CHAPTER 7: GENERAL DISCUSSION AND RECOMMENDATIONS

7.1 Analytical method

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

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

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

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

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

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

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

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

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

7.2 Further recommendations

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

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

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

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

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

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

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

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

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

7.4 Final conclusion

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

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

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

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

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

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