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Distinguishing between toxic and non-toxic pyrrolizidine alkaloids and quantification by liquid chromatography–mass spectrometry

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are important plant toxins causing poisoning in livestock, leading to significant financial and production losses each year. It may also enter the human food chain as contaminants of grains, via animal products such as milk, eggs and honey or herbal remedies. Not all PAs are toxic and it is important to be able to distinguish between toxic and non-toxic PAs. We developed a sensitive and selective analytical method to determine toxic 1,2-unsaturated PAs concentrations in plant extracts by liquid chromatography–mass spectrometry (LC–MS) with electrospray ionization and precursor ion experiments. Multi-reaction-mode experiments were used to quantify the concentrations of the different unsaturated PAs and results were expressed as μg/g retrorsine equivalents. The results obtained on *Crotalaria sphaerocarpa* contaminating maize crops have important implications.

1. Introduction

Generally, pyrrolizidine alkaloids (PAs) are esters of hydroxylated methyl pyrrolizidines, consisting of a necine base- and necic acid moiety. The necine base can either be 1,2-unsaturated or saturated. The unsaturated necine bases are further classified as two types, *viz*. retronecine- and otonecine-type alkaloids (Ober and Hartmann, 1999). Pyrrolizidine alkaloid bases can also exist as *N*-oxides, which are often present with the basic alkaloids in plants. Together with the *N*-oxides more than 640 pyrrolizidine alkaloid structures are possible, of which at least 350 types have already been found in nature and their structures elucidated (Mattocks, 1986).

Not all PAs lead to the synthesis of the toxic metabolite. Only the esters of 1,2-unsaturated retronecine- and otonecine type PAs, which are bioactivated by hepatic cytochrome PF mixed function oxidases to toxic dehydropyrrolizidines (DHP) are toxic (Prakash et al., 1999). These bi-functional DHP molecules can react with a variety of nucleophilic intracellular macromolecules, resulting in liver and other tissue damage associated with different types of PA poisonings (Fu et al., 2002). According to Wiedenfeld (2011) "Pyrrolizidine alkaloids (PAs) possessing a 1,2-double bond in their base moiety (necine) are hepatotoxic, carcinogenic, genotoxic, teratogenic and sometimes pneumotoxic".

Various analytical techniques have been used to separate, identify and quantify PAs in plants. However, for most of these procedures authenticated reference materials are needed, of which only a few are currently commercially available. Analytical methods that can selectively detect the 1,2-unsaturated necine bases of toxic PAs in complex mixtures are essential to estimate potential toxicity and to confirm a diagnosis of suspected poisonings (International Programme on Chemical Safety).

Lin et al. (1998) developed a LC–MS–MS method for the determination of known PAs. Spectra were obtained with in-source collision as well as with collision induced dissociation (CID) in the collision cell. All PAs analyzed by electrospray ionization (ESI) in the positive mode had an abundance of the $[M+H]^+$ pseudo-molecular ion. CID spectra of retronecine-type 1,2-unsaturated PAs produced characteristic fragments at *m/z* 120 and 138. Other fragments characteristic of this type of PA were an ion at *m/z* 94 and a fragment corresponding to $[MH\text{-}CO]^+$. Triple quadrupole MS–MS detectors can be used in precursor scan mode, where the fragments produced after CID are used to determine the compound of origin. For diverse compounds like toxic PAs, where all the 1,2-unsaturated structures yield such distinguished fragments, this would be the ideal screening method for the presence of 1,2-unsaturated toxic PAs in natural products or food. Our aim was to develop a quantitative screening method to search for unknown

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toxic PAs in various plants. In an attempt to evaluate the method four South African plant species were investigated:

1. *Crotalaria sphaerocarpa* seeds, associated with grain contamination (Eloff et al., 2003).
2. *Crotalaria dura*, commonly accepted as a poisonous plant in South Africa.
3. *Crotalaria laburnifolia*, not known to be toxic.
4. *Senecio inaequidens*, analyzed after suspected poisoning of cattle (Dimande et al., 2007).

2. Materials and methods

2.1. Chemicals

Unsaturated retronecine-type PAs, i.e. retrorsine (CAS: 480-54-6) and monocrotaline (CAS: 315-22-0) were purchased from Sigma–Aldrich (South Africa). Acetonitrile, ammonium acetate, hydrochloric acid, ethanol, zinc powder, ethyl acetate, ammonia solution 25%, hexane and methanol were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

A Waters Alliance 2796 HPLC gradient pump system (Microsep, South Africa) was used with a Phenomenex Luna C18 5 μm 250 mm × 2.0 mm (Separations, South Africa) analytical column. 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 and 10% 25 mM ammonium acetate buffer (pH 3.84). Gradient elution was 0–5 min 98% A; 5–15 min 40% A (linear); 15–20 min 98% A (linear). The flow rate was 0.2 mL/min. Total runtime was 30 min with a 5 min equilibration time at the end of the run. A Quattro Micro Triple Quad instrument (Micromass, Microsep, South Africa) mass spectrometer with electrospray interface in the positive mode was used with Argon as the collision gas for MS–MS experiments. The software used was MassLynx® version 4.0.

