Rapid Multiresidue Screen for Alkaloids in Plant Material and Biological Samples

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A multiresidue screen for the quantitative and qualitative determination of alkaloids in plant material, animal ingesta, tissue, and biological fluid is described. The alkaloids were extracted with 5% ethanol in ethyl acetate (v/v) after the addition of aqueous NaOH. The organic extract was cleaned up by partitioning with 0.5 N HCl. The aqueous phase was made alkaline; the alkaloids were retained on a polymeric C-18 minicolumn and subsequently eluted with ethyl acetate. The concentrated extract was analyzed quantitatively by GC/NPD. The alkaloids coniine, nicotine, atropine, retrorsine, solanidine, and strychnine were tested to measure the performance of the method. Fortifications at 10 and 1 μg/g of the six model alkaloids in alfalfa hay, bovine rumen content, liver, urine, and serum were prepared. The method recovered the six alkaloids in the range of 113–82% at the 10 μg/g level and 113–72% at the 1 μg/g level. Qualitative screening methods using a modified commercial TLC system and GC/MS were developed. Theoretical detection limits for qualitative screening ranged from 0.2 to 1.0 μg/g, while theoretical detection limits for GC/NPD ranged from 0.025 to 0.2 μg/g for the compounds studied using a nominal 5 g sample. The method was used successfully in a diagnostic case to identify lupine alkaloids in rumen contents.

Keywords: *Alkaloid; multiresidue screen; gas chromatography; thin layer chromatography*

INTRODUCTION

A general need in a veterinary diagnostic lab is to screen for numerous potentially toxic compounds with a minimum of analyses. This is best accomplished by using multiresidue screening methods. Multiresidue methods (MRMs) must be rapid and test diverse sample types for numerous toxicants at relevant concentrations. While MRMs exist for many classes of toxic agents, a satisfactory MRM to screen for plant alkaloids does not exist.

Alkaloids are widespread plant natural products which contain a heterocyclic nitrogen, are basic in character, and are usually toxic. They are otherwise very diverse, with varying structures, volatility, stability, and polarity. Alkaloids are of interest in a veterinary diagnostic setting because many can cause acute and chronic toxicity in animals (Cheeke and Shull, 1985).

A MRM for alkaloids has several key requirements. It must be applicable to this chemically diverse class of compounds in a wide variety of sample matrices, including animal tissue, plant material, biological fluids, and ingesta. A sample extract must be produced which has not undergone evaporative steps, due to the volatility of some alkaloids. Extreme conditions which could hydrolyze esters must be avoided. For diagnostic use, the method should be rapid, providing a result within the same day. Cleanup must be sufficient to provide diagnostically relevant detection limits qualitatively, with good precision and accuracy quantitatively. Typical MRMs have defined retention times or R_fs, with each compound on the screen validated through recovery studies. Analytical standards for all alkaloids are unavailable, so an alkaloid MRM must be able to detect rare or unknown alkaloids, even those which have not been characterized using the analytical method.

Reported here is a MRM for alkaloids of potentially broad applicability. Six model alkaloids were selected to represent the diverse chemistry of this class of compounds (Figure 1). They included the piperidine alkaloid coniine which has high volatility; atropine, a polar tropane alkaloid which is readily hydrolyzed to tropine; retrorsine, a polar pyrrolizidine alkaloid; solanidine, a low-volatility steroidal alkaloid; strychnine, a large alkaloid of low polarity; and nicotine, a pyridine alkaloid with no polar functional groups and a good degree of thermal stability. The AOAC method for total alkaloids is based on the behavior of nicotine as a standard (AOAC, 1990). A method that quantitatively recovers these six alkaloids is likely to give adequate recovery for most alkaloids of diagnostic interest.

Current MRMs for alkaloids typically involve extraction with a polar solvent such as ethanol, evaporation to remove the solvent, and a series of acid/base liquid–liquid partition steps for cleanup (Thienes, 1972; Jackson, 1974). An alternate method is extraction with aqueous acid followed by addition of base and extraction into organic solvent (Stahr, 1991; Wagner et al., 1984) or the use of solid phase extraction (SPE) columns (Peng and Chiou, 1990). Direct solvent extraction has also been used for rapid toxicological screening (Jackson, 1974).

Traditionally, alkaloids are detected using TLC on silica gel plates, modified by base in the mobile phase or impregnated on the plate with visualization using alkaloid sensitive reagents such as Dragendorff's reagent or iodoplatinate (Stahr, 1991; Wagner et al., 1984). Multiresidue drug screens which include alkaloids have also used TLC for rapid analysis of extracts (Harper et al., 1989).

Alternatively, reverse phase HPLC may be used with (Lin, 1993; Parker et al., 1990) ion-pairing reagents or

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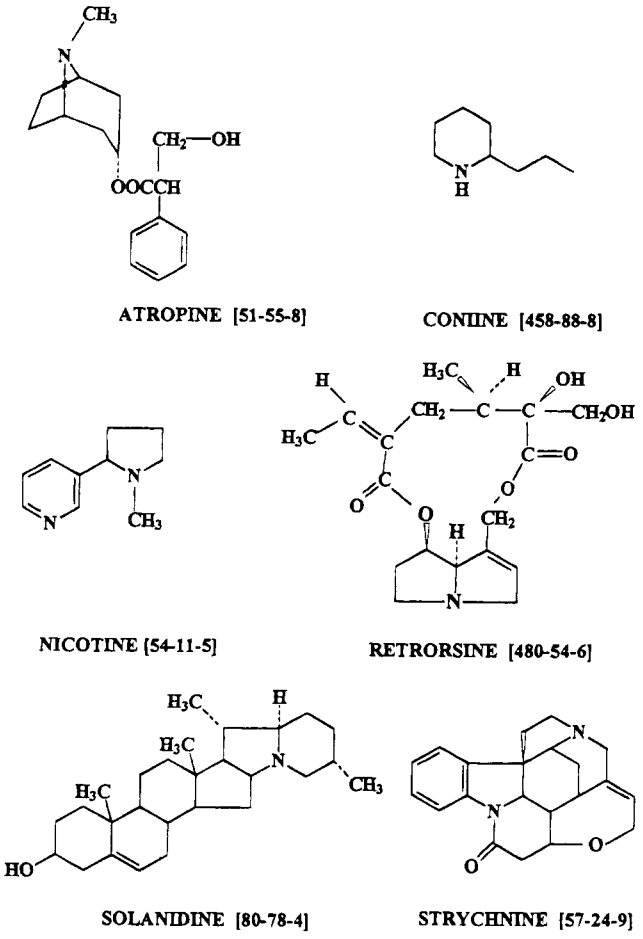

