Multiresidue Screen for Cardiotoxins by Two-Dimensional Thin-Layer Chromatography

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A two-dimensional thin-layer chromatographic method was developed for the qualitative determination of the cardiotoxins oleandrin, gitoxin, digitoxin, gitoxigenin, and grayanotoxins I, II, and III in gastrointestinal contents (stomach, rumen, colon, and cecum contents), feces, and plant material. The cardiotoxins were extracted with dichloromethane. The extract was cleaned up by charcoal and reverse phase solid-phase extraction columns. Analysis was performed by two-dimensional thin-layer chromatography on silica gel plates and visualized by aluminum chloride followed by chloramine T spray. The method detection limits were 0.05 μg/g for oleandrin, 0.1 μg/g for gitoxin, and 0.2 μg/g for the other toxicants in gastrointestinal contents and feces and were 5 times higher in plant material. Four replicate fortifications of bovine rumen contents, bovine feces, and alfalfa at these levels were all well recovered. The diagnostic utility of the method was tested by analyzing samples submitted to the veterinary toxicology laboratory.

Keywords: *Oleandrin; digitoxin; gitoxin; grayanotoxin; thin-layer chromatography*

INTRODUCTION

Cardiotoxic plants of interest in a veterinary diagnostic laboratory include *Nerium oleander* L. (oleander), *Digitalis purpurea* (foxglove), *Kalmia latifolia* (mountain laurel), and *Rhododendron* spp. (rhododendrons and azaleas). These plants can cause acute toxicity in animals, and the clinical signs produced by them are similar. Animals exposed to these plants often may be presented "suddenly dead" or with very similar signs of gastrointestinal upset, weakness, and, in many cases, abnormalities in cardiac rhythms. To diagnose possible animal exposure to these plants, a test to screen for the active cardiotoxins is desirable. This multiresidue method must be rapid and able to detect cardiotoxins at the nanograms per gram level in plant material, gastrointestinal contents, and feces.

Oleander (*Nerium oleander* L.) is a shrub with red, white, or pink flowers that grows in tropical and subtropical climates and is used in California and the southern regions of the United States primarily as a drought-resistant ornamental (Cheeke, 1998; Kingsbury, 1964). *N. oleander* contains cardiac glycosides, oleandrin being found in the highest concentration (Yamauchi et al., 1983). Fresh oleander leaves have been found to contain 2200 μg/g oleandrin (Tor et al., 1996). Exposure to oleander can cause sudden death, diarrhea, pulmonary edema, cardiac arrhythmias, colic, and lethargy (Galey et al., 1996; Langford and Boor, 1996). Several methods have been used for the analysis of oleandrin at the nanograms per gram level. Oleandrin has been analyzed by two-dimensional thin-layer chromatography (TLC) (Galey et al., 1996), high-performance liquid chromatography (HPLC) (Tor et al., 1996), liquid chromatography/mass spectrometry (LC/MS) (Rule et al., 1993; Tracqui et al., 1998), and cross-reactivity with digitoxin fluorescence polarization immunoassay (Dasgupta and Emerson, 1998; Datta and Dasgupta, 1997).

Foxglove, or *D. purpurea*, is a cardiac glycoside-containing plant found in Europe and in the Pacific Northwest of the United States. Symptoms of exposure are similar to those of oleander (Langford and Boor, 1996; Cheeke, 1998; Kingsbury, 1964). *D. purpurea* contains many cardiac glycosides, including the secondary glycosides digitoxin, gitoxin, and gitaloxin and the more polar primary glycosides purpurea glycoside A, purpurea glycoside B, and glucogitaloxin (Fujii et al., 1990). Digitoxin and gitoxin are present in the dried leaves around the 1000 μg/g level (Fujii et al., 1983). The cardiac glycosides in *N. oleander* and *D. purpurea* inhibit Na^+- and K^+-ATPases, causing elevation of intracellular Ca^{2+} levels (Langford and Boor, 1996). These compounds have been screened at the micrograms per gram level by numerous techniques, such as TLC (Fujii et al., 1990; Matysik, 1994), two-dimensional TLC (Clarke and Cobb, 1979), and HPLC (Weigrebe and Wichtl, 1993; Ikeda et al., 1995). Methods capable of detecting cardiac glycosides at the nanograms per gram level include HPLC with postcolumn derivatization (Belsner and Buchele, 1996; Maekawa and Morimoto, 1992), HPLC with pulsed amperometric detection (Kelly et al., 1995), LC/MS (Tracqui et al., 1997), and HPLC immunoassay (Jortani et al., 1997).

