Analysis of the Toxic Amino Acid Indospicine by Liquid Chromatography-Tandem Mass Spectrometry

Dale R. Gardner[1] and Franklin Riet-Correa[2]

[1]USDA-ARS Poisonous Plant Research Laboratory, Logan, UT, USA
[2]Center for Health and Rural Technology, Federal University of Campina Grande, Patos, Paraiba, Brazil

Corresponding author: Dale Gardner, dale.gardner@ars.usda.gov

Abstract

Some *Indigofera* species contain a toxic non-protein amino acid known as indospicine. Indospicine-containing plants are toxic to livestock, and cases of secondary poisoning have been documented in dogs that consume indospicine-containing meat. For the analysis of indospicine in the plant material, a method was developed based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) of the phenylisothiocyanate derivative of indospicine. Indospicine was extracted from the plant with ethanol/0.01N HCl (70:30). The sample was then derivatized with a phenylisothiocyanate solution and then analyzed by LC-MS/MS. The method was linear over the range of 1.0-25 µg/mL, recovery was 86% and the intra-day precision was 4.7% (RSD). Indospicine was confirmed in samples of *I. lespedezioides* collected in Brazil with a concentration range of 60-1400 µg/g (dry weight basis). No indospicine was detected in samples of *I. praticola* and *I. tinctoria* from Zimbabwe, Africa.

Keywords: indospicine, *Indigofera lespedezioides*, poisonous plants, LC-MS

Introduction

The *Indigofera* genus contains approximately 700 different species, many of which are agronomically important plants that are used as grazing forages and feed supplements (Aylward et al. 1987). Some *Indigofera* species, however, contain a toxic non-protein amino acid known as indospicine (2,7-diamino-7-iminoheptanoic acid; figure 1). Indospicine is thought to be hepatotoxic in cattle, sheep, mice, rats, and rabbits (Norfedlt et al. 1952, Hutton et al. 1958a,b, Hegarty and Pound 1968, 1970, Christie et al. 1969, 1975) and may be neurotoxic in horses (Hegarty and Pound 1968, Hooper et al. 1971). Dogs appear to be highly susceptible to indospicine hepatotoxicity, and there are now several reported cases of secondary poisonings occurring from dogs eating indospicine-contaminated meat (Hegarty et al. 1988, Kelly et al. 1992, FitzGerald et al. 2011). Indospicine is structurally similar to arginine and differs only in the C-6 methylene group versus the amino group that is found in arginine (figure 1). The compound was first isolated by Hegarty and Pound (1968) from *I. spicata* and its toxicity is attributed to the inhibitory action of arginine incorporation into proteins, inhibition of arginase activity, and inhibition of nitric oxide synthase (Madsen and Hegarty 1970, Madsen et al. 1970, Pass et al. 1996).

In the past, indospicine has been detected using an amino acid analyzer consisting of ion-exchange chromatography and post-column derivatization with ninhydrin (Hegarty and Pound 1970, Miller and Smith 1973, Aylward et al. 1987). Analysis times

