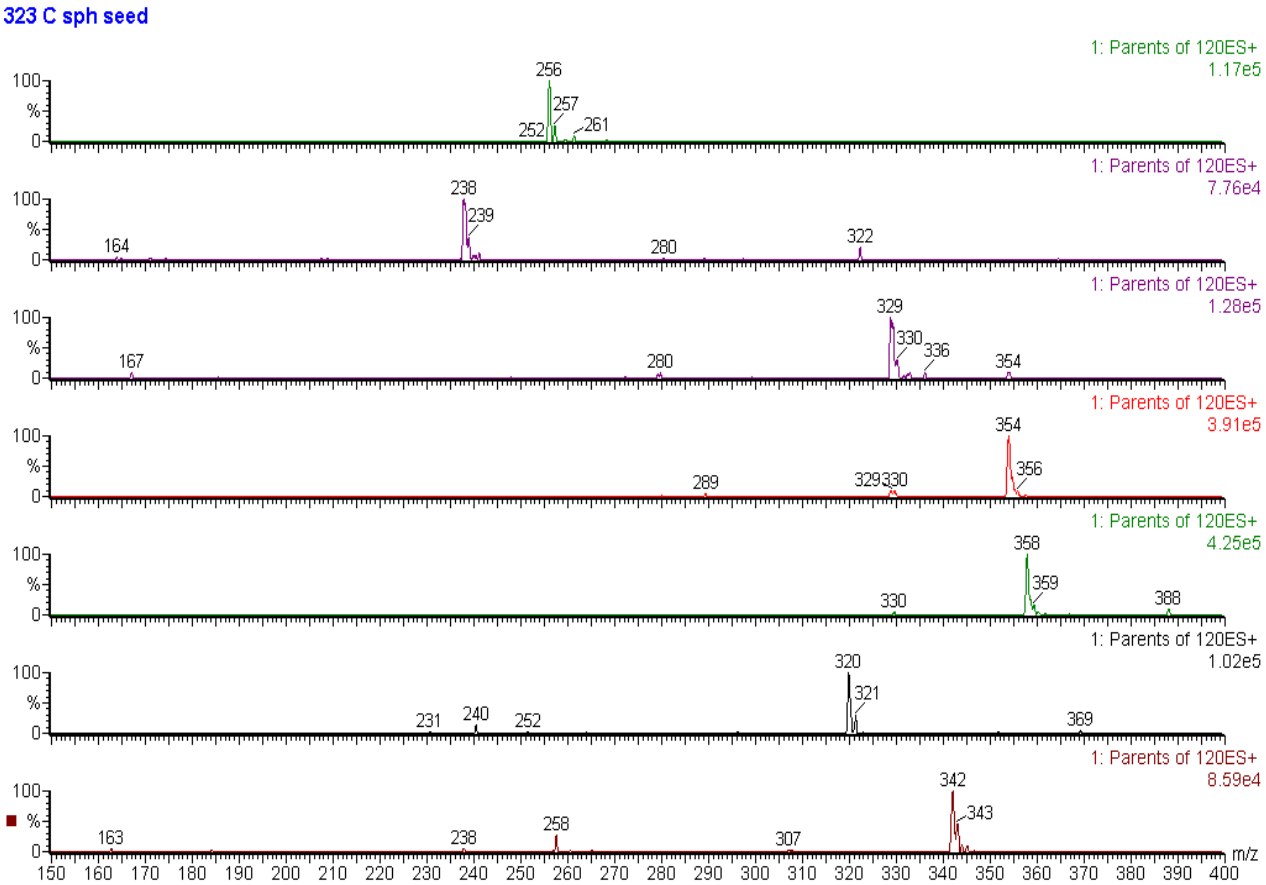

Figure 3-11: Reconstructed precursor ion $[M+H]^+$ masses of the fragment *m/z 120* obtained in Fig 3-10 at their corresponding retention times

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.

