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.

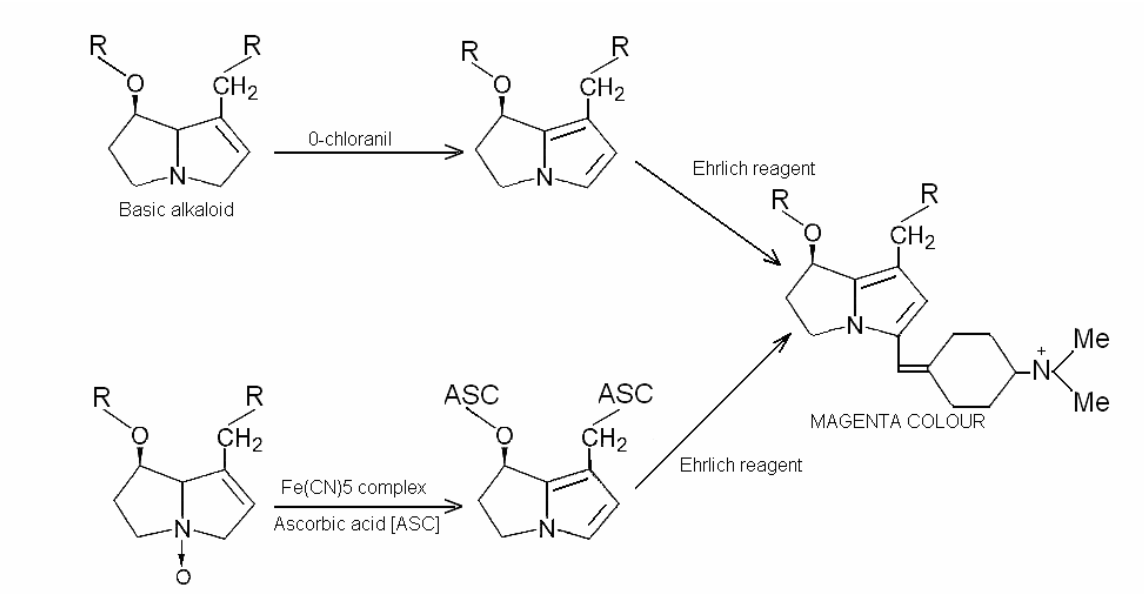


Figure 2-1: Reactions of Ehrlich's reagent with basic PA and *N*-oxide structures

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)

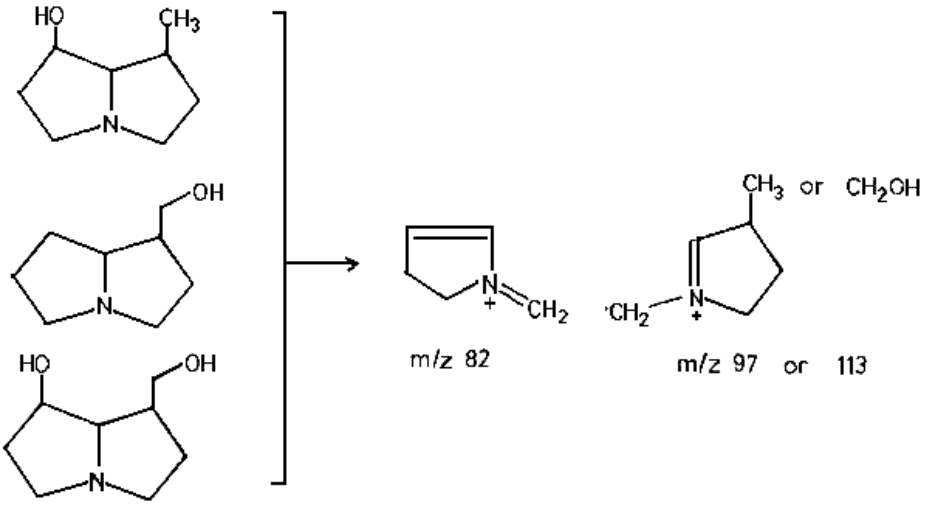
NMR spectroscopy provides detailed information on PA structures and stereo-chemical orientations and can even be used for quantification of compounds. Röder (1990) discussed the role of ^{13}C-NMR in structural elucidation of PAs. The major disadvantage of this technique is the large quantity of purified 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.

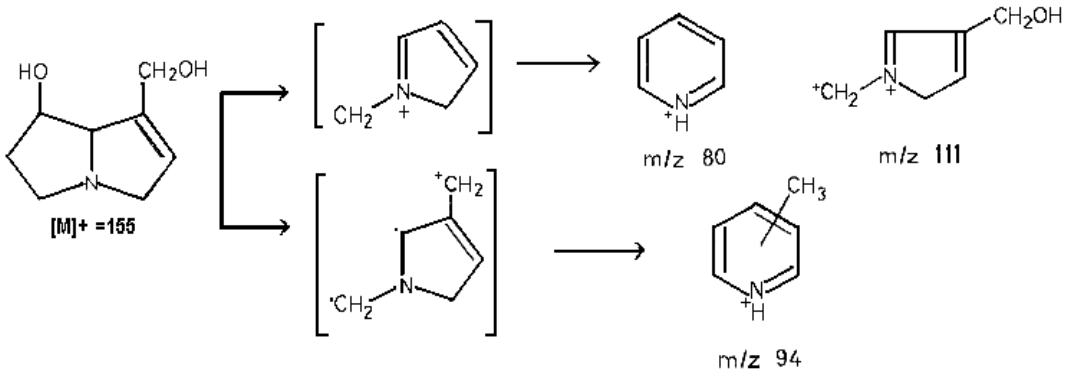
2.3.3.2 Classical electron impact mass spectra (EI-MS)

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).

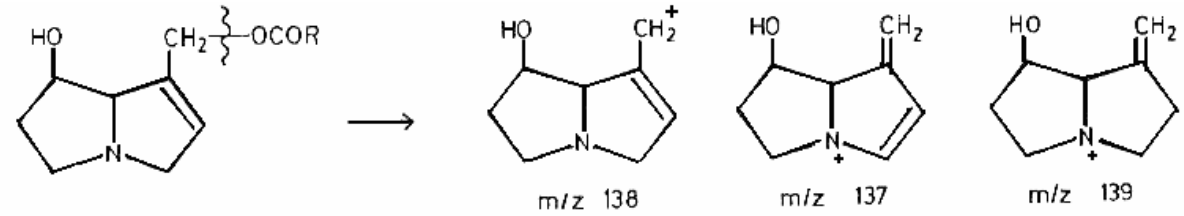
With classical EI fragmentation, saturated necines give typical fragments in the ranges *m/z* 95-97, 113-115, 122-123 and 138-140; with a characteristic base peak at *m/*z 82:



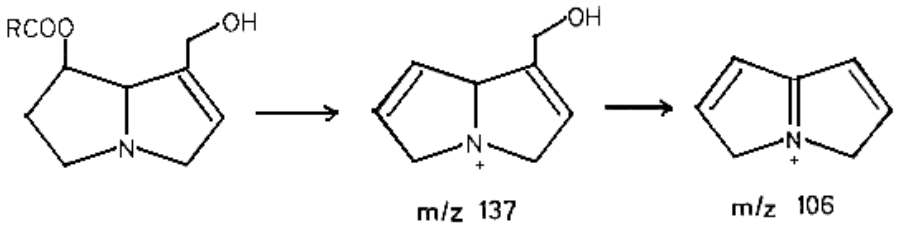
Corresponding fragments from unsaturated necines are two mass units lower with major fragments at *m/z* 80, 94, 111 and 120.



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.