Several members of the Ericaceae (heath) family are cardiotoxic, including rhododendrons and azaleas (*Rhododendron* spp.), laurels (*Kalmia* spp.), black laurel (*Ledum* spp.), and Japanese pieris (*Pieris japonica*) (Cheeke, 1998; Kingsbury, 1964). Intoxication is indicated by bradycardia, hypotension, cardiac arrhythmia, gastrointestinal pain and spasm, and muscular weakness and paralysis (Cheeke, 1998; Onat et al., 1991; Hough,

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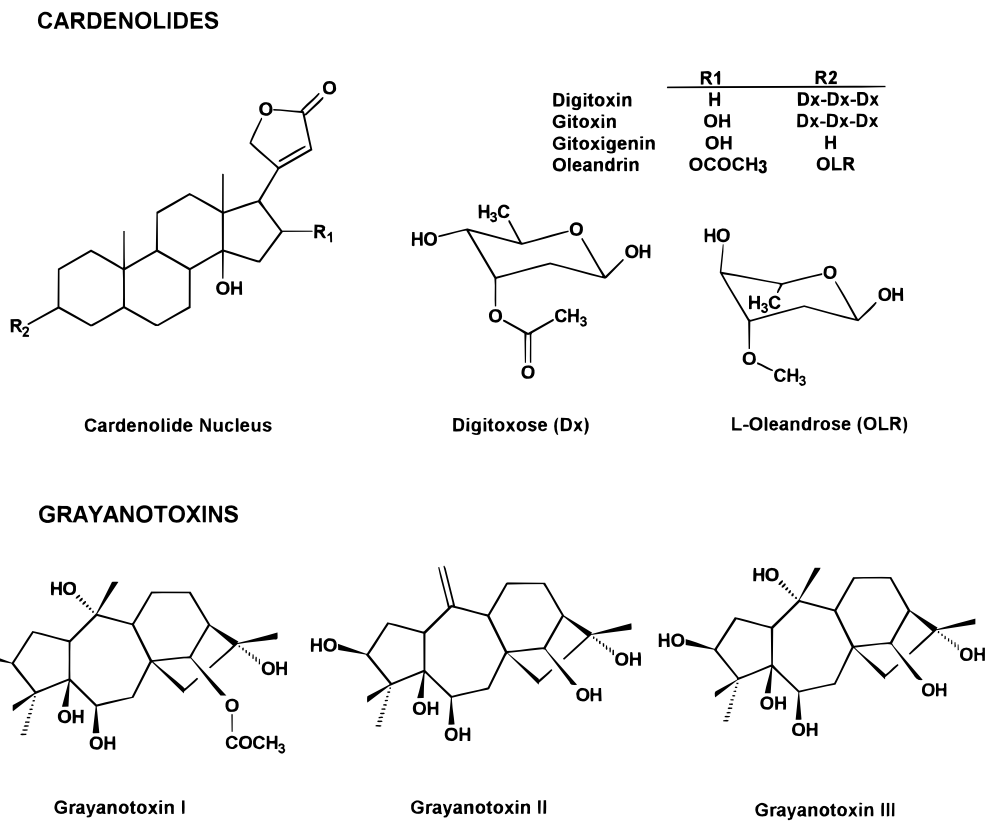

Figure 1. Structures of the seven cardiotoxins studied.

1997). Toxicity is related to the presence of diterpene polyalcohols called grayanotoxins (Hikino et al., 1976; Burke and Doskotch, 1990; Cheeke, 1998; Smith, 1978). Grayanotoxins act on excitable cells by increasing membrane permeability to Na^+, resulting in sustained depolarization (Yakehiro et al., 1997; Zushi et al., 1983). Although numerous grayanotoxins have been found in these species, grayanotoxins I, III, and IV are the most toxic, with the ip LD_{50} in mice ranging from 0.87 to 1.3 mg/kg (Hikino et al., 1976). Grayanotoxins have been analyzed in plant material and honey by direct spectrometric methods (Terai, 1983), by gas chromatography (GC) as the trimethylsilane (TMS) derivative (Terai et al., 1994), and by TLC (Terai, 1984; Kinghorn et al., 1978; Sutlupinar et al., 1993; Kerkvliet, 1981). *Rhododendron maximum* has been reported to contain >50 μg/g of both grayanotoxins I and III (Jawad et al., 1977).

