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.

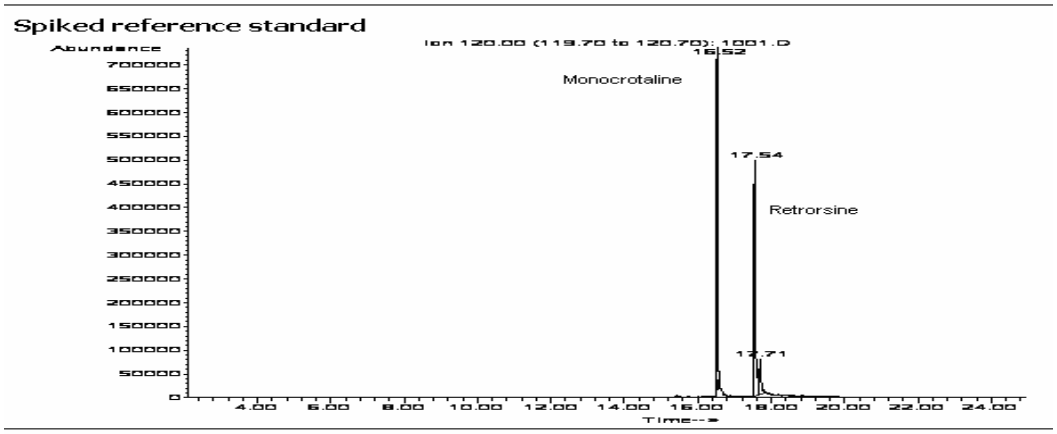

Figure 5-1: Reconstructed EI-MS chromatogram of the extracted ion *m/z* 120, of a reference standard containing monocrotaline and retrorsine in lucerne extract

