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Analysis of Swainsonine: Extraction Methods, Detection, and Measurement in Populations of Locoweeds (*Oxytropis* spp.)

Dale R. Gardner,^{*,†} Russell J. Molyneux,[‡] and Michael H. Ralphs[†]

Poisonous Plant Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 1150 East 1400 North, Logan, Utah 84341, and Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

An analytical method has been developed to measure the locoweed toxin, swainsonine, in locoweed plant material. Dry ground plant samples were extracted using a small-scale liquid/liquid extraction procedure followed by isolation of the swainsonine by solid phase extraction with a cation-exchange resin. Detection and quantitation of the swainsonine were accomplished using reversed phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionization tandem mass spectrometry (LC-MS²). The limit of quantitation was estimated to be 0.001% swainsonine by weight in dry plant material, which corresponds to the lower threshold for toxicity of locoweeds. The method of analysis was applied to the analysis of *Oxytropis sericea* (white locoweed) and *Oxytropis lambertii* (Lambert locoweed) plant samples to measure the variability of individual plant swainsonine levels within populations and within species. Individual plant variability was found to be highly significant for both *O. sericea* and *O. lambertii* populations. The combined three-year mean swainsonine values taken from three populations of *O. sericea* ranged from 0.046% in Utah to 0.097% in a New Mexico population. Sixteen individual populations of *O. lambertii* were sampled from eight different U.S. states. Swainsonine was detected at levels >0.001% in only 5 of the 16 collection sites. Those populations of *O. lambertii* found to contain higher swainsonine levels were restricted to the most southern and western portion of its distribution, and all were identified as belonging to var. *bigelovii*, whereas var. *articulata* and var. *lambertii* samples contained swainsonine at levels <0.001%.

Keywords: Swainsonine; locoweed; *Astragalus*; *Oxytropis*; LC-MS²; solid phase extraction; cation exchange

INTRODUCTION

Locoweed poisoning is the most widespread poisonous plant problem in the western United States (1, 2). Locoweeds are presently defined as only those species of the genera *Astragalus* and *Oxytropis* (family Leguminosae) that specifically contain the trihydroxyindolizidine alkaloid, swainsonine (3) (Figure 1). Other species may be toxic due to accumulation of selenium or because they contain nitrotoxins, but these plants do not produce the characteristic signs of locoweed poisoning (4). In contrast, many species do not contain toxins of any type and may provide useful forage. The *Oxytropis* locoweed species are sometimes considered to be the most destructive because of their wider geographical distribution rather than an inherently greater toxicity (4). Locoism is a chronic disease, and develops in livestock only after grazing of locoweeds for an extended period of several weeks. The disease is characterized by neurological changes, reproductive disturbances, and emaciation (5). Swainsonine inhibits cellular mannosidases, resulting in a phenotype of the genetic lysosomal storage disease, mannosidosis (6–9). When grazed at high altitudes, the locoweeds can cause congestive right-heart failure (10).

The locoweed toxin, swainsonine, was first isolated from the Australian legume *Swainsona canescens* (11)

and subsequently identified in a number of other *Swainsona* species (12). Although locoweeds do not occur in Australia, *Swainsona* species (sometimes known as “poison peas”) cause a disease similar to locoism in which sheep are referred to as being “peastruck” (13, 14). Locoweed intoxication is not confined to North America, and there are a number of *Astragalus* and *Oxytropis* species that poison livestock in South America and parts of Asia (15, 16). Reviews of the toxicology, pathology, and grazing aspects of locoweeds have recently been published (17–19).

Obtaining knowledge and understanding of where locoweeds grow, the environmental conditions under which they become a threat, and the level of the toxin in locoweed plants is important to the management of livestock and to avoid poisonings. The distribution and levels of swainsonine in locoweeds have not been extensively investigated. Earlier analyses of swainsonine in locoweeds documented conclusively the occurrence of swainsonine in several *Astragalus* and *Oxytropis* species most commonly incriminated as locoweeds from field observations (20–22). It was recognized at that time that there was significant variability in swainsonine levels found within species and among individual plant parts and that further investigations would need to be completed.

The purpose of this research was therefore threefold: (1) to refine the analytical method for analysis of swainsonine in plant material; (2) to document the individual plant swainsonine levels and the variability

* Corresponding author [telephone (435) 752-2941; fax (435) 753-5681; e-mail gardnerd@cc.usu.edu].

[†] Poisonous Plant Research Laboratory.

[‡] Western Regional Research Center.