A two-dimensional TLC method for qualitative analysis of oleandrin, digitoxin, gitoxin, gitoxigenin, and grayanotoxins I, II, and III (Figure 1) is described. These compounds were selected due to their high concentration in plant material and their availability. The method was designed for rapid analysis of samples and diagnosis of exposure to cardiotoxic plants and has been applied to samples presented to the veterinary diagnostic laboratory.

MATERIALS AND METHODS

Reagents. All solvents were of Optima grade (Fisher Scientific). Sodium sulfate (Fisher Scientific) was of ACS reagent grade. Aluminum chloride hexahydrate and chloramine T were from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride solution was prepared by dissolving 20 g of aluminum chloride hexahydrate in 50 mL of H_2O and then adding 50 mL of ethanol. Chloramine T solution was prepared by mixing 10 mL of aqueous 0.3% chloramine T (w/v) with 40 mL of 25% ethanolic trichloroacetic acid (v/v).

Plant Material. *N. oleander* L., *P. japonica*, and *D. purpurea* were obtained from W. Roberts, University of California arboretum. *Rhododendron* samples were obtained from M. J. Holstege, Port Angeles, WA, and C. V. Booth, Ashland, OR. *K. latifolia* and *K. augustifolia* were obtained from Dr. M. Barkworth, Intermountain Herbarium, Utah State University, Utah.

Preparation of Standard Solutions. Oleandrin, oleandrigenin, digitoxin, gitoxin, gitoxigenin, and digitoxigenin were obtained from Sigma Chemical Co. Grayanotoxins I, II, and III were kindly provided by Dr. W. H. Tallent, U.S. Department of Agriculture (Beltsville, MD). Stock solutions of 1000 μg/mL were made in methanol. Gitoxin was first dissolved in pyridine prior to dilution to 1000 μg/mL with methanol. Subsequent dilutions of the standards were made in methanol from the stock solutions. Standards at concentrations $\geq$10 μg/mL were stable for 6 months when stored at 5 °C.

Procedure. *(a) Extraction*. Ten grams of well-mixed frozen sample (GI contents, feces) or 2 g (frozen plant material) was weighed into a 250 mL French squared homogenization vessel (Fisher Scientific). Dichloromethane (100 mL) and sodium sulfate (50 g) were added, and the samples were homogenized for 1 min at 9500 rpm using a tissue homogenizer (model Ultra-Turrax T-25, IKA-Labortechnik/Tekmar Co., Cincinnati, OH). The extracts were centrifuged at 500 rpm (65 g) for 5 min using an IEC Centra-7 centrifuge (International Equipment Co.). Aliquots (25 mL) were taken, 2−3 mL of methanol was added, and the extracts were evaporated to dryness using a nitrogen evaporator (N-Evap, Analytical Evaporator, Organomation Associates Inc., Berlin, MA) set at 60 °C. The dried extracts were immediately redissolved in 2 mL of dichloromethane.

(b) Charcoal SPE Cleanup. Charcoal SPE columns, MycoSep (Romer Labs., Inc., Union, MO), were prewashed with 20 mL of dichloromethane. Samples were loaded onto the column in 2 mL of dichloromethane, and the column was washed with 20 mL of dichloromethane. The wash was discarded. The