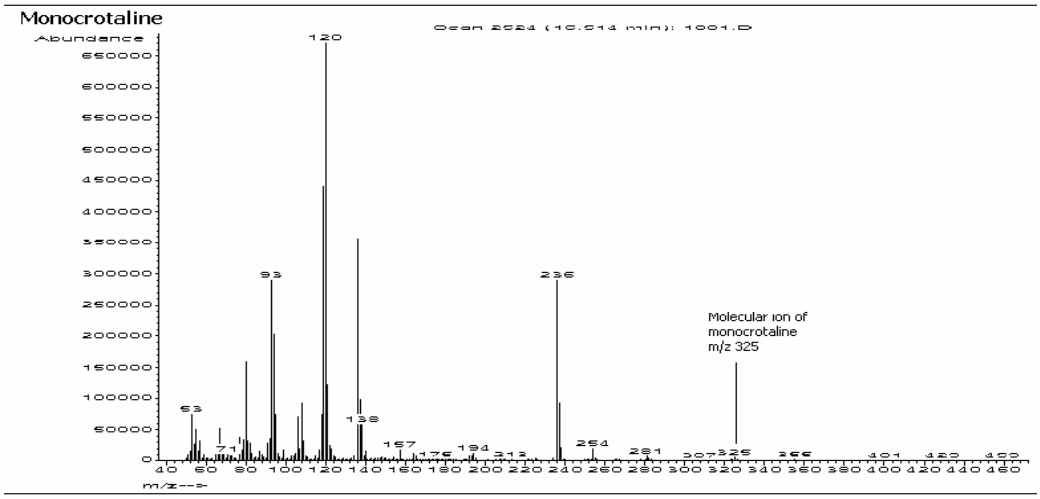

Figure 5-2: EI-MS spectrum of monocrotaline

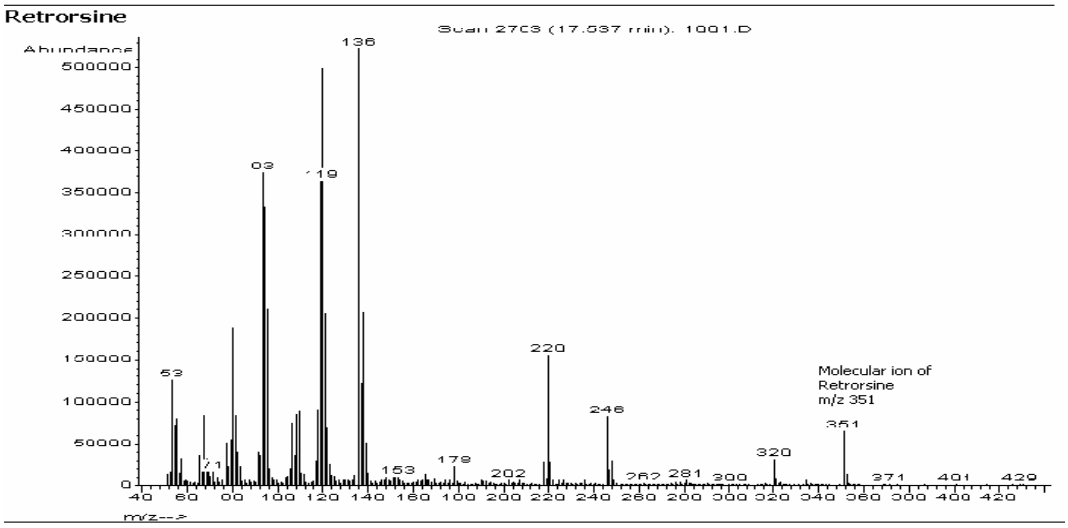

Figure 5-3: EI-MS spectrum of retrorsine

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.