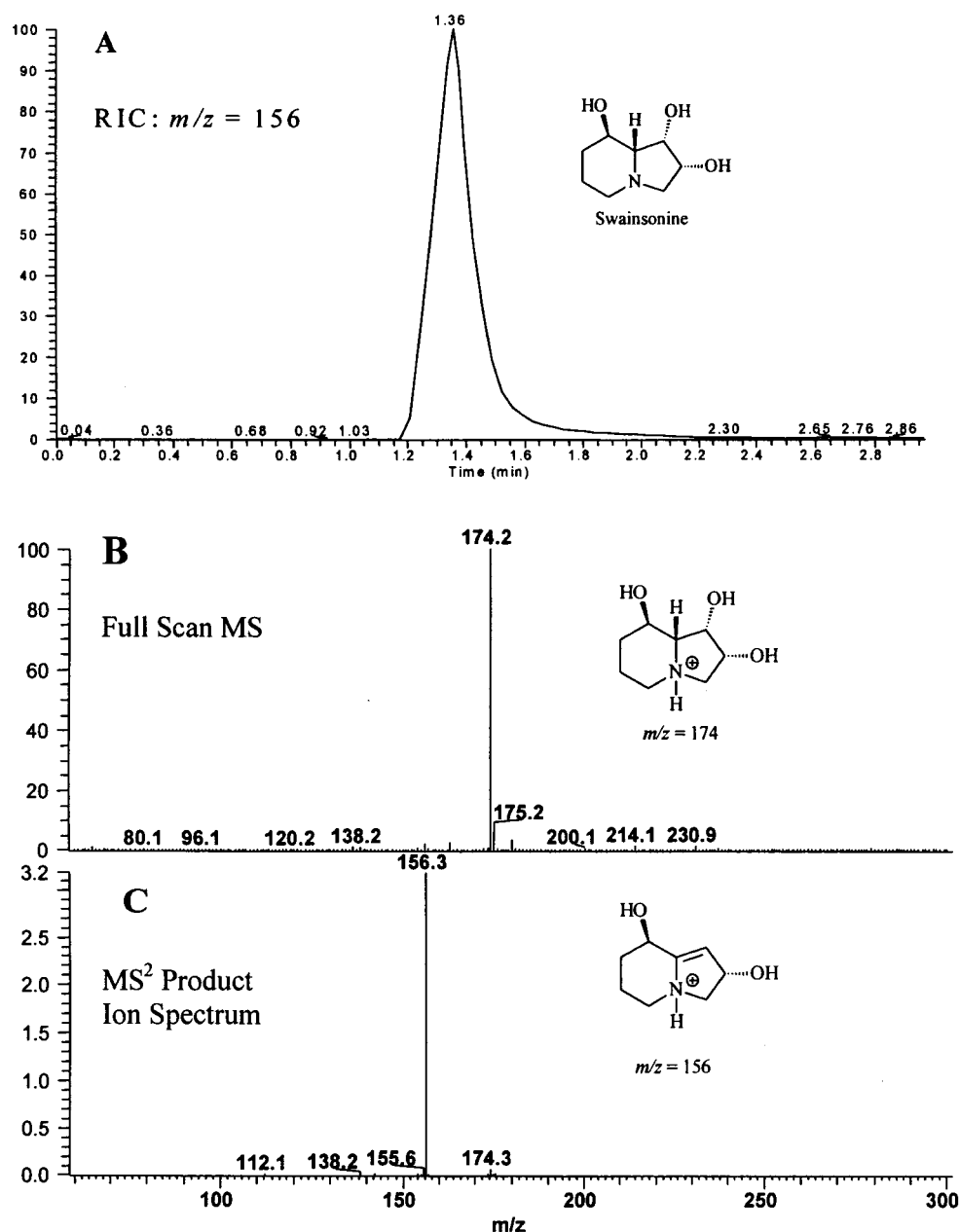


Figure 1. (A) Reconstructed ion chromatogram from the injection of swainsonine standard (1.24 $\mu\text{g/mL}$); (B) full-scan mass spectrum of swainsonine under APCI ionization; (C) MS² product ion spectrum collected after isolation of m/z 174.2 (MH^+) and subsequent collision-induced fragmentation.

within locoweed populations; and (3) to document the distribution and occurrence of swainsonine in a single species over a wide geographical area. For the analysis of individual variation in plant swainsonine levels, three populations of *Oxytropis sericea* were examined. In the analysis of intraspecific variation, three varieties of *Oxytropis lambertii*, from eight different states, were sampled at 16 different locations.

MATERIALS AND METHODS

All solvents or chemicals were of analytical reagent grade unless otherwise stated. Acetic acid was 99.99% glacial (Aldrich Chemical Co.), water was distilled deionized ($>18.0\text{ M}\Omega$), and methanol used in the LC-MS analysis was of HPLC grade (Burdick and Jackson). The cation-exchange resin was Dowex 50WX8-100 (Aldrich Chemical Co.).

Plant Materials. White locoweed (*O. sericea* Nutt.) samples ($n = 8/\text{location}$) were collected at three sites in Colorado, New Mexico, and Utah during the flower stage of growth in 1995 through 1998 (Table 1) as part of an overall investigation

designed to study the effects of clipping on vigor and alkaloid concentrations in locoweed plants (M. H. Ralphs, unpublished results). At each location plants were marked by placing a numbered tent peg in the ground next to them so that the same plants could be sampled in subsequent years. The aerial plant material was collected by clipping the plant stems just above the ground. The flower head/stem were separated from leaf/stem material, and only leaf/stem material was used for swainsonine analyses.

The first site was 5 km west of DesMoines, NM, at an elevation of 2200 m ($36^\circ 45' 00''\text{N}$; $103^\circ 52' 14''\text{W}$). Soils were silty clay loam with interspersed outcroppings of volcanic basalt rock with a gentle southern exposure. Major grasses consisted of blue grama [*Bouteloua gracilis* (H.B.K.) Lag. ex Steudel], sideoats grama [*B. curtipendula* (Michx.) Torr.], little bluestem [*Schizachyrium scoparium* (Michx.) Nash], and western wheatgrass [*Elymus smithii* (Rybd.) Gould]. White locoweed was the dominant forb.