Figure 1. Structures and CAS Registry Numbers of the six model alkaloids.

without (Roos and Lau-Cam, 1986). While HPLC can chromatograph many alkaloids (Chu et al., 1993; Pacifici et al., 1993; Lewis et al., 1990; Mandel et al., 1991; Duez et al., 1985; Bushway et al., 1986), there are no multiresidue means of detecting alkaloids. Several important alkaloids do not absorb UV light at discriminating wavelengths (Popl et al., 1990).

Gas chromatography using a nonpolar column has been used in the multiresidue analysis of drugs, including numerous alkaloids (Musumarra et al., 1987; Stahr, 1991). GC has also been used for single classes of alkaloids (Galey et al., 1992; Plumlee et al., 1993; Manca et al., 1989; Witte et al., 1987, 1993; Saady and Poklis, 1989; Deutsch et al., 1992; Thompson et al., 1982; Lawson et al., 1992). While GC/NPD offers a fairly selective means of detecting alkaloids, numerous alkaloids are not sufficiently volatile for GC analysis (Popl et al., 1990). However, GC/NPD does offer the opportunity to quantitate low levels of volatile alkaloids for which analytical standards are available. GC/MS analysis, with electron ionization (EI) in the scanning mode, can be used to identify unknown alkaloids, by producing library searchable EI spectra (Witte et al., 1987, 1993).

The present paper describes a MRM for alkaloids in plant material, ingesta, tissue, and biological fluid suitable for rapid diagnosis of alkaloid-induced intoxication in animals. The samples are extracted with 5% ethanol in ethyl acetate and cleaned up by partitioning with 0.5 N HCl followed by polymeric C-18 column cleanup. The alkaloids are analyzed by GC/NPD (quantitative analysis) and by gas chromatography/mass selective detection and thin layer chromatography (qualitative analyses). The MRM was tested with the six model compounds by means of a recovery study from alfalfa hay, bovine rumen content, liver, serum, and urine at fortification levels of 10 and 1 μg/g.

MATERIALS AND METHODS

Materials. Standards were purchased from Sigma Chemical Co. (St. Louis, MO). Neat free base alkaloid (25 mg) was dissolved in 25 mL of methanol to make a 1000 μg/mL standard solution. Subsequent dilutions were made using methanol for spiking standards or ethyl acetate for analytical standards. All solvents were pesticide grade (Fisher). Sodium sulfate was ACS regent grade (Fisher).

Extraction. Samples were thoroughly mixed in order to obtain a representative subsample. Plant samples were prepared as follows: 10–25 g of sample was placed in a Stein mill sample cup (Model M-2; Seedburo Equipment Co., Chicago, IL). Sufficient liquid N_2 was added to freeze the sample, and the plant sample was immediately fractured by Stein milling for 1 min. Fractured plant material (100 g) was prepared prior to subsampling. Rumen content and liver (100 g) were chopped while frozen, and serum and urine were thawed and mixed by shaking, prior to subsampling.

A 5.0 g sample was weighed into 250 mL square French homogenization vessels with Teflon-lined caps (Fisher Scientific). Water (10 mL) was added to dry samples such as hay. Ethanol in ethyl acetate (100 mL, 5%, v/v) was added followed by 1 mL of 10 N NaOH and 50 g of sodium sulfate. Samples were homogenized (Polytron Model PT 10/35; Brinkman Instruments Inc.) for 1 min at 50% power. Extracts were centrifuged at 90*g* (IEC CU-5000 centrifuge; International Equipment Co.) for 3 min. A 40 mL aliquot of extract was pipetted into a 250 mL separatory funnel for additional cleanup.

Cleanup. Hexane (100 mL) was added to 40 mL of extract in a 250 mL separatory funnel. The organic layer was extracted with 10 mL of 0.5 N HCl followed by 5 mL of 0.5 N HCl. The aqueous fractions were combined into a 50 mL test tube, and 2.5 g of NaCl was added. The test tube was placed on a temperature-controlled nitrogen gas evaporator (N-Evap analytical evaporator; Organomation Assoc. Inc.). N_2 was bubbled through the extract using a $^1/_{16}$ in. Teflon tube placed to the bottom of the test tube for 15 min, while heating the sample at 40 °C; 1.2 mL of 10 N NaOH was added to the sparged extract. The resulting solution was then pulled through a C-18 SPE column (Polymeric MP-1 100 mg C-18 solid phase extraction minicolumn, preconditioned with 1 column volume (CV) of ethyl acetate, 1 CV of methanol, 1 CV of water, and 1 CV of 0.1 N NaOH; Interaction Chemicals Inc., Mountain View, CA) at a rate of 5 mL/min using a vacuum. The SPE was centrifuged at 1200*g* to dry the column and eluted with 2 mL of ethyl acetate at 5 mL/min. The ethyl acetate extract was dried over 0.5 g of Na_2SO_4 for 1–2 min, and aliquots were taken for subsequent analyses.

Gas Chromatography. *GC with a Nitrogen–Phosphorus Detector (GC/NPD).* The conditions for GC/NPD (Autosystem; Perkin Elmer) were as follows: packed column injector and autosampler; 5 m × 0.53 mm × 1.0 μm DB-5 capillary column (J&W Scientific); glass insert with 0.5 cm of loosely packed silanized glass wool; carrier gas flow, helium at 12 mL/min; detector gas flow, H_2 at 1.8 mL/min and air at 100 mL/min; temperature program, 60 °C for 0.5 min, 10 °C/min–120 °C, 20 °C/min–280 °C, and hold for 5.5 min; run time, 20 min; splitless injection; injector temperature, 240 °C; detector temperature, 300 °C; volume of injection, 2.0 μL; chromatography data system (Turbochrom data system with Link interface; Perkin Elmer-Nelson). Alkaloid residues were quantitated by injecting 2 μL of analytical standard (0.5–5 μg/mL) and sample extracts at 1 g/mL. Calibration was based on injections of external alkaloid standards.