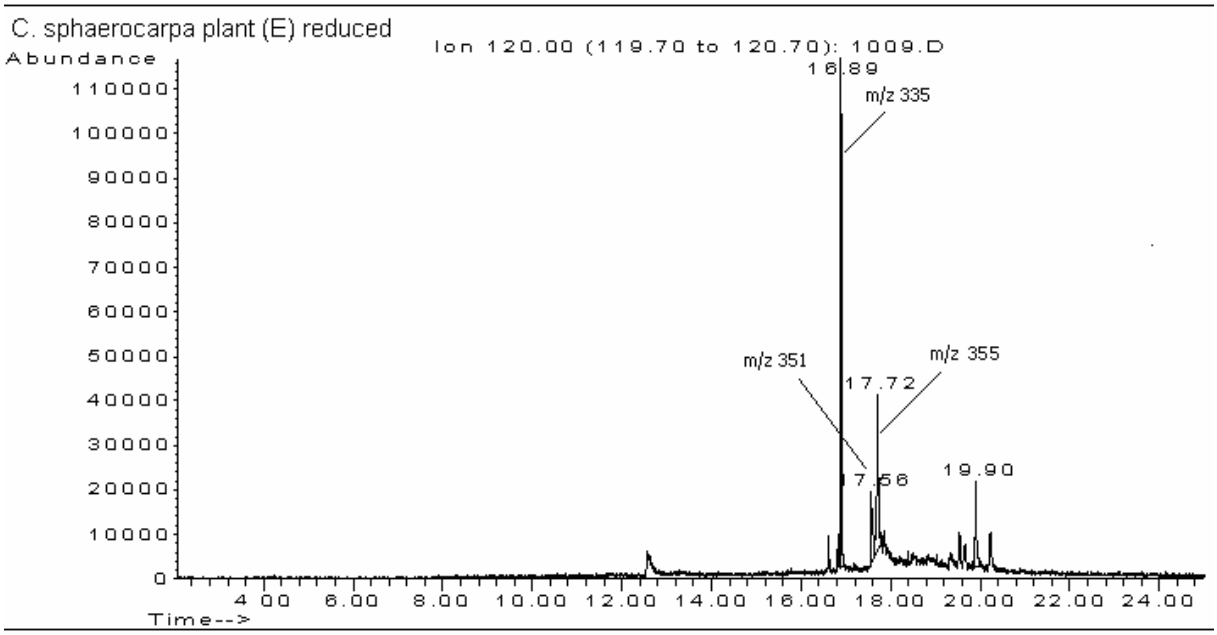

Figure 5-4: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of sample E

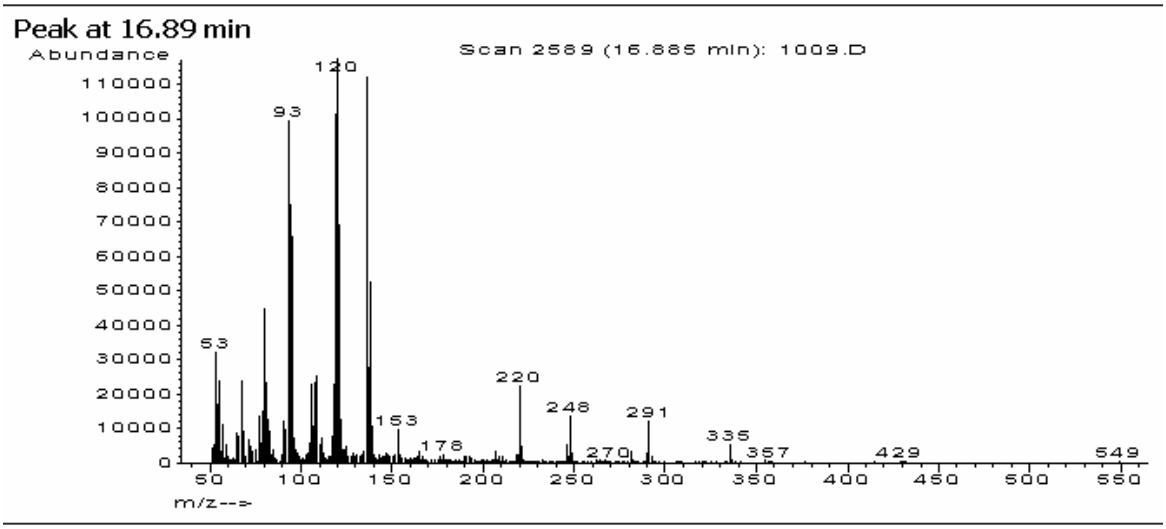

Figure 5-5: EI-MS spectrum of the peak $[M]^+$ 335 at 16.9 minutes, identified as integerrimine