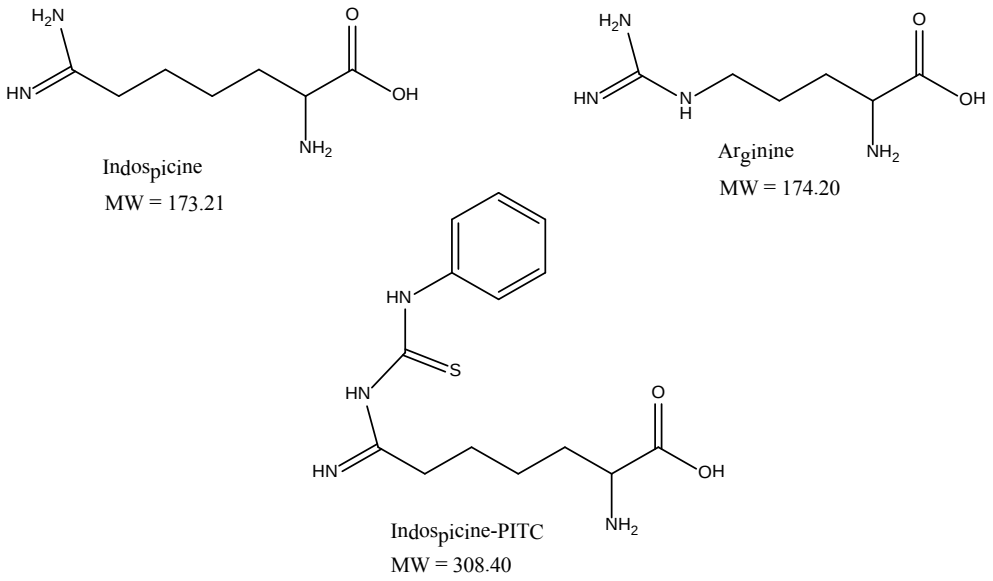

Figure 1. Chemical structures of indospicine, arginine, and the phenylisothiocyanate derivative of indospicine (indospicine-PITC).

using this methodology are lengthy (over 1 hour/sample). More recently, a reversed-phase HPLC (high performance liquid chromatography) method was reported for the detection of indospicine in horse sera and tissue samples (Pollitt et al. 1999). The method was based on pre-column derivatization with phenylisothiocyanate, HPLC separation, and detection by UV (Heinrikson and Meredith 1984). The method of Pollitt et al. (1999) appeared to work well, although to achieve the required resolution of indospicine from arginine and other amino acids in the samples, the chromatographic conditions had to be carefully selected. Concurrent with the work reported here, indospicine was reported to be analyzed by liquid chromatography-tandem mass spectrometry of the underivatized compound with detection in canine sera, liver, and muscle tissue as well as commercially prepared camel mince; however, the exact details of the analysis were not provided (FitzGerald et al. 2011).

The method presented here modifies the method of Pollitt et al. (1999) through the use of binary gradient reversed-phase chromatography and then detection by mass spectrometry. The use of tandem mass spectrometry, though not required, assured an extra level of specificity to the method. The method was used to detect indospicine in a number *Indigofera lespedezioides* plant samples originating from suspected cases of poisoning of horses in Brazil. Secondarily, samples of two different species of *Indigofera* (*I. practicola* and *I. tinctoria*) from Africa, which are associated with floppy trunk syndrome in elephants (Fowler and Mikota 2006), were analyzed for indospicine as additional plants from same genera.

Material and Methods

Plant Material and Chemicals

Astragalus lentiginosus was obtained from the general plant collections of the U.S. Department of Agriculture, Agricultural Research Service Poisonous Plant Research Laboratory (PPRL), Logan, UT, USA, and was used for the indospicine negative control, as indospicine has never been reported in *Astragalus* species. *Indigofera spicata* was a gift from Dr. Alan Seawright collected in western Queensland, Australia; it was used as the indospicine positive control sample. *Indigofera lespedezioides* associated with nervous disease in horses was collected from the state of Roraima, northern Brazil (Lima et al. 2011). *Indigofera tinctoria* and *I. praticola* were diagnostic samples (PPRL) received from Zimbabwe, Africa, and associated with floppy trunk disease in elephants. All samples had been air dried at ambient temperature and then ground to pass a 1-mm screen. A standard sample of indospicine was a gift from Dr. Steven Colegate, obtained from the collection of the Plant Toxin Research Unit of CSIRO Australia. Methanol and ethanol were reagent grade; triethylamine (99.5%) and phenylisothiocyanate (99%) were purchased from Aldrich Chemical; acetonitrile was HPLC grade (Burdick and Jackson); and water was Milli-Q-purified (Waters Millipore).