GC with Mass Selective Detector (GC/MSD). The conditions for GC/MSD (Model HP 5890 with HP 5970 MSD; Hewlett-Packard) were as follows: autosampler (Model 7673; Hewlett

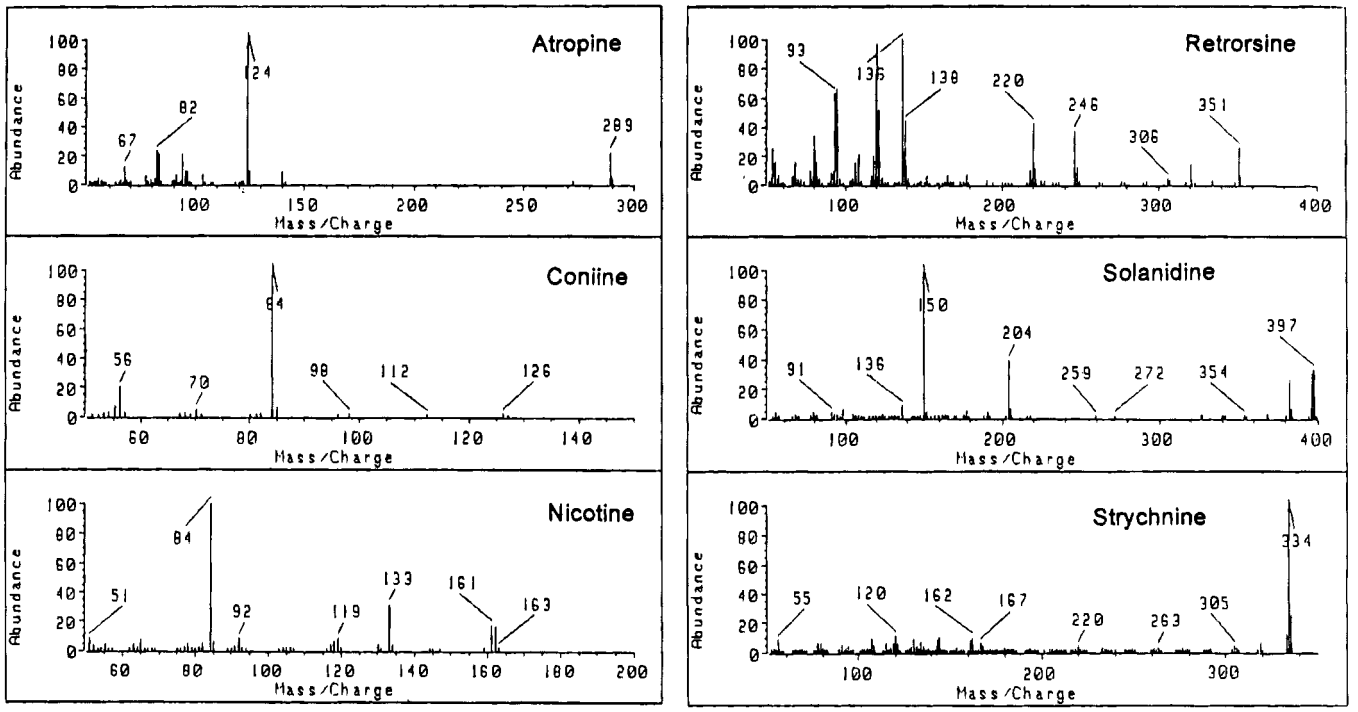

Figure 2. EI mass spectra of the six model alkaloids produced by the HP 5970 mass spectrometer.

Packard); 12 m × 0.2 mm × 0.33 μm HP-1 capillary column (Hewlett-Packard); glass insert with 0.5 cm of loosely packed silanized glass wool; He carrier gas flow, 10 psi (1.5 mL/min at 50 °C); He purge flow, 2 mL/min; He total flow, 70 mL/min; temperature program, 50 °C for 0.5 min, 10 °C/min–200 °C, 30 °C/min–290 °C, and hold for 6.5 min; run time, 25 min; splitless injection, valve off at 0.5 min; injector temperature, 240 °C; interface temperature, 280 °C; volume of injection, 2.0 μL; detector scan from 50 amu to 550 amu at 0.86 scans/s; detector on at 2 min.

An aliquot (0.5 mL) of ethyl acetate extract (1 g/mL) was evaporated to dryness at 40 °C with a stream of nitrogen and redissolved in 50 μL of ethyl acetate. Qualitative analysis was performed by injection of 2 μL of sample extract (10 g/mL) and analytical standard (1–100 μg/mL). Peaks were identified by comparison of peak spectra and retention times to those of external alkaloid standards.

Thin Layer Chromatography. Thin layer chromatography (TLC) was performed using a modified commercial TLC system (Toxi-Lab Division of Marion Laboratories, Laguna Hills, CA) for qualitative screening. A 1 mL aliquot of extract (1 g/mL) was evaporated to dryness at 40 °C with a stream of nitrogen and redissolved in 25 μL of ethyl acetate. The 25 μL of extract was spotted in 2.5 μL increments on a blank Toxi-Disc A and heated (Toxilab heated warmer) at 40% of full power (Variac autotransformer; General Radio Co., Concord, MA) to evaporate the solvent. Discs were placed in six-hole normal phase chromatograms impregnated with vanadium salt (blank Toxi-Gram A). Position six was filled with a disc containing a propietary red dye and miscellaneous drugs including nicotine (Toxi-Disc A-2). The plate was then developed with ethyl acetate–diethylamine (95:5, v/v) until the red dye marker reached an R_f of 0.95, indicating that the solvent had reached an R_f of 1.0. Alkaloids were visualized by dipping the dried plate twice in potassium iodide/iodine/bismuth subnitrate solution (Toxi-Dip A-3 reagent, Dragendorff's reagent). The alkaloids appeared as brown or purple spots. The spots were recorded, and the plate was dipped twice in 5% $NaNO_2$ in water (w/v). After 1–2 min the spots were recorded.