The second site was on the Colorado State University Research Foundation Maxwell Ranch, located 40 km northwest

Table 1. Mean Swainsonine Levels (\pm SD) in *O. sericea* from Three Locations and Three Years

location	1995	1996	1997	1998	1999	mean ^a	mean diff ^b	range
New Mexico	0.11 \pm 0.02	0.11 \pm 0.05	0.07 \pm 0.03			0.097 ^a	0.07	0.03–0.21
Colorado	0.04 \pm 0.03	0.09 \pm 0.06	0.06 \pm 0.05	0.09 \pm 0.08	0.05 \pm 0.03	0.062 ^b	0.06	0–0.18
Utah		0.07 \pm 0.03	0.04 \pm 0.02	0.03 \pm 0.02	0.05 \pm 0.03	0.046 ^b	0.05	0–0.12

^a Means identified by the same letter are not significantly different ($P > 0.05$). The balanced ANOVA required the same number of years to be used for each location. The first three years for each location were used (New Mexico, 1995–1997; Colorado, 1995–1997; Utah, 1996–1998). Samples from all years were used to calculate mean range and minimum and maximum values. ^b Mean difference in individual plants between years.

Table 2. Balanced ANOVA Model for *O. sericea* and *O. lambertii*

model	df ^a	SS ^b	F value	$P > F^c$	% of total variance
<i>O. sericea</i>					
location	2	0.032	5.6	0.01	21
plant (loc)	21	0.060	3.97	<0.0001	39
year	2	0.011	7.33	0.0019	7
loc \times year	4	0.019	6.46	0.0004	12
error	42	0.030			20
<i>O. lambertii</i>					
location	4	0.01337	5.88	0.0007	35
plant (loc)	45	0.02557			66

^a Degrees of freedom (df). ^b Sum of squares (SS). ^c Test for significance at $P < 0.05$ ($P > F$).

of Fort Collins, CO, at 2000 m elevation (40° 56' 39" N; 105° 15' 33" W). Soils were gravelly loam with a gently sloping west exposure. Associated grasses included needle-and-thread (*Stipa comota* Trin. & Pupr.), squirreltail (*Elymus elymoides* Raf. Swezey), prairie June grass [*Koeleria macrantha* (Ledeb.) Schultes], and blue grama.

The third site was on top of the Raft River mountains in northwest Utah at 3000 m elevation (41° 54' 24" N; 113° 20' 55" W). Soils were shallow loam (10–25 cm to bedrock), with 35–65% coarse fragments. The plant community was dominated by alpine sagebrush (*Artemisia scopulorum* Gray), muttongrass [*Poa fendleriana* (Steud.) Vasey], and Idaho fescue (*Festuca idahoensis*).

Lambert locoweeds (*O. lambertii* Pursh.) samples ($n = 10$ /location) were collected from 16 different locations during June 5–10, 2000; coordinates for each site were measured with a GPS receiver (Table 3). The phenological growth stage of the plants varied at each location and are listed in Table 3. Voucher specimens were collected at each location and deposited in the Monte L. Bean Herbarium (Brigham Young University, Provo, UT). The aerial plant parts were dried in a forced-air oven at 60 °C for 48 h and then ground to pass through a 1 mm screen.

Sample Extraction. Samples (100 mg of dry, ground plant material) were weighed and placed into 15 mL screw-cap glass test tubes. Chloroform (4 mL) and 2% acetic acid (5 mL) were added and the tubes sealed with Teflon-lined caps. Samples were then extracted by continuous mechanical mixing (tube inversion) for 16 h (overnight). The samples were then centrifuged for 5 min to separate solvent layers and plant material. The upper acetic acid solution was then removed with a Pasteur pipet and added to a prepared ion-exchange extraction tube. Although commercial cation-exchange extraction tubes are available, we prepared our own, as follows. Approximately 0.7 g of resin (Dowex 50WX8-100) was placed into a 5 mL disposable pipet tip in which a small amount of glass wool was placed into the tip. A rubber septum was used to plug the tip end. After the acetic acid sample solution was added, a small rubber stopper (size 00) was used to plug the top end. The resin and solution were then mixed for 15 min using mechanical rotation, allowing the swainsonine cations present in the sample solution to bind to the resin. After mixing, the acid solution was removed by spinning the sample tubes in a centrifuge or by vacuum filtration. The ion-exchange tubes were then set aside, and 5 mL of 2% acetic acid was again added to the original sample/chloroform mixture; the samples were then extracted for an additional 15 min and

centrifuged, and the acetic acid layer was removed and added to the ion-exchange tubes, which were then mixed for 15 min. The acetic acid solution was removed. The ion-exchange tubes were then washed twice with deionized distilled water (5 mL) by mixing for 5 min and then using the centrifuge or vacuum to remove the liquid solution. Acetic acid solutions and wash solutions were discarded. To remove the swainsonine from the resin, ammonium hydroxide (1 M, 5.00 mL) was added using a volumetric pipet, and the tubes were mixed for 15 min. The samples were placed into the centrifuge with an 8 mL vial to catch the filtered solution and centrifuged for ~1 min. The aqueous ammonium hydroxide sample solutions were capped and stored (–20 °C) until analysis for swainsonine by LC-MS².