Sample Extraction and Preparation

An aliquot of plant material (0.100 g) was placed into a screw cap test tube or vial and 5.0 mL of 70% ethanol (in 0.01N HCl) (Aylward et al. 1987) added and the samples sonicated for 30 min. Samples were centrifuged for 5 min and the supernatant decanted into a screw cap test tube. The samples were then extracted two additional times with 5.0 mL of the extraction solvent (70% EtOH/0.01N HCl) in the same manner as above and the decanted extracts were combined. The final volume with no additional adjustment was measured at 15.0 mL. An aliquot (2.0 mL) of the combined extracts was added to a clean 7 mL screw cap glass vial, placed in a heat block (60°C), and the solvent removed by evaporation under a flow of nitrogen. To aid the evaporation of water in the samples, absolute ethanol (~1 mL) was added to each vial after the original volume had been reduced to approximately 0.5 mL. The samples were then evaporated to dryness. The phenylisothiocyanate (PITC) reagent was prepared from a mixture of methanol/water/ triethylamine/phenylisothiocyanate (80/10/5/5) as a slight modification to that previously reported by Pollitt et al. (1999). To each sample was added 0.250 mL of freshly prepared PITC reagent and the samples mixed by mechanical rotation for 15 min. The samples were dried under nitrogen flow on the heat block (60°C) and the dry residue was reconstituted in 1.00 mL of 50% acetonitrile/water and transferred to autosampler vials for analysis.

Indospicine Calibration Standards

A stock solution of indospicine was prepared at 1.74 mg/mL in methanol. From this was prepared a 50 µg/mL solution by dilution of 58 µL stock into 1.94 mL of methanol. Six aliquots at 0.500, 0.400, 0.300, 0.200, 0.100, and 0.020 mL were placed into 8 mL screw cap vials and the solvent removed by evaporation under nitrogen flow in a heated block (60°C). Each calibration standard was then derivatized with the PITC reagent as described above to give calibration standards in the range of 1.0-25 µg/mL.

LC-MS/MS Analysis

Analysis of indospicine was accomplished using a Finnigan Surveyor liquid chromatography system coupled to a Finnigan LCQ Advantage Max ion trap mass spectrometer and electrospray (esi) ionization source. A Thermo Betasil C18 column (100 × 2.1 mm), and guard column of equivalent phase, were used with a gradient flow of acetonitrile (MeCN) and water containing 20 mM ammonium acetate (0.300 mL/min). The programmed gradient flow was 5% MeCN (0-2 min); 5%-60% MeCN (2-10 min); 60%-95% MeCN (10-11 min); 95% MeCN (11-15 min); 95%-5% MeCN (15-16 min) and equilibration at 5% MeCN for 5 min for a total cycle time of 21 min. Flow from the column was directly coupled to the esi-source and the mass spectrometer was operated in the MS/MS mode with a selected parent mass of 309, an isolation width of *m/z* 1.5, a relative collision energy of 27%, an activation Q of 0.25, and an activation time of 30 ms. Parent ion fragments were scanned in the range of *m/z* 85-800. Indospicine detection and peak areas were made from reconstructed ion chromatograms using the selected MS/MS fragment ions at *m/z* 164 and 216. The precision of the method was measured from the repeat intra-day analyses (n = 5) of samples of *I. spicata*. Extraction efficiency was determined from a repeat single extraction of a sample that had already been previously extracted three times with 5 mL of 70% ethanol (0.01N HCl). Accuracy of the method was determined from spike recovery where % recovery = $[(C_F - C_U)/C_A] \times 100\%$: C_F = concentration of analyte measured in fortified sample (n = 3); C_U = Concentration of analyte measured in unfortified sample as measured by analysis in triplicate; and C_A = concentration of analyte added in fortified sample (50 µL of 1.74 mg/mL stock = 87 µg added to 100 mg sample). The unfortified sample was prepared by mixing the *I. spicata* control sample with approximately 50% by weight of the *Astragalus* negative control.