Validation Study. Pooled alfalfa hay, bovine rumen content, urine, serum, and liver were fortified with each model alkaloid at 1 and 10 μg/g. Four replicate fortifications for each matrix type at each spike level were prepared. These were analyzed quantitatively via GC/NPD and qualitatively via TLC and GC/MS.

RESULTS AND DISCUSSION

Recovery for the six alkaloids studied are presented in Table 1. The method quantitatively extracted and recovered the alkaloids from a wide variety of sample types. There was good recovery and precision for fortifications in highly pigmented (alfalfa hay, rumen content), lipid-containing (liver), protein-containing (serum, liver), and aqueous (urine, serum) matrices. Matrix coextractives were effectively removed without loss of volatile, easily hydrolyzed, or polar alkaloids. Retrorsine and solanidine gave slightly lower recoveries and higher percent coefficients of variation in liver and serum, especially at the 1 μg/g level. However, these compounds were well detected via qualitative screening at this low level.

The use of polymeric C-18 SPE columns provided a rapid, nonevaporative concentration step. GC/NPD analysis gave a quantitative means of determining alkaloid content in the final extract. This technique has utility when low levels of a known alkaloid are to be quantitated. TLC analysis provided a means of rapidly screening the extract for the presence of alkaloids, with GC/MS analysis to confirm the identity of suspect compounds.

Extraction. Extraction of alkaloids with a water miscible solvent such as methanol or ethanol or extraction with acidified water has several disadvantages. These techniques generate large volumes of aqueous extract. The water must be removed in a subsequent step. Lengthy filtration steps are required, as aliquots of extract without measurement of the total volume of extract are not representative of the extract due to variable water content. Extraction with a water miscible solvent requires evaporation of the solvent to yield an aqueous extract, with possible loss of volatile alkaloids. Extraction of aqueous extracts with organic solvent produces a larger volume of extract, counter to the requirement to concentrate the sample extract without evaporation. Extraction of tissue with water tends to yield an extract which is difficult to filter and

Table 1. Summary of Validation Study Results, Including Fortification Level, Average Recovery (n = 4), and Percent Coefficient of Variation

fortification level (ppm)	atropine	coniine	nicotine	retrorsine	solanidine	strychnine
			Alfalfa Hay			
10	92 (3%)	106 (5%)	113 (4%)	102 (4%)	101 (4%)	108 (5%)
1	73 (8%)	113 (3%)	101 (4%)	102 (4%)	106 (6%)	99 (3%)
			Bovine Rumen Content			
10	97 (3%)	93 (7%)	86 (14%)	92 (1%)	86 (7%)	100 (4%)
1	103 (4%)	104 (4%)	103 (3%)	112 (4%)	85 (4%)	108 (3%)
			Bovine Liver			
10	92 (2%)	91 (3%)	87 (13%)	82 (10%)	90 (4%)	101 (1%)
1	98 (3%)	83 (8%)	103 (2%)	98 (8%)	74 (10%)	110 (3%)
			Bovine Serum			
10	99 (4%)	97 (4%)	96 (6%)	82 (14%)	88 (2%)	102 (4%)
1	98 (3%)	80 (2%)	90 (6%)	75 (10%)	74 (4%)	103 (4%)
			Bovine Urine			
10	91 (3%)	102 (2%)	108 (2%)	90 (5%)	99 (2%)	110 (1%)
1	109 (11%)	85 (8%)	102 (2%)	96 (9%)	84 (8%)	87 (9%)

Table 2. Summary of Typical TLC and GC/NPD Retention Data, Sensitivities, and Theoretical Detection Limits (DL)[a]

	TLC				GC/NPD		
alkaloid	R_f	DL (μg)	DL (ppm)	color	t_R (min)	DL (ng)	DL (ppm)
atropine	0.4	0.25	0.25	purple	11.2	0.2	0.1
coniine	0.7	1	2.5	purple	1.9	0.05	0.025
nicotine	0.8	0.25	0.5	purple	5.8	0.1	0.05
retrorsine	0.25	0.5	0.5	brown/purple	12.6	0.2	0.1
solanidine	0.9	0.25	0.25	brown	14.4	0.2	0.1
strychnine	0.45	0.25	0.25	brown	14.6	0.2	0.1

[a] Detection limit is based on spotting 1.0 g of sample (TLC) or injection of 2 μL of 1 g/mL sample extract (GC/NPD).

generally forms emulsions on subsequent partitioning steps. These difficulties were overcome by extraction with a water immiscible solvent after making the sample alkaline. The choice of extraction solvents from common solvents was small. Solvents with high disposal costs, which tend to form emulsions or are too volatile to allow a representative aliquot to be taken, were not examined in this study. Thus, methylene chloride, toluene, and diethyl ether were not considered. Studies centered on the use of ethyl acetate, examining the addition of a more polar solvent, such as ethanol or acetone, to increase the overall polarity of the extraction solvent. Holstege et al. (1994) demonstrated the utility of 5% ethanol in ethyl acetate as an extraction solvent for insecticides, including several polar pesticides. Ethyl acetate, 5% ethanol in ethyl acetate, and 20% acetone in ethyl acetate were compared for extraction efficiency of hay fortified at 5 ppm. The addition of Na_2SO_4 was also examined. EtOH (5%) in ethyl acetate with Na_2SO_4 gave the best overall extraction efficiency for the model compounds (91% ± 8%), more than 5% ethanol in ethyl acetate without Na_2SO_4 (87% ± 9%). Ethyl acetate with (82% ± 15%) and without (82% ± 18%) Na_2SO_4 gave lower recovery, while 20% acetone in ethyl acetate gave the lowest average recovery (76% ± 13%). Ethanol (5%) in ethyl acetate with the addition of Na_2SO_4 produced a clear extract, allowing a clean, representative aliquot to be taken. Laborious filtration steps were not required.