Determination of Swainsonine Concentration by LC-MS². A stock solution of swainsonine standard (Toronto Research, Toronto, Canada) was prepared at a concentration of 0.50 mg/mL in water. A 20 μ L aliquot of the swainsonine stock solution was added to 2.00 mL of water to make a 4.95 μ g/mL standard. Subsequent standards (2.47, 1.24, 0.62, and 0.31 μ g/mL) were prepared by serial dilution. The swainsonine calibration standards were transferred to autosampler vials and capped.

An aliquot (100 μ L) of each extracted sample solution was added to a 2 mL autosampler vial and the solvent removed under a flow of nitrogen at 60 °C. Water was then added (1.00 mL), and the samples were capped and mixed. Samples and calibration standards were then loaded into the autosampler vial holder for analysis.

The LC-MS system consisted of an HP 1100 binary solvent pump and autosampler, a Betasil C18 reversed phase HPLC column (100 \times 2 mm, Keystone Scientific) and a Finnigan LCQ mass spectrometer. Swainsonine was eluted using an isocratic mixture of 5% methanol in 20 mM ammonium acetate at a flow rate of 0.5 mL/min. Sample injection size was 20 μ L. Ionization was achieved using an atmospheric pressure chemical ionization (APCI) source with a vaporizer temperature of 450 °C and corona discharge current of 5 μ A. The capillary inlet temperature and voltage were 200 °C and 16 V, respectively. Operational parameters of the mass spectrometer were optimized using the standard "auto tune" procedure to optimize the signal (m/z 609) from a standard solution of reserpine infused into a flow (0.5 mL/min) of 50% methanol in 1% acetic acid. The mass spectrometer was run in an MS² mode, scanning product ions over a mass range of 70–300 amu after fragmentation of the swainsonine protonated molecular ion ($MH^+ = 174.2 \pm 0.75$ amu) using a relative collision energy setting of 25%. Maximum ion trap inject time was 500 ms, and three microscans were averaged for each data point.

Swainsonine eluted from the column at a retention time of ~1.4 min (Figure 1). The swainsonine peak area was measured from the reconstructed ion chromatogram (m/z 156) and quantitation based on an external calibration standard. The resulting swainsonine concentration (micrograms per milliliter) of the injected sample was converted to percent dry weight of the original plant material.

Swainsonine Assay below 0.001%. For those populations of *O. lambertii* in which swainsonine was not detected during the initial assay at a concentration of 0.001%, or greater, a composite sample was made from all individual plant samples collected at the site. From this composite sample, a 200 mg aliquot was extracted using the standard procedure outlined above. A 0.5 mL aliquot of the resulting extract was then dried in the autosampler vial, 0.5 mL of water added, and the sample

Table 3. *O. lambertii* Populations and Mean Swainsonine Concentration

location	<i>O. lambertii</i> var.	stage ^a	GPS coordinates	voucher no. ^b	mean ^c (% dry wt \pm SD)	range
Meade, KS	<i>articulata</i>	pod	37° 10' 09 N; 100° 23' 03 W	27698	<0.0001	
Knowles, OK	<i>articulata</i>	early pod	36° 55' 15 N; 100° 18' 23 W	27689	<0.0001	
Buffalo, OK	<i>articulata</i>	pod	30° 48' 44 N; 99° 46' 22 W	27697	<0.0001	
Flagstaff, AZ	<i>bigelovii</i>	vegetative	35° 23' 41 N; 111° 34' 46 W	27665	0.054 \pm 0.027	0.022–0.106
Springerville, AZ	<i>bigelovii</i>	vegetative	34° 00' 49 N; 109° 10' 48 W	27667	0.026 \pm 0.021	0.0–0.065
Kingston, NM	<i>bigelovii</i>	vegetative	32° 52' 51 N; 107° 51' 55 W	27668	0.016 \pm 0.013	0.0–0.043
Winston, NM	<i>bigelovii</i>	flower	33° 21' 43 N; 107° 34' 41 W	27669	0.038 \pm 0.035	0.0–0.068
Kanab, UT	<i>bigelovii</i>	vegetative	37° 06' 19 N; 111° 51' 28 W	27661	0.008 \pm 0.016	0.0–0.047
Ferron, UT	<i>bigelovii</i>	flower	39° 06' 57 N; 111° 17' 36 W	440983	<0.0001	
Fort Collins, CO	<i>bigelovii</i>	flower	40° 56' 39 N; 105° 15' 33 W	440980	0.0002	
Ocate, NM	<i>bigelovii</i>	pod	36° 15' 11 N; 105° 02' 32 W	27672	0.0006	
Capulin, NM	<i>bigelovii</i>	flower	36° 41' 25 N; 104° 08' 35 W	440981	0.0001	
Sophia, NM	<i>bigelovii</i>	flower	36° 28' 06 N; 103° 59' 54 W	440982	<0.0001	
Sidney, NE	<i>lambertii</i>	flower	41° 09' 18 N; 103° 05' 27 W	27704	0.0007	
Hot Springs, SD	<i>lambertii</i>	flower	43° 24' 35 N; 103° 26' 23 W	27717	0.0001	
Lusk, WY	<i>lambertii</i>	flower	43° 05' 12 N; 104° 19' 36 W	27721	<0.0001	

^a Phenological growth stages. ^b Voucher specimens deposited in Monte L. Bean Herbarium, Brigham Young University, Provo, UT.