Results and Discussion

In the previously reported HPLC analysis of the phenylisothiocyanate (PITC) derivative of indospicine (Pollitt et al. 1999), it was critical to establish proper pH, solvent component ratios, and temperature conditions of the method to ensure proper resolution of the analyte from other amino acids in the sample. In the method reported here, and in using the mass spectrometer for the detector, such detail to resolution of the components on the chromatographic time scale was not as critical because the mass spectrometer would be used to resolve the co-eluting amino acid components based on their parent mass and the mass of their

corresponding fragment ions. Therefore, for the current method we chose chromatographic conditions that would be easy to reproduce and give a reasonable analysis time with acceptable chromatographic qualities. The resulting method used a simple binary gradient of water and acetonitrile with a standard reversed-phase (C18) column. Under the chromatographic conditions established, indospicine-PITC was found to elute at approximately 5.4 min (figure 2). Arginine-PITC was found to essentially co-elute with indospicine-PITC at 5.44 min and thus analysis of indospicine using a UV detector is not possible under these chromatographic conditions.

The esi-MS spectra of indospicine and arginine produce the expected MH^+ ions at 309 and 310, respectively, for their PITC derivatives (figure 2). It was possible to resolve amino acid derivatives based solely on the MH^+ ions in the analysis of the standard compounds. The analysis of an indospicine negative sample (*Astragalus lentiginosus*) presented no false positive as evident from the RIC at MH^+ = 309, even in the presence of arginine (figure 3A). Indospicine was easily detected in the positive control sample (*I. spicata*) under the same analytical conditions (figure 3B). However, the use of their MS/MS fragment ions could provide an additional level of specificity to ensure no possible contamination from extraneous 309 ions. The MS/MS data for indospicine-PITC and arginine-PITC are presented in figure 4. Fragment losses were similar for the two compounds with major losses of 18, 34, 51, 93, and 135 Da with the exception of an extra M-35 ion for indospicine-PITC. The two most abundant MS/MS fragment ions at *m/z* 174 and 216 were therefore used for detection and quantitation of indospicine (figure 5) with no false positive peaks resulting from the co-eluting arginine-PITC component.