Cleanup. Many of the numerous cleanup schemes used for single alkaloid analysis are not applicable to a multiresidue method. The ionizability of alkaloids in acids is the one feature shared by the whole class of compounds, and so acid/base cleanup was chosen. While the alkaloids were readily extracted from organic solvent into acidic water, extracting them back from water into organic solvent at high pH posed some difficulty. Liquid extraction with a large volume of organic solvent was unacceptable because of the subsequent need for an evaporation step to remove this solvent. The use of common reverse phase SPE columns did not work because the silica gel backbone of the packing dissolved at the high pH required to deprotonate the alkaloids. Ion exchange SPE columns appeared to function as mixed mode ion exchange/reverse phase adsorbent columns, perhaps because of the polymeric backbone. A single small volume of elution solvent at suitable pH and solvent strength was not found to elute all six of the model compounds from ion exchange columns.

The use of polymeric C-18 SPE columns allowed the use of elevated pHs without column degradation. Hexane added to extract prior to extraction with aqueous acid decreased the amount of solvent in the aqueous extract prior to the C-18 column. Sparging the aqueous extract with nitrogen removed additional solvent. Of the six model alkaloids, solanidine was the least well retained on the C-18 column. More than 90% of each alkaloid was eluted from the column with 1 mL of ethyl acetate. The solanidine recovery from the SPE column was consistently less than 70% until ethyl acetate was included in the SPE column conditioning. The small amount of water retained on the SPE was removed via centrifugation, rather than by pulling air through the column, to avoid the loss of coniine. Trace amounts of water in the ethyl acetate were removed with Na_2SO_4.

Gas Chromatography/Nitrogen–Phosphorus Detector. The GC/NPD gave linear responses for the alkaloids and was able to detect as low as 0.05 ng of some alkaloids at 10 times signal to noise (Table 2). Detection limits are theoretical, based on injection of 2 μL of 1 g/mL extracts. Samples fortified at 1 ppm were readily detected on the GC/NPD (Figure 3). The response of retrorsine decreased when trace amounts of water remained in the final extract, with the detector response for 1 ng dropping to less than 10% in five sample injections. This was remedied by drying the extract with Na_2SO_4. Evaporation of the extract to dryness followed by addition of ethyl acetate also

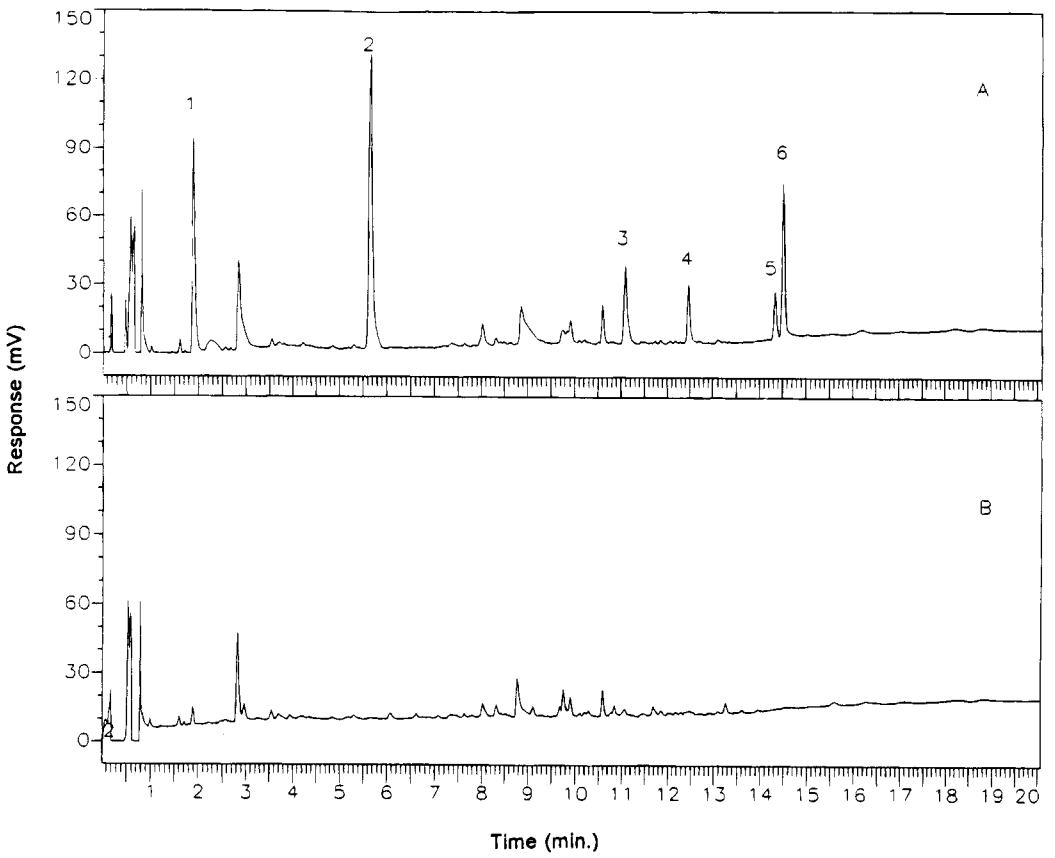

Figure 3. GC/NPD chromatograms of (A) rumen content fortified at 1 ppm with six model alkaloids and (B) control rumen content; 2 μL of 1 g/mL sample extract injected under standard GC conditions; (1) coniine, (2) nicotine, (3) atropine, (4) retrorsine, (5) solanidine, and (6) strychnine.