^c For those samples with initial swainsonine levels at <0.001%, a separate bulk sample was analyzed with quantitation down to 0.0001% (1 ppm) and the presence of swainsonine confirmed by GC-MS.

analyzed a second time by LC-MS² (providing a 20 \times concentration with detection down to 0.0001% or 1 ppm of swainsonine).

To confirm trace swainsonine levels in the plant material, a second 0.200 mL aliquot was placed into a 1.0 mL reactive vial and dried under a flow of nitrogen at 70 °C, after which the sample was silylated by the addition of 0.200 mL of pyridine and 0.050 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) silylation reagent (Pierce) and heating at 70 °C for 30 min. This sample was then analyzed by GC-MS², providing confirmation of swainsonine presence using a second method. GC-MS² analysis was accomplished using a Finnigan GCQ system with a J&W DB-5MS capillary column (30 m \times 0.25 mm). Helium was used as the carrier gas at a flow rate of 40 cm/s. Samples (2.0 μ L) were injected splitless with a injector temperature of 225 °C. The column temperature was programmed at 120 °C for 1 min, raised from 120 to 200 °C at 5 °C/min and from 200 to 300 °C at 20 °C/min, and then held at 300 °C for 8 min. The mass spectrometer was set to scan the product ions from a parent ion of 299 [a major fragment ion from swainsonine—triTMS; M – (CH₃)₃SiOH]. Swainsonine was detected from the selected ion chromatogram [*m/z* 210; loss of OSi(CH₃)₃ from the parent 299] at a retention time of 16.17 min.

Statistical Analysis of Swainsonine Data. *O. sericea*. Data were analyzed by analysis of variance (ANOVA) in a split plot in time model. Locations were the main plot and were tested by the plant-within-location interaction. Year was the split plot in time, and along with the location-by-year interaction, was tested by the residual error.

Variability in swainsonine concentration among individual plants was estimated by partitioning the sources of variance from the statistical model. The proportion of sums of squares for locations, plants-within-locations, year, location-by-year, and the residual error was calculated. The amount of variability between plants was compared to the other factors of the model.

O. lambertii. Data were analyzed by one-way ANOVA to test differences between locations. Variability in swainsonine concentration among individual plants was estimated by partitioning the sources of variance from the statistical model. The proportion of sums of squares for locations and plants-within-locations was calculated.

RESULTS AND DISCUSSION

Analytical Methodology. Typically, methods of analysis for swainsonine in plant material have used a solvent extraction procedure followed by partial purification of the swainsonine using ion-exchange chromatography and then detection and quantification of swainsonine using thin-layer chromatography (21),

capillary gas chromatography of the tri-TMS (8, 20, 22) or the triacetate derivative (23, 24), or inhibition of α -mannosidase (24). For example, Molyneux et al. (21) used a Soxhlet extraction of the plant material (0.5 g) with methanol, followed by solvent removal and then liquid/liquid extraction between chloroform and 1 N HCl. The resulting acid solution was then passed through a small prepared ion-exchange column from which the swainsonine was eluted with 0.5% aqueous ammonium hydroxide. These procedures for extraction and isolation of swainsonine were essentially retained, with some minor modifications in solvents and equipment. The sample size was reduced to 0.1 g, and the plant extraction and liquid/liquid extraction were combined into a single step by extracting the plant material with a mixture of chloroform and 2% acetic acid. In a comparison of the two methods, no difference was found in the efficiency to extract swainsonine from the plant material. However, when the ion-exchange resin is mixed with the acid extract as described here (see Materials and Methods), as opposed to passing the solution through a small column (21), it is important to use 2% acetic acid because the partition coefficients of swainsonine ([μ g/mL]_{resin}/[μ g/mL]_{aq}) are very different for the two acids. When a solution of swainsonine, in 2% acetic acid, was mixed with a known amount of ion-exchange resin for 15 min, we found swainsonine to be completely bound to the resin. However, for a solution of swainsonine in 1 N HCl we found approximately half of the swainsonine remaining in solution after mixing with the resin (*K* = 1.1), and thus for efficient retention of swainsonine when using 1 N HCl, it is best to pass the solution through a column of resin as previously described (21).

For detection and quantitation of the isolated swainsonine, reversed phase liquid chromatography coupled to atmospheric pressure chemical ionization (APCI) and sequential tandem mass spectrometry (MS²) was used. It is noted that GC-MS would be an acceptable method for detection and quantitation of swainsonine for those laboratories not equipped with LC-MS instrumentation. However, we have experienced some difficulty in quantitative derivatization of swainsonine, which we believe is related to the type of container in which the derivatization was carried out, and it is therefore noted that the derivatization conditions need to be carefully established before routine quantitative analyses. The use

