Analysis of pyrrolizidine alkaloids in *Crotalaria* species by HPLC-MS/MS in order to evaluate related food health risks

GM Rösemann

A thesis submitted in fulfilment of the requirements for the degree of

Philosophiae Doctor

Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria

Promoter: Professor JN Eloff
Co-Promoter: Professor CJ Botha

DECLARATION

The work described in this thesis was conducted on a part time basis at FARMOVS-Paraxel, Bloemfontein between January 2004 and March 2005, and at Drs DuBuisson, Bruinette and Kramer, Pretoria, between April 2005 and December 2006.

I hereby declare that the data included in this thesis are the results of my investigations and that the thesis was written by me. References made to published literature have been duly acknowledged.

Magda Rösemann

30 December 2006

University of Pretoria

Abstract

Analysis of Pyrrolizidine Alkaloids in *Crotalaria* species by HPLC-MS/MS in order to evaluate related Food Health Risks

By Magda Rösemann

Promoter: Professor JN Eloff

Co-Promoter: Professor CJ Botha

Phytomedicine Programme

Department of Paraclinical Sciences

Pyrrolizidine alkaloids (PAs) are one of the most significant groups of plant toxins in the world and are an important cause of poisoning in livestock, resulting in significant financial and production losses each year (Kellerman *et al.* 1996). Pyrrolizidine alkaloids may also enter the human food chain as contaminants of grains, via animal products such as milk, eggs and honey or may be consumed as constituents of herbal medicines (ANZFA 2001).

Not all PAs are toxic. Pyrrolizidine alkaloids affecting human health are the esters of 1,2-unsaturated hydroxymethyl dehydropyrrolizidines (DHP). Before it can be converted to DHP, PAs need to have certain essential features, which include an unsaturated 3-pyrrole ring, one or two hydroxyl groups attached to the ring, one or two ester groups and a branched acid moiety (Mattocks 1986). These compounds can be metabolized in the liver to nucleophillic pyrroles which cause damage to hepatocytes (Winter and Segall 1989).

Although the involvement of PAs in the development of hepatic veno-occlusive disease is well established (Bras *et al.* 1961), there is still uncertainty concerning the consequences of long-term, low-dose exposure in humans. Exposure to PAs through the use of herbal remedies

may also be a contributing factor to the high rates of liver cancer and cirrhosis seen in Africa (Steenkamp *et al*. 2000).

Crotalaria spp. are known to contain toxic PAs and various incidences of human poisoning through contaminated grains have been recorded in the scientific literature (IPCS 1989). Legislation controlling the allowable levels of toxic seeds in grains in South Africa is generally much stricter than in many other grain producing countries. The Soybean and Sunflower Forum recently commissioned a study (Eloff *et al.* 2003) to review published and unpublished information on toxic seed that could affect human health in South Africa and to make recommendations accordingly.

Crotalaria sphaerocarpa is one of the problem plants discussed in the review and is apparently the only species which regularly contaminate grain in certain areas in South Africa. There is uncertainty at present about the number of these seeds that should be allowed in grains and the threat that this may pose to human health. Based on the review a provisional recommended level of 10 seeds of *C*. *sphaerocarpa* per 10 kg of grain was proposed as an approximated safe level in the report. As emphasized by the authors (Eloff *et al.* 2003), this absolute level is based on assumptions that must still be tested.

As a follow-up on the report, a sensitive LC-MS/MS method for the determination of toxic PAs in plants was developed in this study. The characteristic fragments produced by 1,2-unsaturated necine bases under specific MS/MS conditions were used to discriminate between the toxic and non-toxic PAs. The concentration of these PAs were then determined using multi-reaction-mode experiments. Quantitative results were calculated against a retrorsine calibration curve and expressed as µg retrorsine equivalents per gram plant material.

Various extraction methods described in the literature were investigated. A final liquid-liquid extraction method was used to extract unsaturated PAs from small amounts (about one gram) of milled plant samples. Recoveries from spiked lucerne samples were 98% for retrorsine and 105% for monocrotaline.