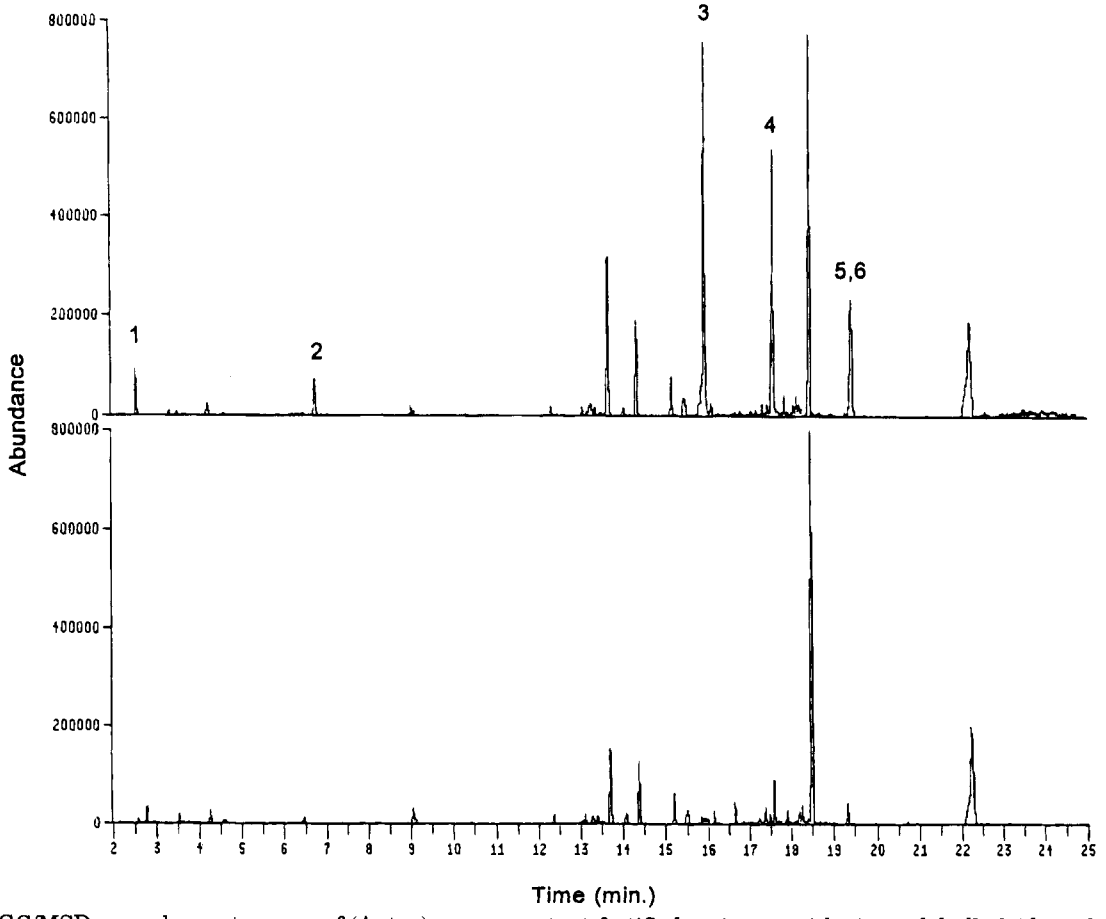

Figure 4. GC/MSD scan chromatograms of (A, top) rumen content fortified at 1 ppm with six model alkaloids and (B, bottom) control rumen content; 2 μL of 10 g/mL sample extract injected under standard GC conditions; (1) coniine, (2) nicotine, (3) atropine, (4) retrorsine, (5) solanidine, and (6) strychnine.

Table 3. Summary of Typical GC/MSD Retention Data, Sensitivities, Theoretical Detection Limits (DL), and Relative Abundance of the Molecular Ion (*) and Five Principal Ions[a]

alkaloid	t_R (min)	DL (ng)	DL (ppm)	primary ions (relative abundance)
atropine	15.9	5	0.25	124 (100), 82 (23), 289* (22), 83 (21), 67 (12), 140 (8)
coniine	2.5	10	1.0	84 (100), 56 (20), 85 (6), 70 (5), 126 (2), 127* (2)
nicotine	6.75	5	0.5	84 (100), 133 (31), 161 (17), 162* (16), 119 (8), 92 (8)
retrorsine	17.65	10	0.5	136 (100), 120 (97), 138 (44), 220 (42), 246 (37), 351* (26)
solanidine	19.5	5	0.25	150 (100), 204 (39), 397* (33), 396 (30), 382 (26), 151 (15)
strychnine	19.5	5	0.25	334* (100), 335 (25), 333 (12), 120 (11), 144 (10), 162 (10)

[a] Detection limit is based on injection of 2 μL of 10 g/mL sample extract.