2.3. Instrument optimization

Solutions of pure reference materials – retrorsine (FW 351) and monocrotaline (FW 325) in methanol (0.5 mg/mL) – were infused to optimize the mass spectrometer settings in the MS (Fig. 1), MS–MS (Fig. 2) and tandem LC–MS–MS modes. LC–MS–MS settings were optimized for the fragment *m/z* 120 and used for precursor scans (Fig. 3), product ion scans and multi-reaction mode (MRM) scans, performed on all the extracts. The MRM transition of the $[M+H]^+ > m/z$ 120 fragments were used for quantification. The LC–MS–MS settings were: capillary 3.2 V, cone 20 V, extractor 3 V, RF lens 0.3 V, source temperature 150 °C, desolvation temperature 350 °C, cone gas 60 L/h, desolvation gas 300 L/h, collision energy 34.

2.4. Preparation of standards

Dried, milled lucerne (*Medicago sativa*) was extracted with ethanol as a blank matrix and spiked with pure retrorsine and monocrotaline (1 mg/mL methanol) reference material. Serial dilutions with the blank matrix were used to prepare a standard curve consisting of 12 different concentrations between 0.01 μg/mL and 100 μg/mL. Aliquots (1 mL) of the standard solutions were evaporated and extracted as described under Section 2.6.

2.5. Plant samples and rumen content

Crotalaria sphaerocarpa seeds were collected from a maize farm 2 km north of Oberholzer in the Free State Province, Republic of South Africa. *Crotalaria dura* and *Crotalaria laburnifolia* plant material were collected in KwaZulu-Natal, South Africa by Prof. TW Naudé, Faculty of Veterinary Science, Onderstepoort. In October 2004 nine adult cows died near Frankfort in the Free State Province after ingesting plants suspected of containing PAs. The plants were identified by the National Herbarium, South African National Biodiversity Institute as *Senecio inaequidens* (DC) (Dimande et al., 2007). Necropsies were performed on cattle that have died and rumen content (500 g) was collected and submitted for analysis together with the collected plant material.

2.6. Sample handling

N-oxides are often present in plants together with the basic PAs. To enhance extractability, the polar *N*-oxides were reduced to the basic alkaloids before extraction. The extract was then divided into two fractions and the *N*-oxides in one of the fractions were reduced by addition of zinc. The total alkaloid content was determined in the reduced fraction, while the second fraction was used to determine the basic alkaloids content. The *N*-oxide content is represented by the difference between the total and the basic alkaloid fractions.

The plant samples were well mixed and milled to obtain a sub-sample of 100–500 g plant material. A portion of the sub-sample was weighed (1.0 g), homogenized with 10 mL 90% ethanol and left

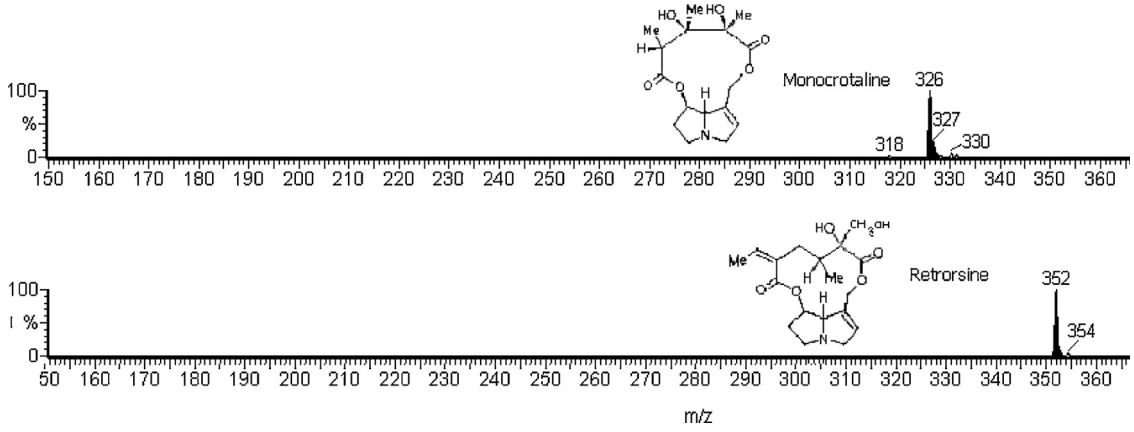

Fig. 1. ESI^+ mass spectra of monocrotaline and retrorsine showing the $[M+H]^+$ ions obtained with the infusion experiments.