To determine the applicability of the LC-MS/MS method the unsaturated PA content of *C. laburnifolia* and *C. dura* were investigated. *Crotalaria laburnifolia,* which is regarded as non-toxic, contained low concentrations (< 20 $\mu g.g^{-1}$) of unsaturated PAs. *Crotalaria dura,* on the other hand, is known to be toxic to livestock and the concentration of unsaturated PAs was

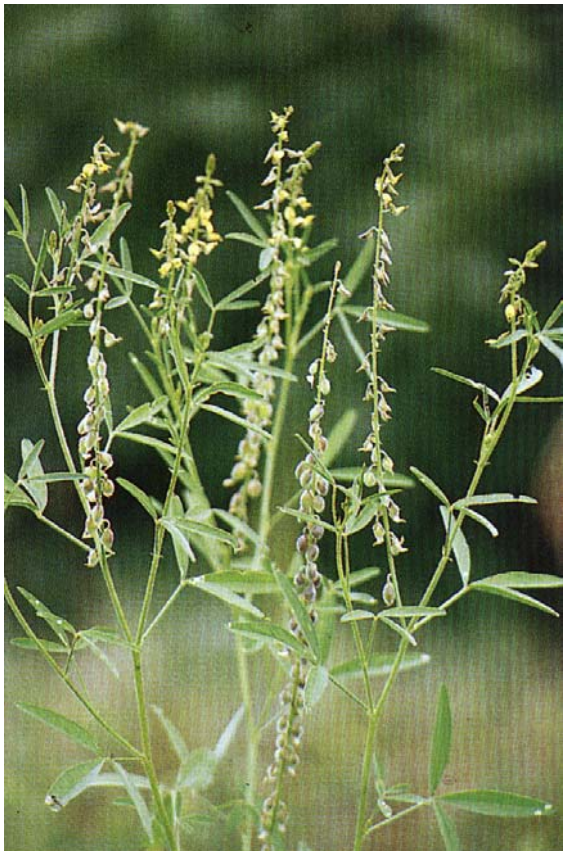
significantly higher (585 µg.g^{-1}). The toxic PA content of *Senecio inaequidens* was also determined after an incident of livestock poisoning. The plant material contained very high concentrations of retrorsine (11.5 mg.g^{-1}) and senecionine (0.5 mg.g^{-1}) which were also present in the rumen content collected post-motally. These results confirmed the suspected toxicity of *S. inaequidens.*

The LC-MS/MS method was also used to follow variations in unsaturated PA content in *C. sphaerocarpa* plants during the growing season. Pyrrolizidine alkaloids were present in the roots of the growing plants as *N*-oxides and also found in the mature aerial parts, where it was present mainly as the basic alkaloids.

The method was used to determine the concentration of unsaturated PAs, in various *C. sphaerocarpa* seeds from different locations, in order to calculate the allowable level of *C sphaerocarpa* seed in maize. Of all the seed samples analyzed, the highest unsaturated PA concentration found was 150 µg.g^{-1}. The allowable level of seed was calculated using this result and was found to be 656 seeds per 10 kg maize, based on the Australian and New Zealand Food Authority level of 0.1 µg.kg^{-1}.day^{-1}. If these results are confirmed with systematic statistical samples of *C. sphaerocarpa* seed from different grain production areas, the allowable level could be increased substantially. This may have an economic benefit to grain producers.

ACKNOWLEDGEMENTS

The author wishes to thank everyone involved in this project:
Prof. Kobus Eloff, Phytomedicine Programme, Onderstepoort, for identifying the project and for his support and encouragement.
Prof. Christo Botha, Faculty of Veterinary Science, Onderstepoort, for his support and for the *Senecio inaequidens* specimens.
Riaan Bruitendag, Bothaville, for providing *Crotalaria sphaerocarpa* plant specimens.
Prof. Theuns Naudé, Faculty of Veterinary Science, Onderstepoort for *C. laburnifolia* and *C. dura* samples.
Dr. Leentjie van Niekerk, AMPATH, and Dr. Ken Swart, Parexel, for the use of their LC-MS/MS instruments.



Crotalaria sphaerocarpa

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADI	Acceptable daily intake
amu	atomic mass unit
C.	*Crotalaria*
CI	Chemical ionization
CID	Collision induced dissociation
DHP	Dehydropyrrolizidine alkaloid
EI	Electron impact
ESI	Electrospray ionization
FMO	Flavin-containing monooxygenase
GC	Gas chromatography
GSH	Glutathione
HPLC	High performance liquid chromatography
LLE	Liquid-liquid extraction
LLOD	Lowest limit of detection
$[M]^+$	Molecular ion (equal to the molecular mass of the compound)
$[M+H]^+$	Pseudo-molecular ion (molecular mass + 1)
MRM	Multi reaction mode
MS	Mass spectrometer
m/z	Mass to charge ratio of the fragment
NMR	Nuclear magnetic resonance
NOAEL	No-observed-adverse-effect-level
PA	Pyrrolizidine alkaloid
PCI	Positive chemical ionization
RI	Retention indices
SCX	Strong cation exchange
SPE	Solid phase extraction
Spp.	Species
TIC	Total ion chromatogram
TLC	Thin layer chromatography
UV	Ultra violet