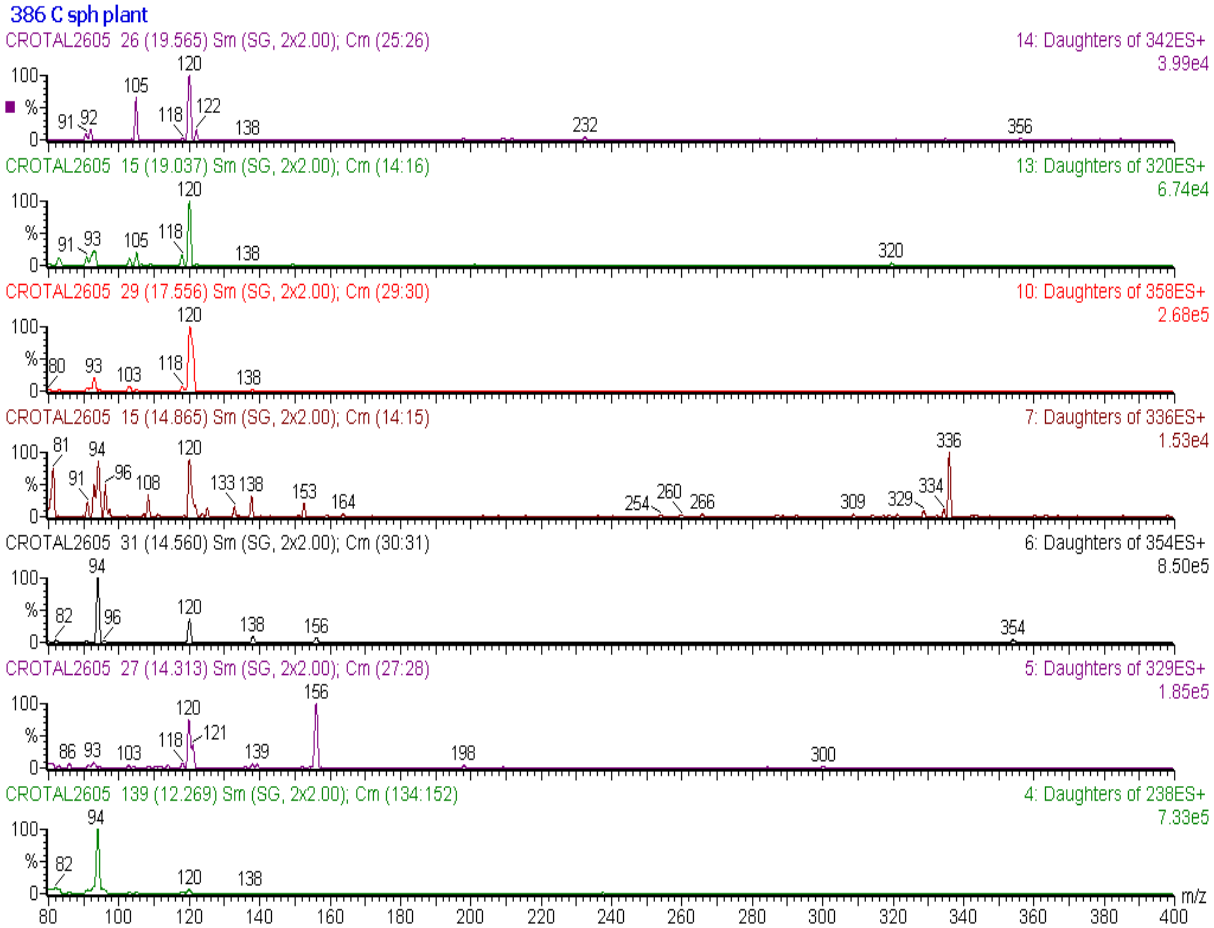

Figure 3-12: MS/MS spectra of the possible toxic PAs found in sample E. (Precursor ion mass listed in each trace as "Daughters of ...")

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.

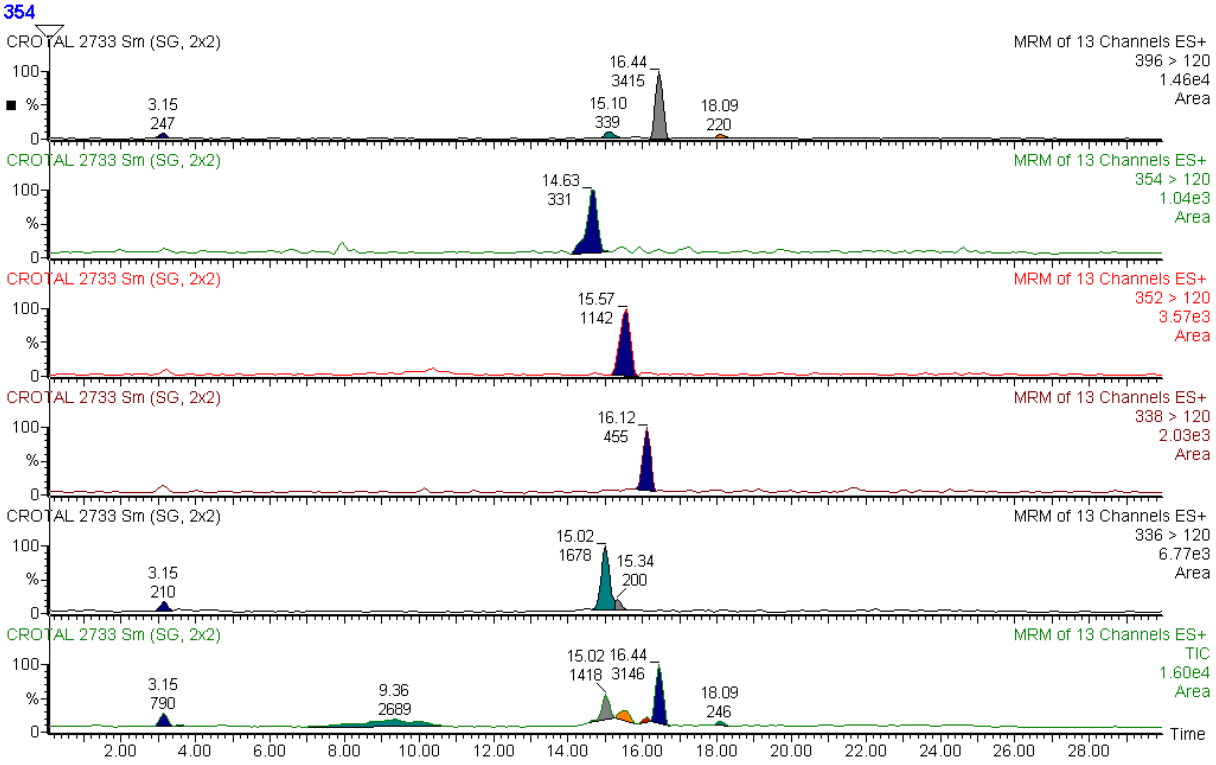

Figure 3-13: MRM chromatogram of the different transitions measured in sample E

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.