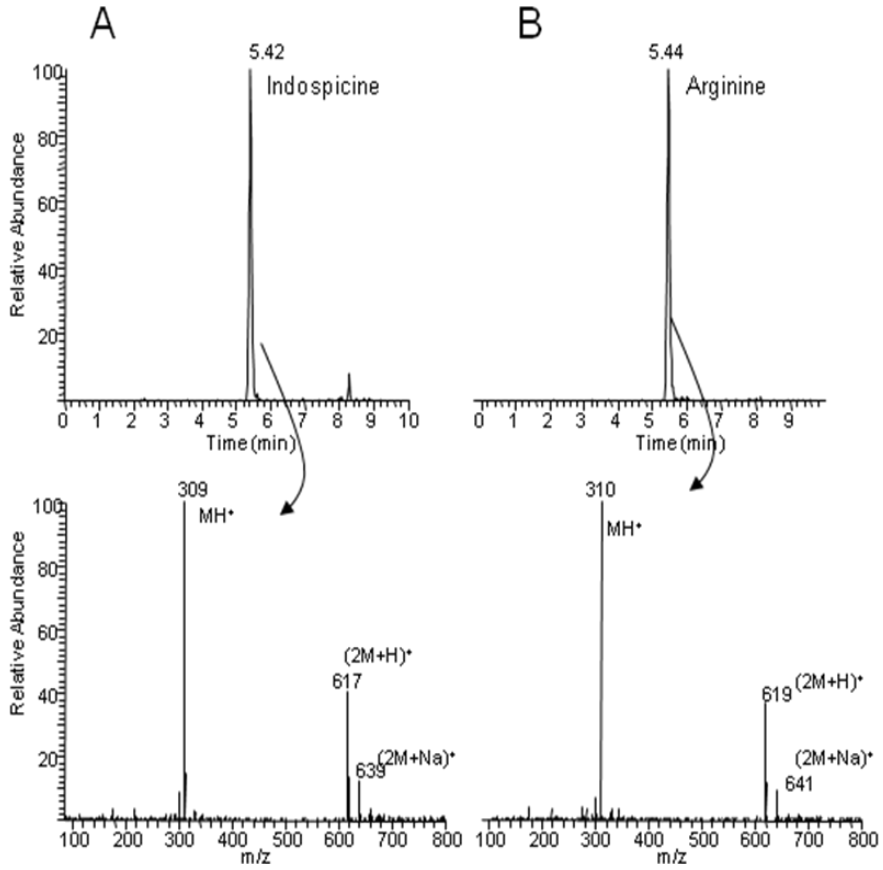

Figure 2. LC-esiMS analysis of standard solutions of indospicine (A) and arginine (B) and their corresponding esiMS spectra.

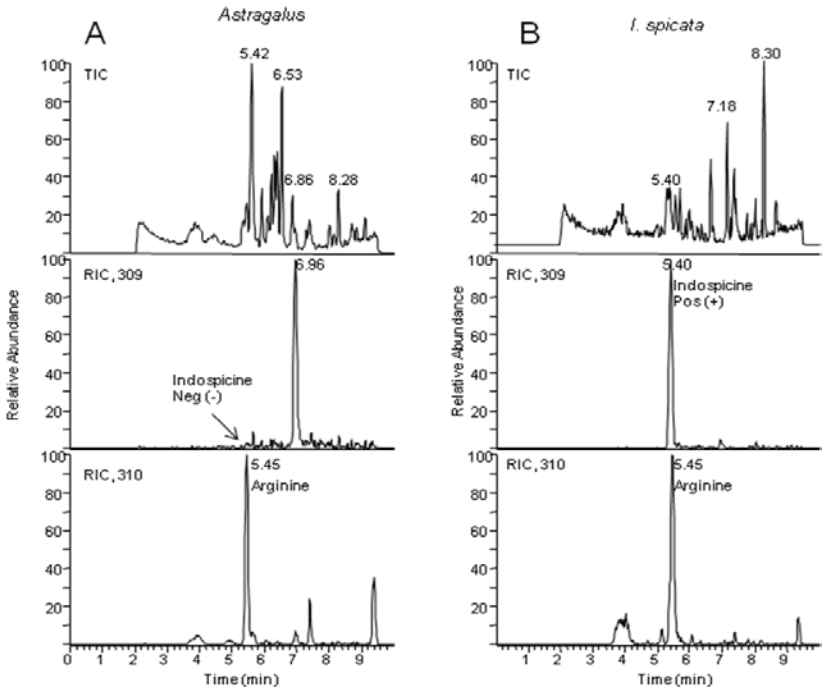

Figure 3. LC-esiMS analysis of *Astragalus* plant material (A) used for indospicine negative control and *Indigofera spicata* (B) used for indospicine positive control. Each sample includes the total ion chromatogram (TIC) and their reconstructed ion chromatograms for *m/z* 309 (indospicine) and *m/z* 310 (arginine).