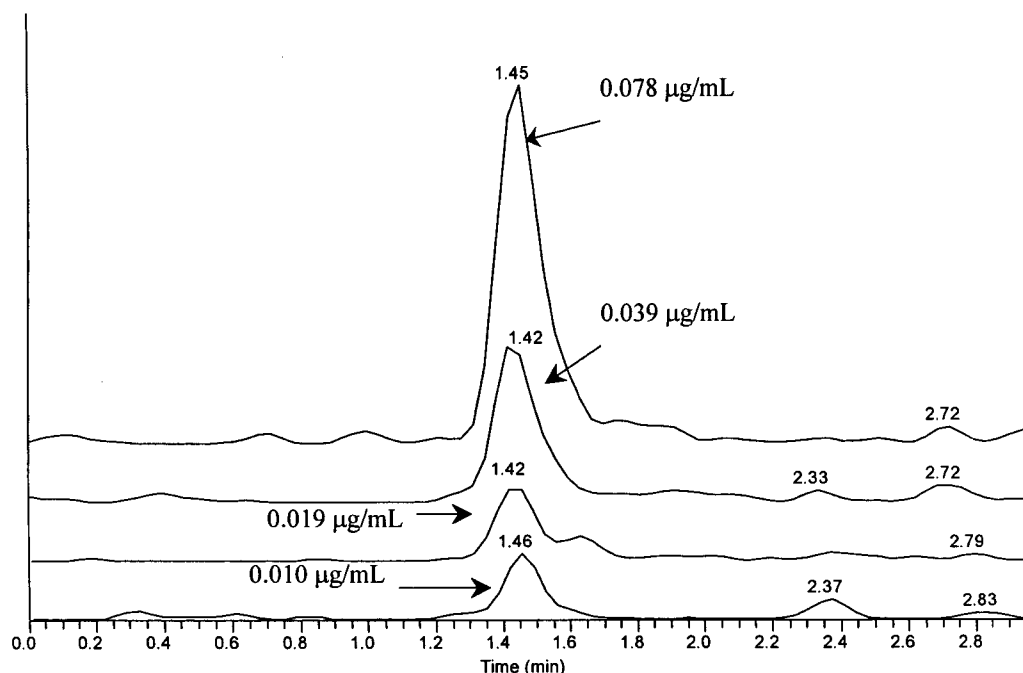


Figure 2. LC-MS² analysis of swainsonine low-level standards. Reconstructed ion chromatograms (m/z 156.2) from MS² product ion detection and swainsonine concentrations are those of the injected standard solution.

of LC-MS² methodology, however, provides a highly sensitive and selective detector for swainsonine (20, 22, 25) and eliminates extra steps of derivatization and the extended analysis times used in GC-MS. It is noted, however, that for *O. lambertii* samples that contained very low levels of swainsonine (<0.001%) GC-MS was used to provide confirmation of the presence of swainsonine in the samples.

The reconstructed ion chromatogram, full-scan mass spectrum, and a product ion spectrum (MS²) for swainsonine are shown in Figure 1. Under APCI conditions the first-order mass spectrum of swainsonine shows an intense protonated molecular ion (m/z 174), and essentially no other major fragment ions are present (Figure 1B). The MS² product ion spectrum (Figure 1C) showed a single loss of one hydroxyl group as water (7, 25). MS³ and MS⁴ product ion spectra (data not shown) show successive loss of hydroxyl groups (as water) characteristic of polyhydroxy alkaloids analyzed by tandem mass spectrometry (25, 26). The use of MS² product ions for quantitation provides a greater degree of specificity and an increase in sensitivity by removing certain background ions. Another advantage is that the raw data file sizes are greatly reduced, making storage of data more convenient when a large number of samples are analyzed.

The combination of LC with tandem mass spectrometry permits swainsonine to be analyzed specifically without the necessity for rigorous purification. The use of ion-exchange chromatography provides an appreciable degree of purification because the only other compounds likely to be present are other alkaloids and basic amino acids. A similar approach to the detection, but not quantitation, of polyhydroxy alkaloid glycosides has been reported; the sample solution, after ion-exchange separation, was analyzed by direct injection and tandem mass spectrometry (26). We attempted a similar procedure for the quantitation of swainsonine, but our results were erratic at times; and insertion of a column in-line and detection from MS² product ions greatly improved the reliability of the method with only

a minimal increase in analysis time from approximately 3 to 5 min between injections. Most recently, an LC-APCI-MS method was reported for the analysis of polyhydroxy alkaloids, including swainsonine, comparable to the method presented here using a carbohydrate column, and a retention time of 2.0 min was reported for swainsonine (25).

For quantitative analysis a five-point calibration (4.95–0.310 µg/mL) was generally used, although linear results could be obtained down to 0.019 µg/mL ($r = 9998$). Below 0.019 µg/mL the signal/noise ratio was poor (Figure 2) and the response nonlinear. The limit of quantitation was therefore estimated to be 0.001% swainsonine, by weight, in dry plant material using the described sample size and dilutions. The recovery of the method was measured from the analysis of fortified and unfortified samples in triplicate and was found to be 94%. The precision of the method was found to be $\pm 10.2\%$ (relative standard deviation) calculated from the standard deviation of a control sample ($x = 0.182\% \pm 0.019$, $n = 20$) that was analyzed with each set of samples over an extended period of sample analyses.

Swainsonine Levels in *O. sericea*. The three sites from which the white locoweeds (*O. sericea*) plants were collected are established study locations with a known history of poisonings in livestock. The locoweeds populations from all three locations were previously shown to contain swainsonine. However, no detailed information was available on swainsonine levels of individual plants and the interplant variability of the populations.