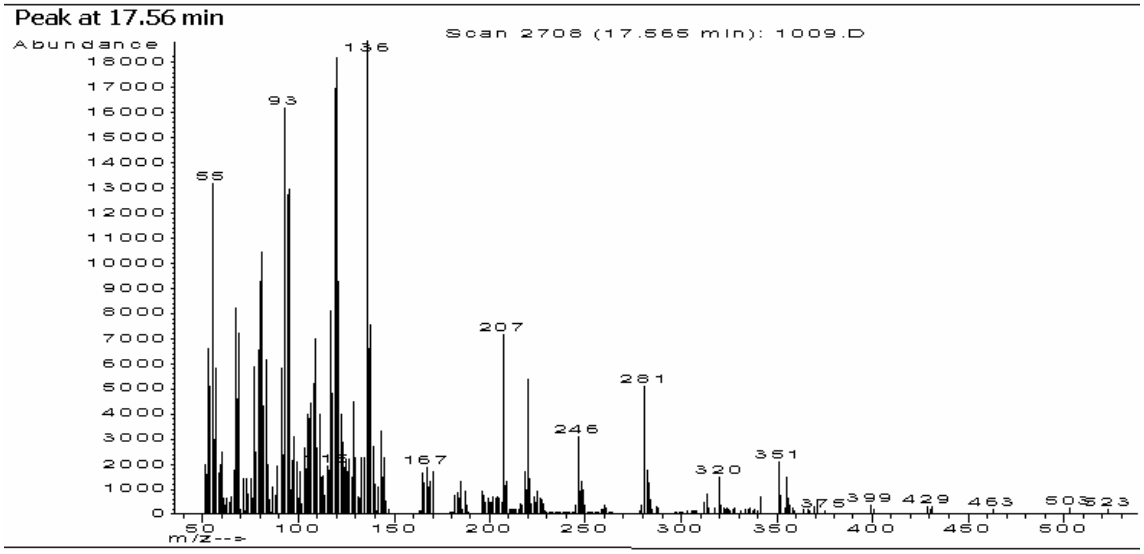

Figure 5-6: EI-MS spectrum of peak $[M]^+$ 351 at 17.56 minutes

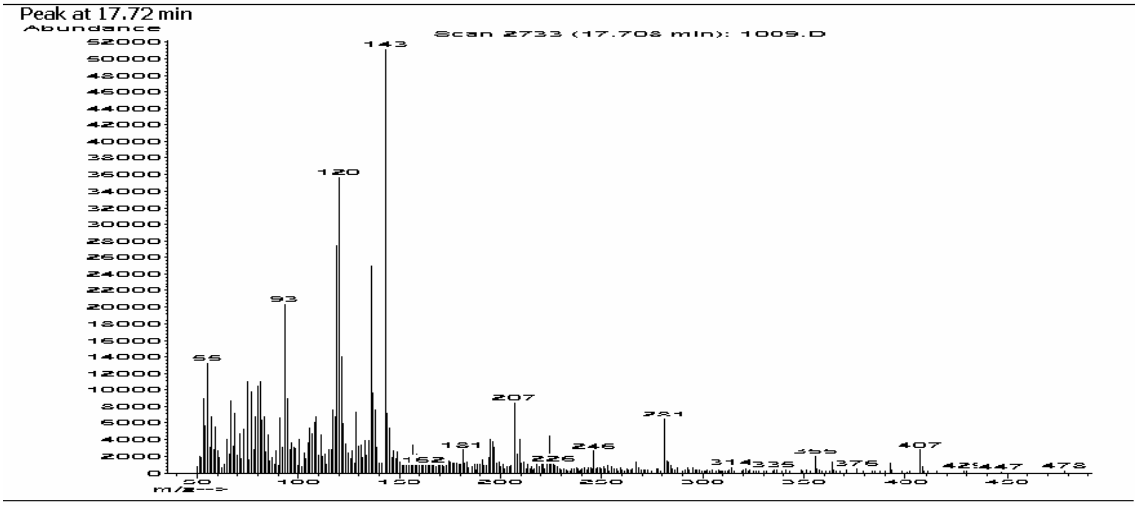

Figure 5-7: EI-MS spectrum of peak $[M]^+$ 355 at 17.72 minutes

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.

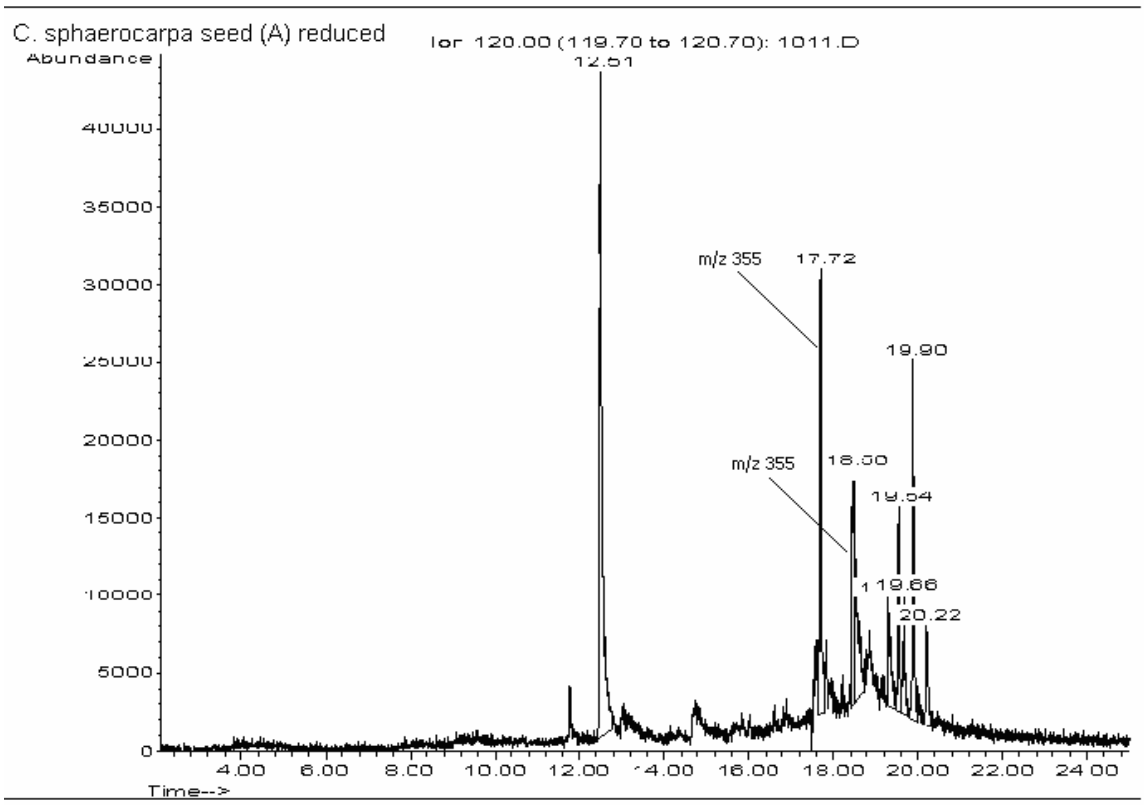

Figure 5-8: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced sample A in EI mode