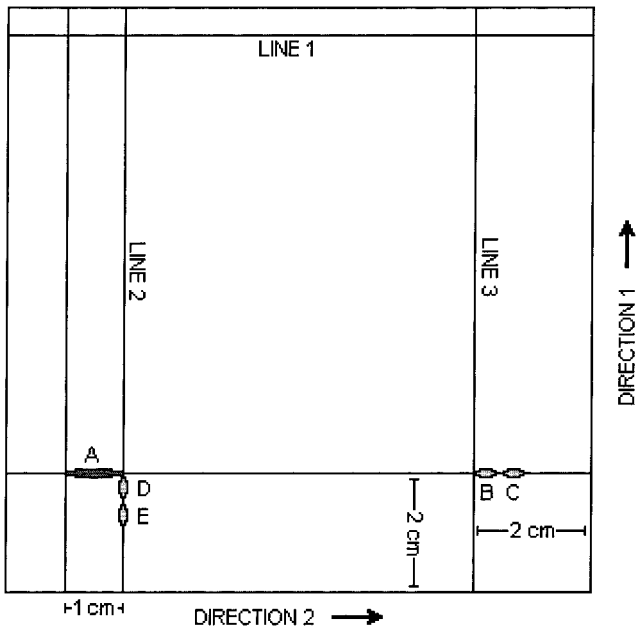

Figure 2. Two-dimensional TLC plate setup. Sample extract is spotted in spot A. Standards are spotted in spots B and C for development in direction 1 and in spots D and E for development in direction 2.

cardiotoxins were eluted from the column with 20 mL of 10% methanol in dichloromethane (v/v). The cleaned up extract was evaporated to dryness using a nitrogen evaporator set at 60 °C, and 0.5 mL of 20% H_2O in methanol (v/v) was added.

(c) C-18 SPE Cleanup. C-18 SPE columns, Oasis HLB 3 cm^3 60 mg extraction cartridges (Waters Corporation, Milford MA), were prewashed with 3 mL of methanol followed by 3 mL of 20% H_2O in methanol. Samples were loaded onto the column in 0.5 mL of 20% H_2O in methanol. The cardiotoxins were eluted from the column with 2.5 mL of 20% H_2O in methanol, which was combined with the loading volume of 0.5 mL. The cleaned up extract was evaporated to dryness using a nitrogen evaporator set at 60 °C, and 50 μL of methanol was added.

(d) TLC Analysis. Twenty microliters of sample, or 1 g equivalent (0.2 g of alfalfa), was spotted in a 1 cm wide band (Figure 2, spot A) in one corner of a 10 × 10 cm silica gel TLC plate (Uniplate silica gel HL, 250 μm, Analtech Inc., Newark, DE). Oleandrin, grayanotoxin I, and grayanotoxin III were spotted in spot B. Digitoxin, grayanotoxin II, gitoxin, and gitoxigenin were spotted in spot C. Table 1 describes spotting volumes and concentrations of standards used. The TLC plate was developed up to line 1 in direction 1 (Figure 2) first with 25% dichloromethane in ethyl acetate (v/v) and, after drying on a heater (Toxi-Lab Division of Marion Laboratories, Laguna Hills, CA), with ethyl acetate/toluene/acetic acid (6:3:1, v/v). After drying, grayanotoxins I, II, and III and oleandrin were spotted in spot D, and digitoxin, gitoxin, and gitoxigenin were spotted in spot E. The plate was developed in direction 2 up to line 2 with methanol, dried, and developed up to line 3 with dichloromethane/acetonitrile/ethanol/ammonium hydroxide (90:20:10:1). The dried plate was sprayed with 20% aluminum chloride solution and heated for 5 min at 110 °C. After the spots were recorded, the plate was sprayed with chloramine T solution and heated for 5 min at 110 °C. Table 1 describes the colors of spots visualized by these sprays.

Method Validation. The method was validated by analyzing four replicates each of negative control bovine rumen content, bovine feces, and alfalfa samples fortified at the method detection limits (Tables 2−4) with the cardiotoxins. The method was also tested by analyzing samples from diagnostic cases.

RESULTS AND DISCUSSION

The method qualitatively extracted and recovered all of the cardiotoxins studied. Despite the greatly different polarities of the analytes, the compounds were well extracted, cleaned up, and analyzed by TLC with one analysis.

The results of the recovery study are presented in Tables 2−4. Slight differences were observed between the migration of the standards and the fortification spots on the plates. Differences in migration distance were typically greatest with oleandrin, with the R_f being off by almost 4 mm in the feces fortifications. Changes in R_f were highly matrix dependent. Method detection limits (MDLs) were based on the amount of sample extract that could be spotted without causing unacceptable changes in R_f. Gitoxigenin was included primarily as a possible internal standard to correct for these small R_f changes using relative R_f values, but this technique did not significantly reduce variability. Migration distances from plate to plate were consistent, with percent coefficients of variation (%CVs) of <13%.