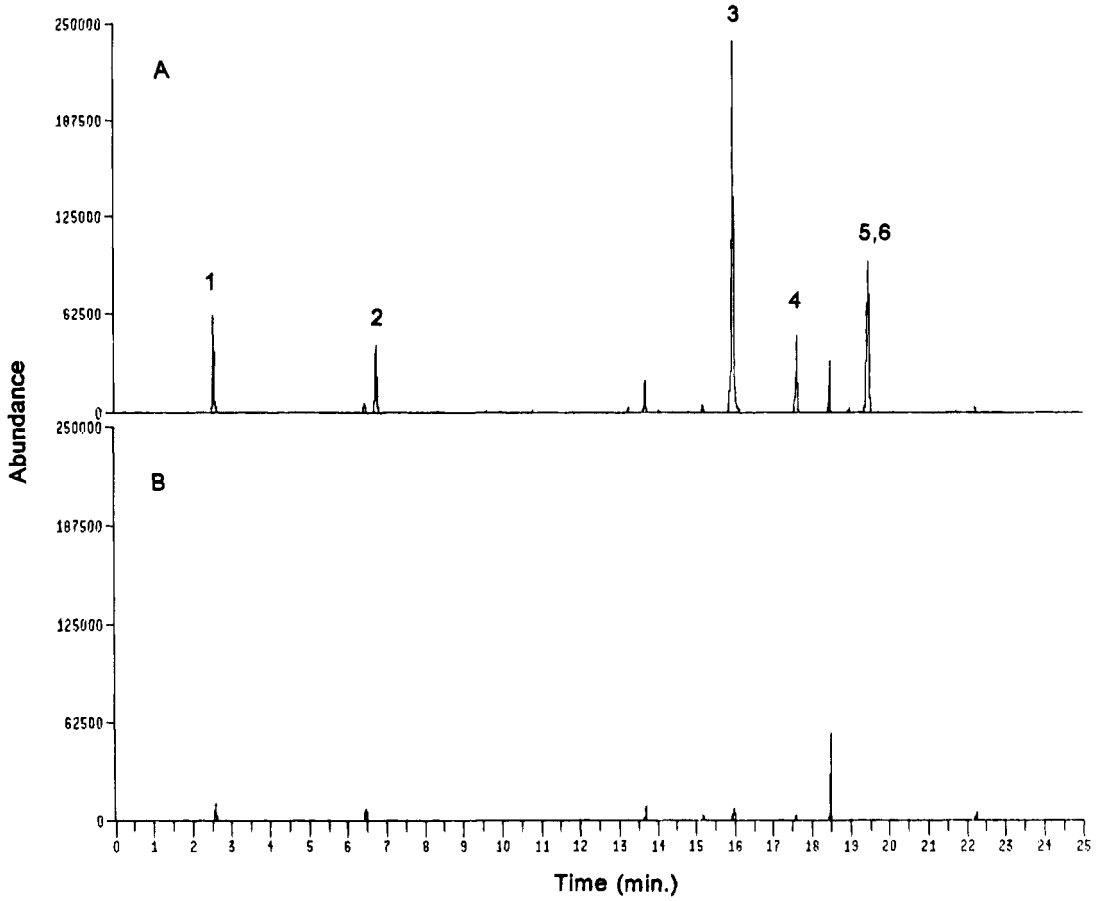

Figure 5. Sum of ion chromatograms of the most abundant ion (Table 3) for each model alkaloid extracted from GC/MSD scan chromatograms of (A) rumen content fortified at 1 ppm with six model compounds and (B) control rumen content; 2 μL of 10 g/mL sample extract injected under standard conditions; (1) coniine, (2) nicotine, (3) atropine, (4) retrorsine, (5) solanidine, and (6) strychnine.

removed the effects of residual water, although up to 70% loss of coniine was observed. If only testing for alkaloids with low volatility, it is recommended that the final extract be evaporated to dryness and reconstituted in ethyl acetate to remove remaining trace amounts of water.

Gas Chromatography/Mass Selective Detector. Initial quantitative work was performed via GC/MSD in the selected ion monitoring mode, using the GC parameters of the GC/MSD qualitative screen. While this instrument was capable of detecting sub-nanogram levels of each model compound, matrix had significant effects on response. Standards in solvent, such as ethyl acetate, toluene, or methylene chloride, gave nonlinear standard curves. Standard curves made from standards in cleaned up extract were more linear, yet without sufficient cleanup detector response degraded by as much as 50% per injection of 1 g/mL sample extracts for the polar alkaloids. Matrix interactions created difficulty in interpreting developmental data, with spike recoveries ranging up to 200% for some compounds. The GC/MSD also gave nonlinear standard curves in the full scan mode. Repeat injections of dilute (1 g/mL) aliquots of cleaned up extracts did not cause response deterioration, but repeat injections of 10 g/mL cleaned up extracts, especially liver and serum, did effect alkaloid response. Liver extracts (10 g/mL) fortified at 10 ppm were repeatedly injected on the GC/MS. After 10 injections the response of solanidine and retrorsine was reduced to less than 1% of the first injection, and the peak width of atropine was doubled. The response of atropine and strychnine decreased by 20%, while coniine and nicotine response was enhanced by 10% and 15%, respectively. The negative effect of injections of concentrated extracts was reduced by injection of 2 μL of 25% *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in ethyl acetate (v/v). This injection returned the sensitivity of all the alkaloids to at least 75% of the original sensitivity, but the peak width of atropine remained at twice the original width. These severe matrix effects were not as significant on a clean injection liner, nor were they significant after plant or rumen