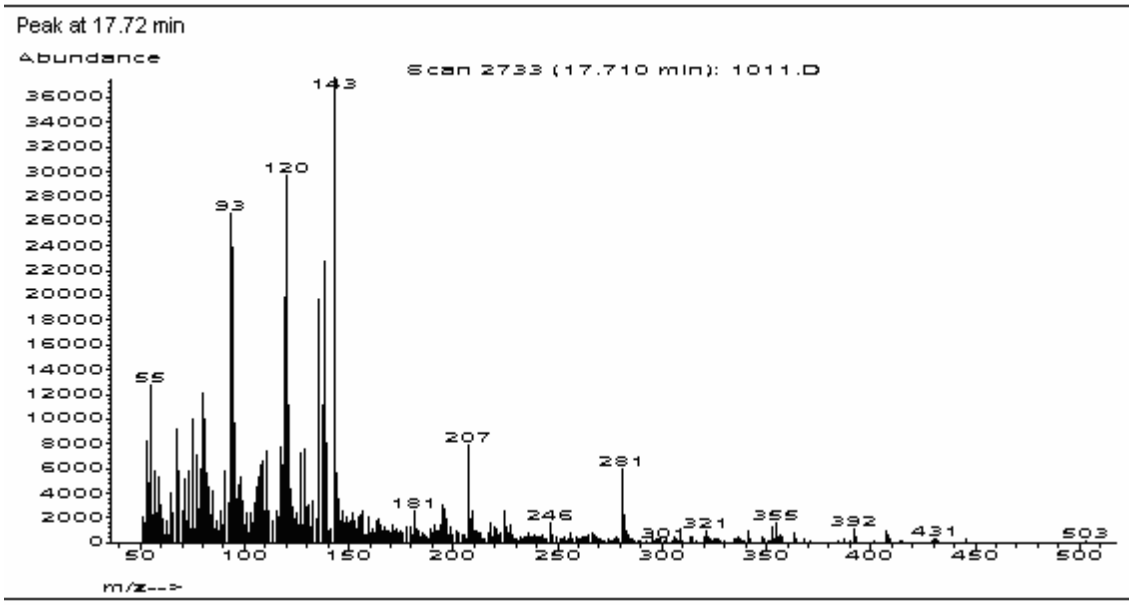

Figure 5-9: EI spectrum of peak $[M]^+$ 355 at 17.71 minutes

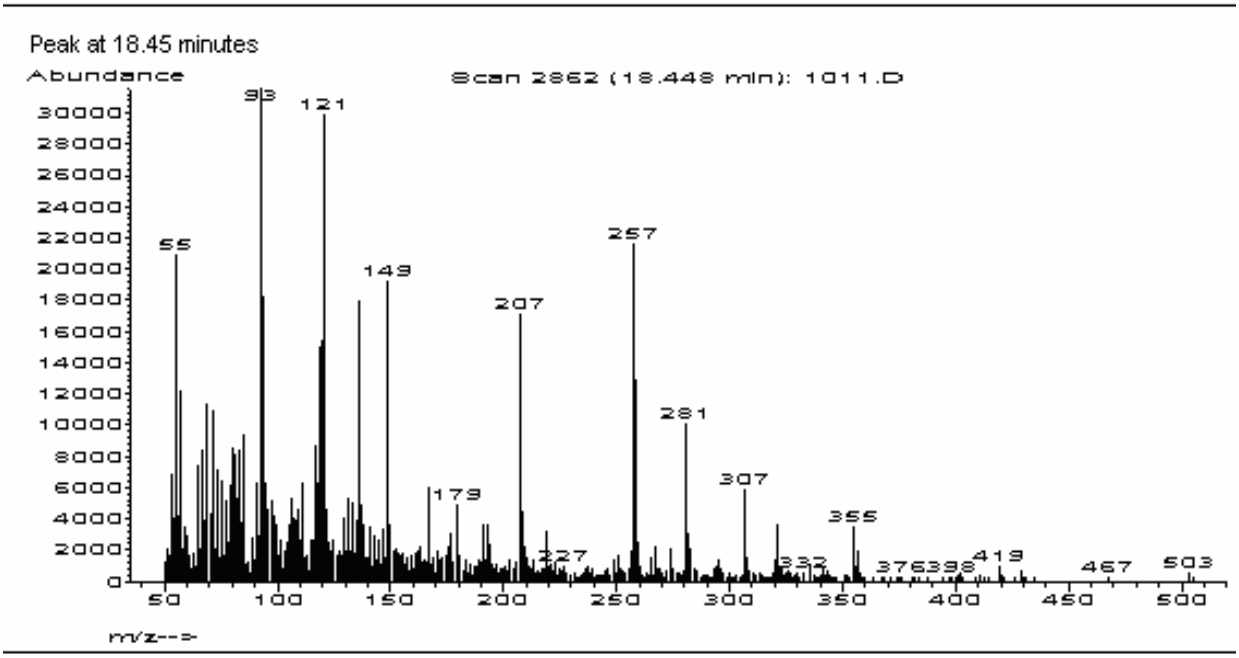

Figure 5-10: EI spectrum of peak $[M]^+$ 355 at 17.71 minutes

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).