Over a three-year period the New Mexico plants contained significantly ($P = 0.01$) higher levels of swainsonine (0.097% swainsonine dry weight) compared to those from the Maxwell Ranch in Colorado (0.062%) or from the Raft River mountains in northwestern Utah (0.046%) (Table 1). At each location there was a significant change in the swainsonine concentration between years ($P = 0.0004$); however, no clear-cut trend was observed in the changes (Table 2). Interplant variation at each location was highly significant ($P < 0.0001$) and also accounted for the highest percent of the total

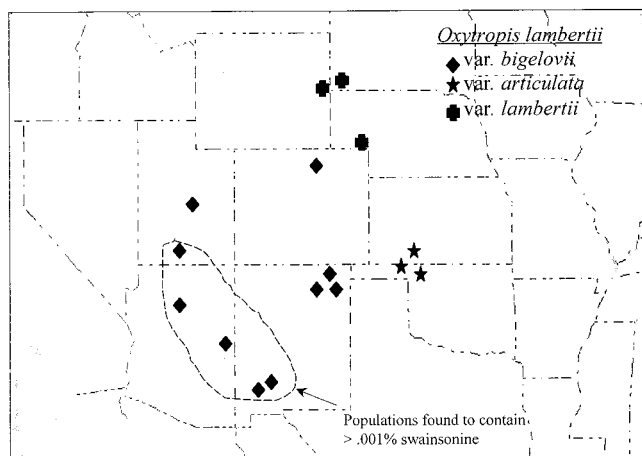


Figure 3. Map showing the 16 locations for collection of *O. lambertii* var. *lambertii*, *articulata*, and *bigelovii* from western United States.

variance of the model. At New Mexico the swainsonine values ranged from 0.03 to 0.21% with a relative average standard deviation of 34%. At the Colorado (0–0.18%) and Utah (0–0.12%) locations the range of values was somewhat lower and there were several plants collected in which no swainsonine was detected. Differences in swainsonine levels among plants within location was the greatest source of variability (39%) in the statistical model (Table 2).

Two important conclusions can be drawn from the data. The concentration of swainsonine in individual plants from year to year is highly variable. The average difference between years in the same plant ranged from 0.05% in Utah to 0.07% in New Mexico. Therefore, the overall level of swainsonine (toxicity) in a locoweed population can change significantly from year to year. Second, because the swainsonine concentration is highly variable between individual plants within a population, an appropriate sampling of individual plants must be taken to obtain an accurate measure of the swainsonine concentration within that population. We calculated the number of samples required to statistically measure $\pm 20\%$ of the true mean for each location and year [$n = (ts/e)^2$, $t = t$ value at 0.05, $s =$ standard deviation, $e = 20\%$ of mean]. The sampling number was highly variable, ranging from a low of 6 samples to a high of 86. The average was 46 samples.

Swainsonine Levels in *O. lambertii*. *O. lambertii* is also known for its association with livestock poisonings, but its swainsonine content has been verified only on a limited basis. During some recent investigations of several *O. lambertii* populations we were surprised to find collections of plant material that were negative for swainsonine. To obtain a better understanding of swainsonine presence or absence in populations of *O. lambertii*, we surveyed a large geographical area (Figure 3) and collected plants from 16 different locations. Collection sites were chosen to cover essentially all of the known varieties of *O. lambertii* (vars. *lambertii*, *bigelovii*, and *articulata*). In the initial analyses, swainsonine was found in only five populations (all var. *bigelovii*), two from New Mexico, two from Arizona, and one from Utah (Table 3). The average swainsonine content varied from a low of 0.008% (Kanab, UT) to a high of 0.054% (Flagstaff, AZ). As with *O. sericea*, interplant variation within a population was high and accounted for 66% of the total variance (Table 2).

Populations containing swainsonine seemed to be geographically restricted to the most southern and western populations (Figure 3). Interestingly, two of the *O. lambertii* var. *bigelovii* populations (Capulin, NM, and Fort Collins, CO), although negative for swainsonine, were collected from sites shared by *O. sericea*, which does contain swainsonine (Table 1).

In those populations of *O. lambertii* in which swainsonine was not detected, we analyzed a second composite sample to verify either the presence or absence of swainsonine in those samples. After sample size and extract volumes had been adjusted, our limit of quantitation was lowered to a level of 1 ppm (0.0001%) using LC-MS², and by using GC-MS² we could detect swainsonine at a level below 1 ppm for confirmation (Figure 4). All *O. lambertii* populations were found to contain swainsonine (Table 3), although the levels were a minimum of 10-fold less than those of the five populations of *O. lambertii* var. *bigelovii* initially found to contain swainsonine, most having only a trace (<1 ppm) detectable level of swainsonine.

The swainsonine levels measured in this study are comparable with those reported previously for locoweeds and *Swainsona* species, with the mean content rarely exceeding 0.1% dry weight of the whole plant. However, it should be noted that the alkaloid level does not have to be particularly high for the plant to be toxic because of the potent nature of swainsonine as an inhibitor of α -mannosidase and because prolonged grazing can result in continuous suppression of the enzyme activity, leading to cellular vacuolation characteristic of locoism. It has been proposed that the threshold of toxicity should be conservatively set at 0.001% swainsonine (dry weight) (27). Plants containing swainsonine below 0.004% should be safe in sheep (28); however, horses, for example, are probably twice as susceptible to locoweed poisoning, and thus a threshold of toxicity approaching 0.001% swainsonine (dry weight) seems to be reasonable. All of the *Oxytropis* samples in this investigation which contained significant levels of the alkaloid (0.001%) exceeded that value by at least an order of magnitude. Moreover, the limit of detection of the general procedure is also 0.001%, so that any sample in which swainsonine is detectable by the designed LC-MS method should be considered to be potentially toxic.