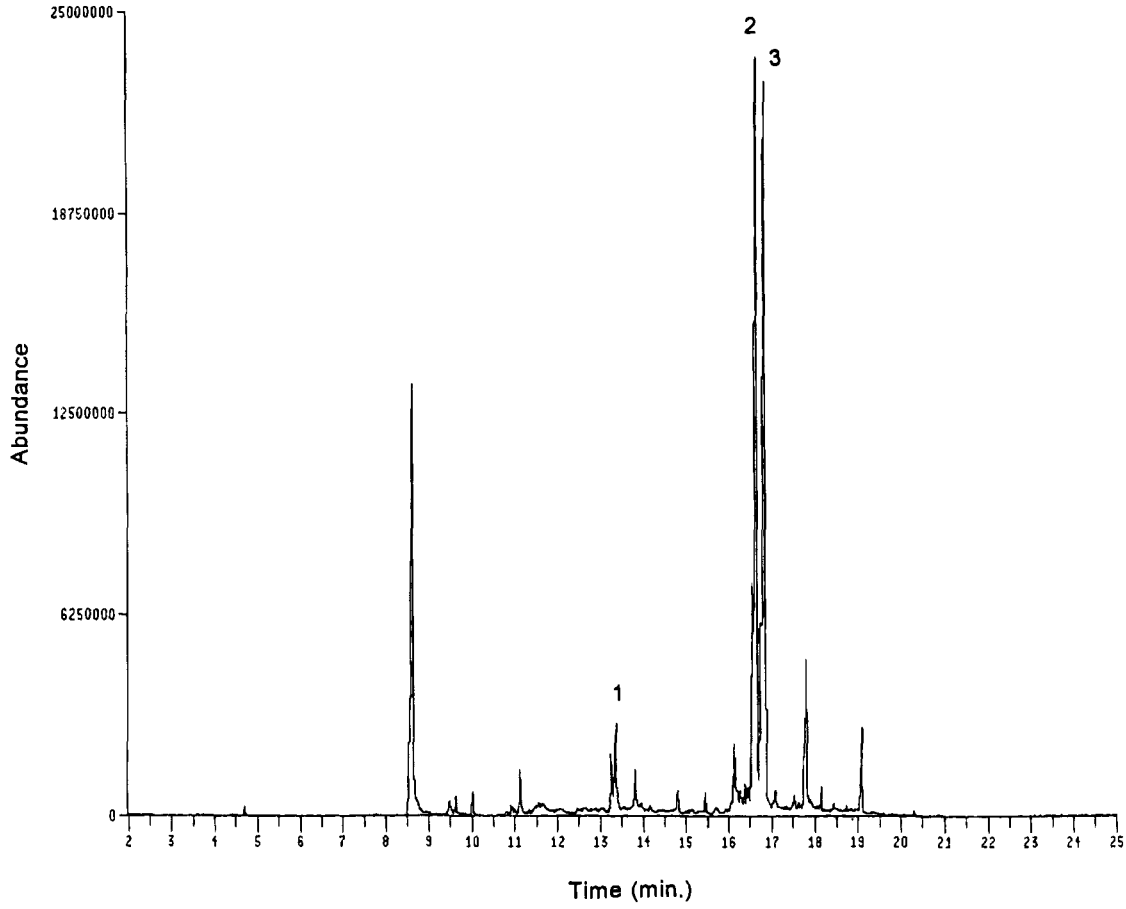

Figure 6. GC/MSD scan chromatograms of a diagnostic rumen content sample positive for lupine alkaloids; 2 μL of 10 g/mL sample extract injected under standard GC conditions; (1) sparteine, (2) lupanine, and (3) anagyrine.

content extract injections. Injections of 25% BSTFA were necessary to maintain adequate response (detection of six primary ions) over more than five liver or serum sample injections at 10 g/mL. Acetylation of the extract with acetic anhydride was unsuccessful in reducing matrix effects.

The GC/MS was used for qualitative results only because of the effect of matrix on response. Alkaloid spectra are shown in Figure 2, and the six main ions and sensitivities on the GC/MS in the scan mode are given in Table 3. Ions were selected based on high abundance and large mass to charge ratio. A requirement of determining detection limits was identification of the compound from the spectral information. These theoretical detection limits are based on 10 times signal to noise detection of all six ions from the MSD scan analysis and injections of 2 μL of 10 g/mL extracts. The detection limit for coniine is high due to the low (2%) relative abundance of the molecular ion and the evaporative losses (as much as 70%) during sample concentration. Despite evaporative losses of coniine, 1 ppm of each alkaloid was detected via GC/MS in the scan mode (Figure 4). These losses could be eliminated using a more sensitive GC/MS, which would probably yield similar detection limits for the alkaloids with injection of the 1 g/mL sample extract. Using the nonpolar HP-1 column, strychnine and solanidine were coeluted. The compounds were identified in extracts using the six principal ions of each (Table 3). By generating ion chromatograms from the scan analysis, the presence of target compounds could be rapidly determined. Figure 5 shows a chromatogram of rumen content control and spike at 1 ppm. This chromatogram is the sum of the ion chromatograms (for most abundant ions, see Table 3) for each compound. The matrix background of the sample was significantly reduced for the target compounds.

Thin Layer Chromatography. The TLC analysis used a modified Toxi-Lab commercial TLC system. This was chosen because it uses a standardized chromatographic system, with reproducible retention factors. The Toxi-Lab system was modified in several ways. To reduce losses of coniine and nicotine, the evaporation was performed at reduced temperature, with the extract spotted directly onto the Toxi-Disc. Of the Toxi-Lab reagents, only Dragendorff's visualizing dip was used, as it is an alkaloid-specific reagent. The use of a second dip in 5% $NaNO_2$ was included to increase visualization of the alkaloids following Dragendorff's reagent (Wagner et al., 1984). The light brown background of the chromatogram was eliminated, and the detectability of solanidine was increased 2-fold, although the detectability of coniine and retrorsine was greatly reduced. The eluting solvent was modified. The Toxi-Lab system of ethyl acetate:methanol:H_2O:NH_4OH (94.5:3.3:1.6:0.6) gave low R_fs and poor separation of the six compounds. For example, the R_f of atropine under standard Toxi-Lab conditions was 0.05. Using 5% diethylamine in ethyl acetate as the developing solvent, the R_f was increased to 0.4. A major difficulty with the TLC analysis was the loss of coniine on evaporation. Sample extracts are evaporated to dryness, redissolved, and then spotted and dried. This led to variable recovery and detectability of coniine. Only 2.5 ppm coniine could be reliably detected after a representative 1 g of sample extract was spotted. The other alkaloids were all detectable at 1 ppm, with theoretical detection as low as 0.25 ppm for some alkaloids (Table 2).

Diagnostic Utility of the MRM. The MRM has utility in the veterinary diagnostic setting. In one diagnostic case, 22 ewes of 800 were found dead near Mono, CA. They had suffered sudden death. Two toxic plants were identified in the immediate area, a *Lupinus* species (lupine) and a *Delphinium* species (larkspur). Neither plant was identifiable in the rumen contents of the animals. The rumen content was tested using the alkaloid MRM. TLC analysis indicated that several alkaloids were present. GC/MS analysis followed by a library search of all peaks identified three lupine alkaloids, lupanine, anagyrine, and sparteine (Figure 6). Thus it was determined that the ewes had consumed the acutely toxic lupine species.

CONCLUSION

This multiresidue alkaloid screen has demonstrated the ability to rapidly extract, cleanup, and analyze six alkaloids, chosen to model the wide variety of alkaloid structures. The MRM worked well in five different matrices, representing different amount of lipid, pigmentation, and moisture content. The method contained the necessary key components of an alkaloid MRM. A concentrated, cleaned up sample extract was produced without losses of volatile or easily hydrolyzed compounds. Polar alkaloids were well extracted without the use of a water miscible solvent. The method was rapid, providing a qualitative TLC result within 4 h, and a GC/MS scan result the same working day. Sample extraction and cleanup provided diagnostically relevant detection limits, with good precision and accuracy using GC/NPD detection.

Good fortification recoveries and coefficients of variation for the alkaloids were obtained in all matrixes, using GC/NPD detection. The procedure is especially suited to veterinary diagnostic laboratory situations where sample size is limited, a large number of difficult samples must be rapidly analyzed, and extremely low detection limits (<1 ppm) in source material are not required. Future work will include testing additional compounds using the MRM, building MS and TLC libraries of alkaloids and plant extracts using this MRM, and animal-dosing trials to evaluate the utility of the MRM at diagnostic levels.

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