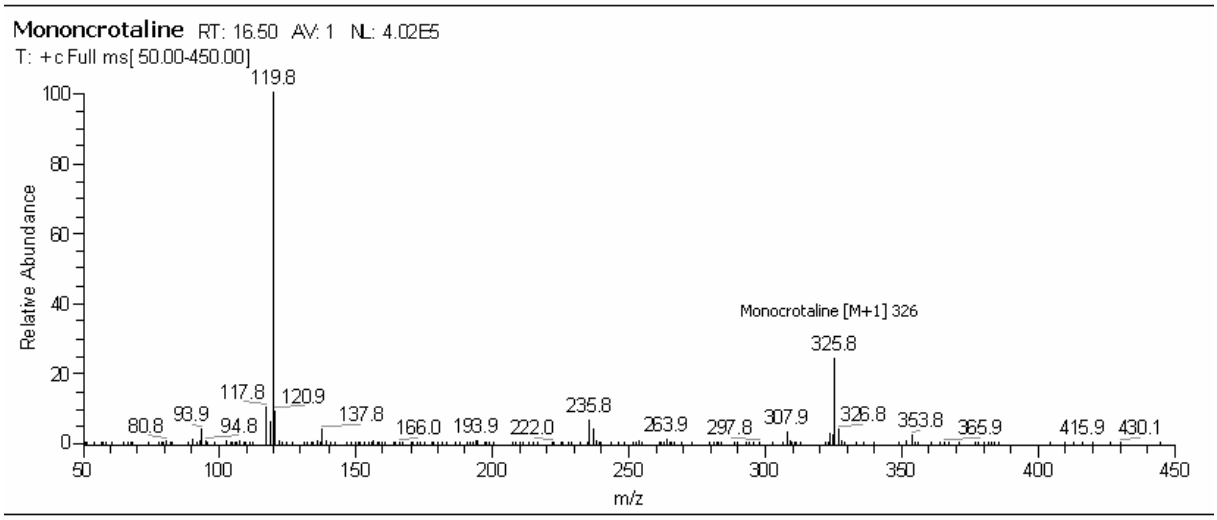

Figure 5-11: MS spectrum of monocrotaline obtained with PCI

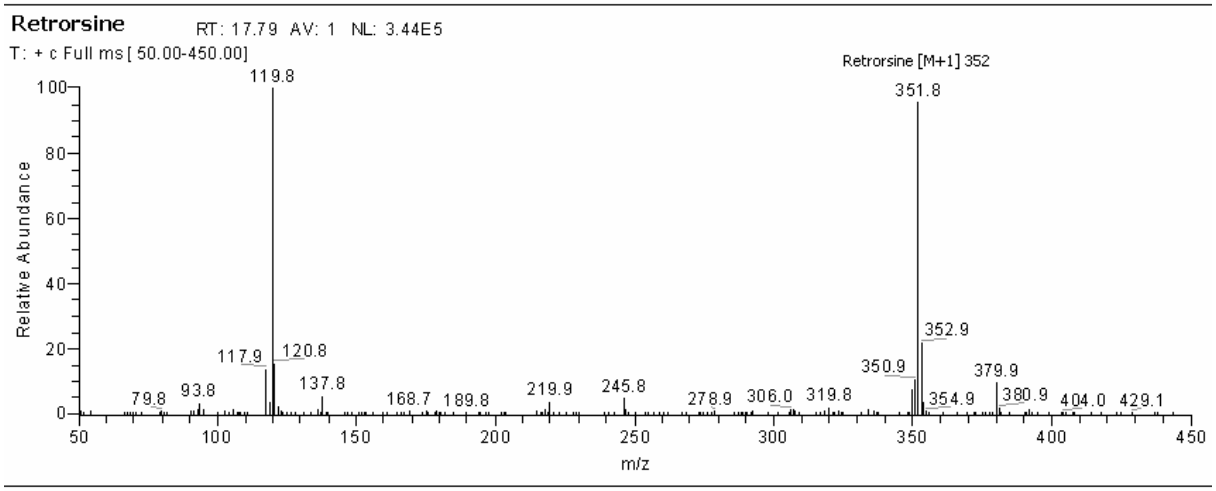

Figure 5-12: CI-MS spectrum of retrorsine

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.