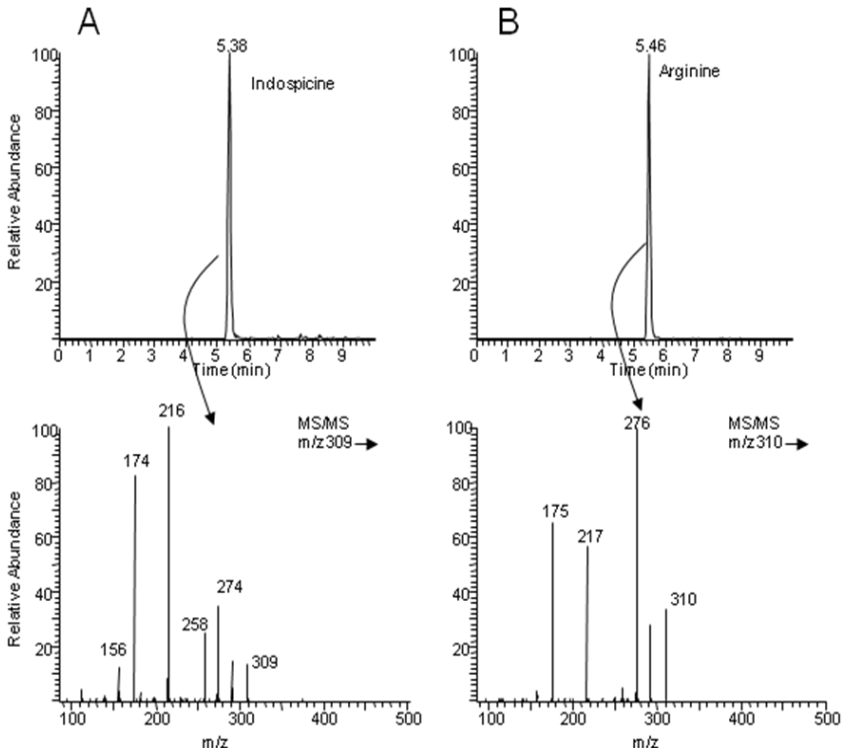

Figure 4. LC-esiMS/MS analysis of standard solutions of indospicine (A) and arginine (B) and their corresponding MS/MS spectra resulting from fragmentation of the parent ions for indospicine ($MH^+ = 309$) and arginine ($MH^+ = 310$).

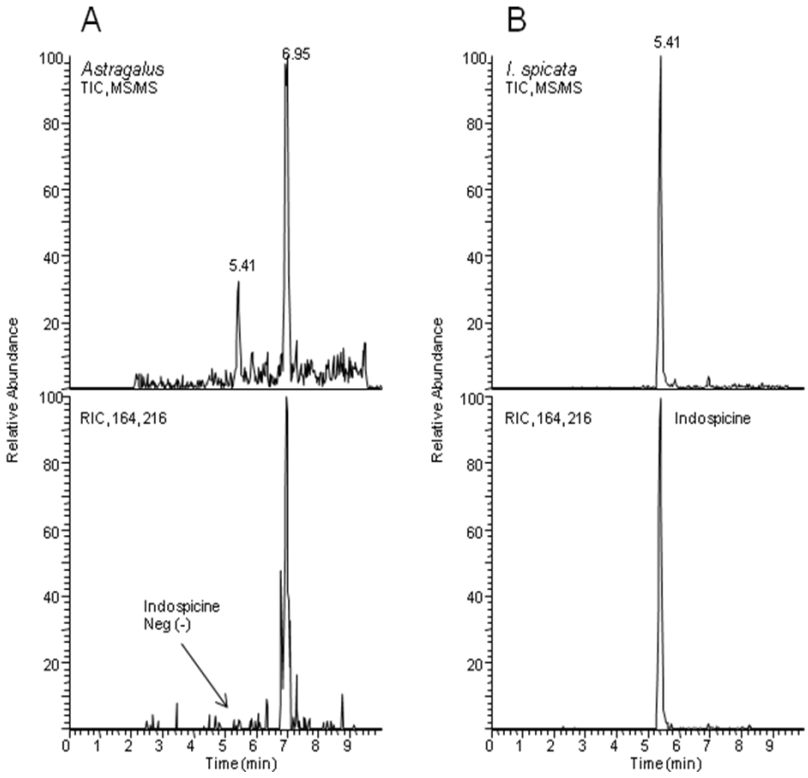

Figure 5. LC-esiMS/MS analysis of *Astragalus* plant material (A) used for indospicine negative control and *Indigofera spicata* (B) used for indospicine positive control. Each sample includes the total ion chromatogram (TIC) and the reconstructed ion chromatograms for *m/z* 309 -›› 174, 216 the selected ions for indospicine.