The compounds were extracted with dichloromethane, which had previously been shown to quantitatively extract oleandrin (Tor et al., 1996). Losses of grayano-

Table 1. Volume of 100 μg/mL Standards Spotted for Each Cardiotoxin, Typical Cardiotoxin HR_f in Both Directions, and Colors Visualized Following the Aluminum Chloride and Chloramine T Sprays

compound	vol spotted (μL)	HR_f direction 1	HR_f direction 2	color after aluminum chloride spray	color after chloramine T spray
oleandrin	2	0.55	0.80	blue-purple	white-blue
grayanotoxin I	4	0.25	0.48	orange	white-orange
grayanotoxin II	4	0.25	0.43	orange	white-orange
grayanotoxin III	4	0.13	0.22	orange	white-orange
digitoxin	4	0.13	0.60	not observed	yellow
gitoxin	2	0.05	0.45	blue-purple	white-green
gitoxigenin	2	0.30	0.65	blue-purple	white-green

Table 2. Summary of Validation Study Results in Alfalfa, Including Fortification Level at the MDL Average HR_f, and %CV for the Fortification and Standard Spots (n = 4) in Both Directions

	MDL (μg/g)	direction 1		direction 2	
		standard HR_f (%CV)	spike HR_f (%CV)	standard HR_f (%CV)	spike HR_f (%CV)
oleandrin	0.25	0.61 (2.7)	0.58 (2.2)	0.79 (4.8)	0.78 (3.2)
grayanotoxin I	1	0.28 (4.2)	0.23 (5.2)	0.46 (10.6)	0.42 (10.6)
grayanotoxin II	1	0.27 (4.0)	0.22 (6.6)	0.43 (10.5)	0.39 (9.0)
grayanotoxin III	1	0.16 (4.1)	0.14 (3.9)	0.23 (12.6)	0.21 (9.3)
digitoxin	1	0.16 (5.3)	0.12 (6.2)	0.58 (6.8)	0.54 (9.6)
gitoxin	0.5	0.07 (4.6)	0.07 (9.6)	0.48 (12.5)	0.46 (11.9)
gitoxigenin	0.5	0.34 (5.2)	0.30 (2.9)	0.65 (6.8)	0.58 (9.6)

Table 3. Summary of Validation Study Results in Feces, Including Fortification Level at the MDL, Average HR_f, and %CV for the Fortification and Standard Spots (n = 4) in Both Directions

	MDL	direction 1		direction 2	
	(μg/g)	standard HR_f (%CV)	spike HR_f (%CV)	standard HR_f (%CV)	spike HR_f (%CV)
oleandrin	0.05	0.56 (2.0)	0.55 (2.5)	0.84 (2.7)	0.78 (5.4)
grayanotoxin I	0.2	0.24 (2.6)	0.23 (7.7)	0.50 (5.7)	0.47 (6.3)
grayanotoxin II	0.2	0.24 (4.8)	0.24 (3.5)	0.40 (4.9)	0.38 (6.2)
grayanotoxin III	0.2	0.14 (3.9)	0.12 (7.0)	0.21 (7.6)	0.19 (10.2)
digitoxin	0.2	0.13 (3.0)	0.13 (5.1)	0.51 (4.5)	0.50 (5.7)
gitoxin	0.1	0.06 (5.7)	0.06 (5.7)	0.39 (6.2)	0.38 (5.2)
gitoxigenin	0.1	0.31 (3.3)	0.30 (2.1)	0.63 (4.4)	0.60 (6.5)

Table 4. Summary of Validation Study Results in Rumen Content, Including Fortification Level at the MDL, Average HR_f, and %CV for the Fortification and Standard Spots (n = 4) in Both Directions

	MDL	direction 1		direction 2	
	(μg/g)	standard HR_f (%CV)	spike HR_f (%CV)	standard HR_f (%CV)	spike HR_f (%CV)
oleandrin	0.05	0.57 (2.8)	0.55 (1.7)	0.78 (2.9)	0.73 (1.6)
grayanotoxin I	0.2	0.24 (1.5)	0.21 (5.9)	0.41 (5.3)	0.40 (1.7)
grayanotoxin II	0.2	0.26 (5.6)	0.22 (5.9)	0.34 (1.4)	0.32 (3.4)
grayanotoxin III	0.2	0.13 (3.1)	0.10 (6.7)	0.16 (6.1)	0.16 (8.0)
digitoxin	0.2	0.13 (13.3)	0.11 (7.2)	0.47 (5.1)	0.45 (3.7)
gitoxin	0.1	0.05 (7.7)	0.05 (8.9)	0.35 (5.3)	0.34 (5.8)
gitoxigenin	0.1	0.31 (2.3)	0.30 (2.4)	0.56 (3.5)	0.54 (0.9)