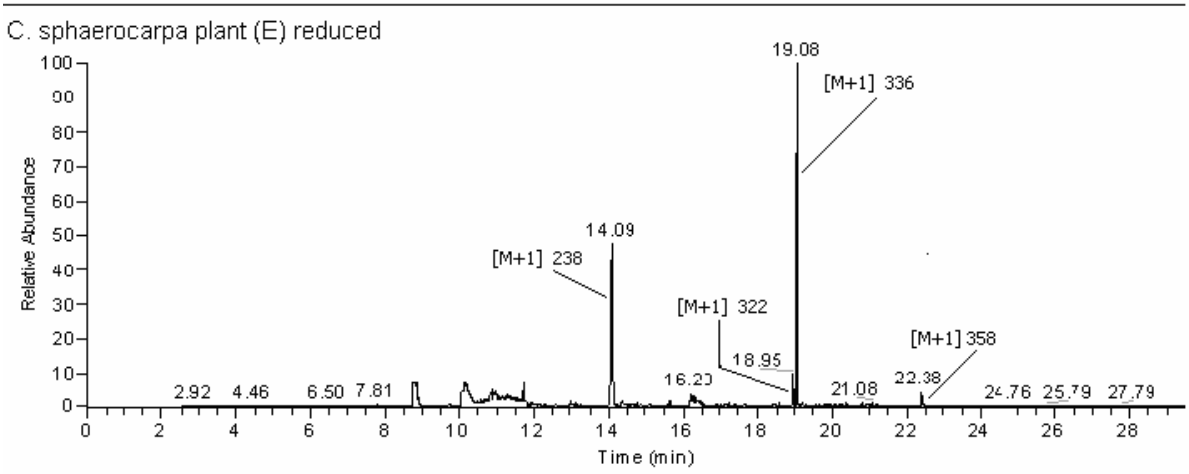

Figure 5-13: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of sample E in PCI mode

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.

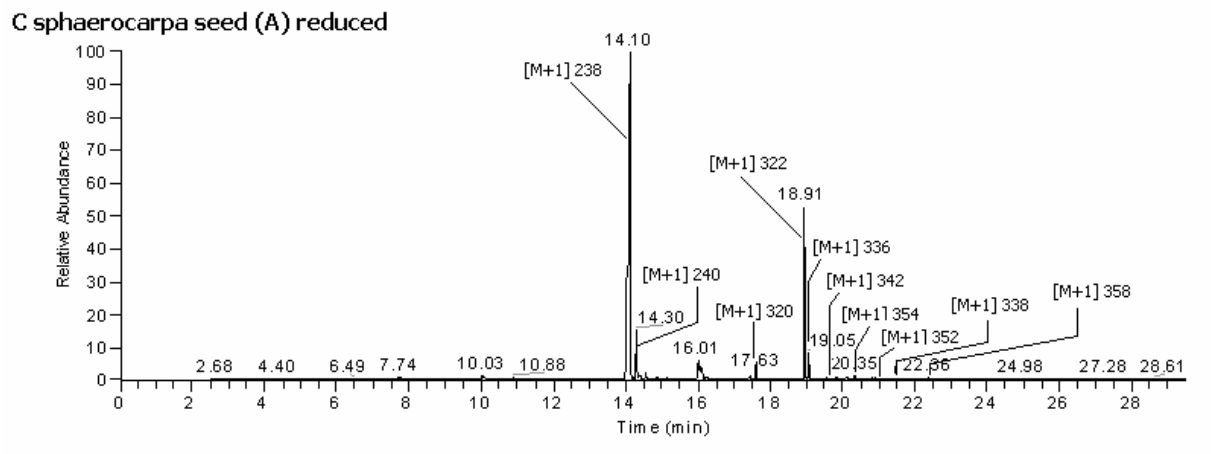

Figure 5-14: Reconstructed chromatogram for the extracted ion *m/z* 120 of a reduced extract of a seed sample (A) in PCI mode

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.