It has been suggested that endophyte infection may impact swainsonine concentration (22). Swainsonine is known to be produced by fungi, including the pathogens *Rhizoctonia leguminicola* (29) and *Metarhizium anisopliae* (30). More specifically, it has recently been reported that some locoweeds are infected by an endophyte belonging to the genus *Alternaria* and that the isolated endophyte produced swainsonine when grown in an artificial medium (31). The exact role of the endophyte is unclear. The biosynthesis of swainsonine in Diablo locoweed (*Astragalus oxyphysus*) and *R. leguminicola* has been shown to proceed by an identical route from pipercolic acid in both the plant (32) and the fungus (33). Endophyte infection may therefore enhance or supplement swainsonine production. The levels of swainsonine in samples of *O. lambertii* from different locations may indicate that the endophyte is present in some populations but not in others. Swainsonine was virtually absent from samples of this species at sites shared by *O. sericea*, which contained relatively high levels of the alkaloid. This difference between species may be due to host specificity of the endophyte, low rates of trans-

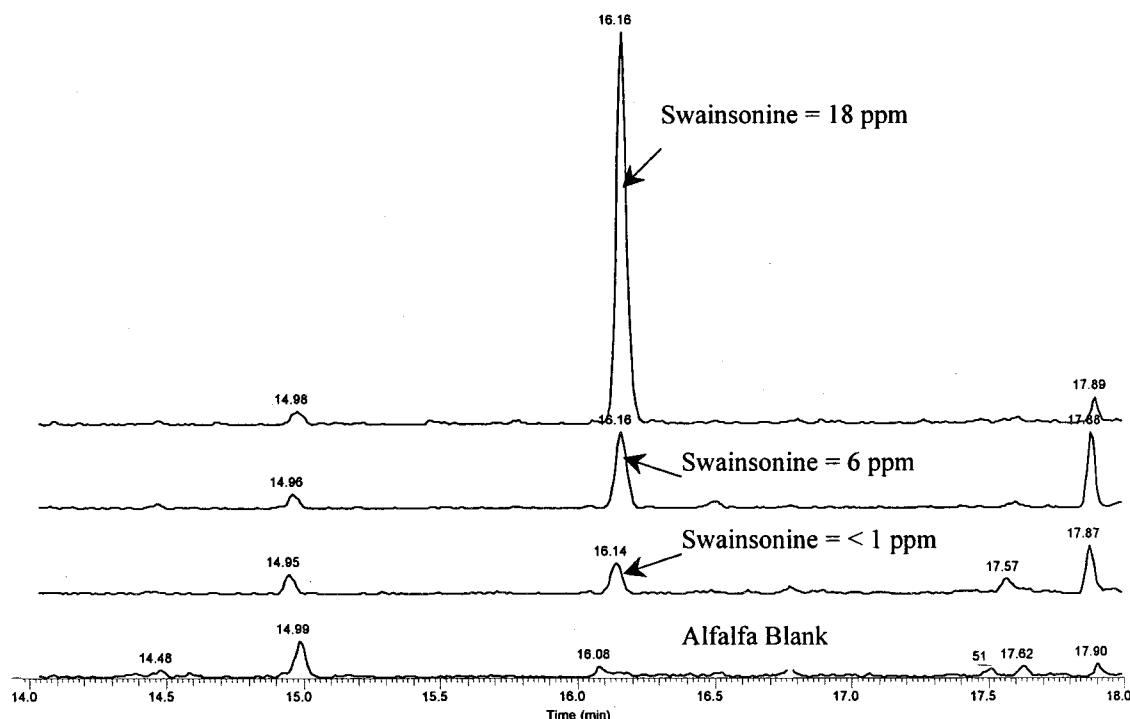


Figure 4. GC-MS² analysis of plant samples with swainsonine concentration near and below 0.0001%. Chromatograms are selected ion chromatograms (m/z 210) from MS² product ions (parent ion 299), and concentrations are as measured in the plant sample on a dry weight basis.

mission of the endophyte between plants, or difficulty in defining varieties of *O. lambertii*, as has been noted by Barneby (34). It is possible that those samples of var. *bigelovii* which contained only trace levels of the alkaloid represent intergradations with the other varieties, *articulata* and *lambertii*, which also lack significant levels of swainsonine. In this respect it is interesting to note that only those samples of var. *bigelovii* from the most southwestern collections, furthest removed geographically from the other varieties, contain swainsonine at significant levels. The analysis of many samples over an extended period may be helpful in resolving this question and determining whether swainsonine may be applied as a chemotaxonomic marker for distinction of varieties.

It is important to understand the source of swainsonine in locoweeds and what affects toxin concentration in the plants. If swainsonine levels are controlled by endophyte, geographical, taxonomic, or environmental factors, such information would be useful in the management of the locoweed-poisoning problem. The simple extraction techniques and LC-MS analytical methodology developed in this study are sufficiently rapid and sensitive to be applied to a much larger range of samples, including both *Astragalus* and *Oxytropis* species. The genus *Astragalus* encompasses a much greater number of species than *Oxytropis* in North America, and the identities of those that contain swainsonine need to be established unequivocally so that productive grazing land is not underutilized simply because *Astragalus* species are present. It is anticipated that this knowledge of the factors controlling the occurrence and levels of swainsonine will ultimately lead to a predictive model for the prevention of locoweed poisoning in livestock.

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