Indospicine was extracted as previously reported by Aylward et al. (1987) with an acidic aqueous ethanol (ethanol/water/0.1N HCl, 70:30:1) solvent mixture. This extraction solution extracted at least three times more indospicine in comparison to a simple methanolic extract of the sample (data not presented). The extraction was estimated to be 98% complete after three extractions and intra-day precision was 4.7%. The calibration was linear over the range of 1-25 µg/mL ($r^2 = 0.9962$). Recovery of the method was found to be 86% from samples spiked with a standard solution of indospicine. The recovery was slightly lower than expected given the simplicity of the extraction procedure and the apparent completeness of the extraction. However, the low recovery may indicate that some binding of indospicine with the plant matrix occurs and that a different extraction solvent or method may be required to increase the extraction recovery. Alternate extraction solvents were not explored other than a simple methanol extraction.

The developed method was applied to a small number of *Indigofera* samples received by the laboratory from Brazil and Africa from areas of possible livestock poisonings. All samples of *I. lespedezioides* were positive for indospicine, ranging from 60-1178 µg/g (dry weight basis) (table 1). We found one previous analysis of *I. lespedezioides* that reported a concentration of indospicine at 0.02% (200 µg/g) (Aylward et al. 1987). The two samples from the state of Roraima (Bom Fim and Amajari), where the disease occurs, had significantly higher concentrations of indospicine than the sample collected from Manaus. Interestingly, the sample from Manaus was harvested from plants originally collected in the state of Roraima (where the disease occurs) and planted one year before in Manaus (state of Amazonas) in a place where the disease does not occur. There are three reported toxic *Indigofera* species for horses (*I. spicata* in Florida, *I. linnaei* (= *I. domini, I. enneaphyla*) in Australia, and *I. lespedezioides* in Brazil. It has not been proven that indospicine is the toxic compound causing nervous disease in horses, and there is some suspicion that a nitrotoxin is responsible (Majak et al. 1992). Nevertheless, indospicine is found in the three species that cause nervous signs in different parts of the world (Australia, United States, and Brazil), and at least *I. lespedezioides* collected in Brazil does not contain nitro-compounds (data not shown).

Table 1. Analysis of *Indigofera* samples for indospicine by LC-MS/MS for method validation and submitted samples from possible livestock intoxications

Sample	Indospicine (µg/g) ± s.d.	Validation parameter
I. spicata/A. lentiginosus (unfortified) n=3	733 ± 31.6	
I. spicata/A. lentiginosus (fortified w/870 µg/g) n=3	1491 ± 54.9	Recovery 87%
I. spicata (positive control) n=5	1136 ± 46.0	Intra-day RSD 4.1%
I. spicata (extraction #4)	21	Extraction~ 98%
I. lespedezioides		
Bom Fim[1]	263	
Manaus[2]	63	
Amajaru[1]	1178	
I. lespedeziodes[3]	488	
I. practicola[4]	n.d.	
I. tinctoria[4]	n.d.	
I. practicola[4]	n.d.	
A. lentiginosus (negative control)	n.d.	

[1] Collected in the state of Roraima, Brazil, February 2010.
[2]Collected in the state of Amazonas, Brazil, February 2010, from plants collected in the state of Roraima (where the disease occurs) and planted 1 year before in Manaus (state of Amazonas) in a place where the disease does not occur.
[3] Collected in the state of Roraima, Brazil, April 2008.
[4] Collected from Fothergill Island, Zimbabwe, Africa, May 2008.

In addition, in Australia the disease in horses was treated and prevented with arginine or arginine-containing substances, and it has been suggested that indospicine may competitively interfere with the utilization of arginine in protein metabolism (Hooper et al. 1971).

Indospicine was not detected in *I. praticola* and *I. tinctoria* samples collected from Zimbabwe, Africa. This was not an unexpected result because indospicine was not suspected to be associated with the floppy trunk syndrome in elephants (Fowler and Mikota 2006).

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