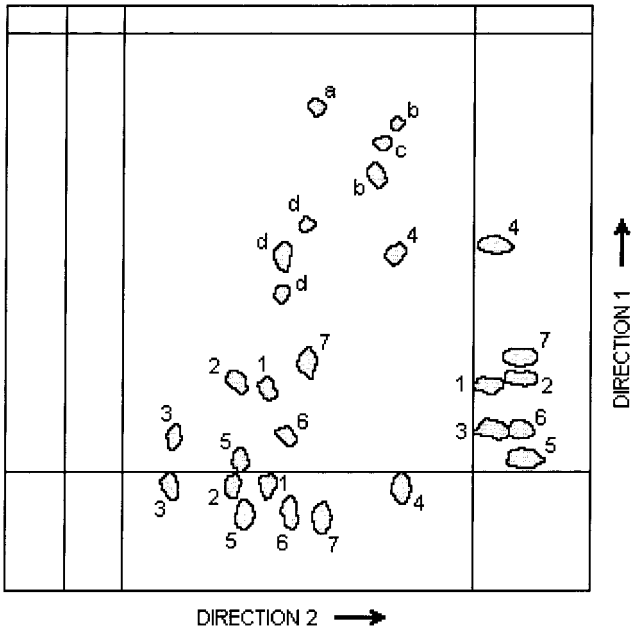

Figure 3. Touched-up digitalized scan of a two-dimensional TLC plate representing 1 g of bovine rumen content fortified with 0.2 μg/g grayanotoxin I (1), 0.2 μg/g grayanotoxin II (2), 0.2 μg/g grayanotoxin III (3), 0.05 μg/g oleandrin (4), 0.1 μg/g gitoxin (5), 0.2 μg/g digitoxin (6), and 0.1 μg/g gitoxigenin (7). Matrix spots are (a) no color after spray 1/white after spray 2, (b) none/orange, (c) none/yellow, and (d) yellow/yellow.

toxins during the subsequent evaporation step were minimized with the addition of methanol to the extract prior to concentration. The charcoal cleanup was effective in removing lipids, pigments, and polar matrix components. The C-18 SPE cleanup was necessary to remove a waxy substance that interfered with the migration of the spots in direction 1. The resulting extract was sufficiently clean to allow for spotting of up to 1 g of feces and rumen content extract and up to 0.2 g of alfalfa extract.

The TLC analysis gave good two-dimensional resolution of all seven cardiotoxins and the matrix compounds remaining after the cleanup steps (Figure 3). Numerous reagents have been used to visualize cardenolides and grayanotoxins. These include aluminum chloride (Galey et al., 1996), 60% H_2SO_4, Godin's reagent, vanillin oversprayed with ethanolic perchloric acid (Kinghorn, 1978), phosphotungstic acid in ethanol (Terai, 1984), phosphoric acid (Clarke and Cobb, 1979), and chloramine T (Matysik, 1994). Aluminum chloride gave good sensitivity for the grayanotoxins and the cardiotoxins, with the exception of digitoxin, and typically resulted in a plate with few matrix spots. A second spray was necessary to visualize digitoxin, but this caused some additional migration and variation in R_f of the digitoxin spot. Chloramine T provided the best sensitivity and lowest background following the aluminum chloride spray, gave the least additional migration of digitoxin, and yielded a plate on which the spots could be easily recorded. The acidic charring sprays tended to weaken the organic binder of the plate, making recording of the spots difficult.

The plants *N. oleander* L., *D. purpurea*, *R. percii*, *K. latifolia*, *K. augustifolia*, and *P. japonica* were analyzed. The samples were all positive for cardiotoxins. When an extract representing 0.1 g of *N. oleander* was spotted, the oleandrin gave a yellow color after the aluminum chloride spray. Spotting less sample resulted in the typical blue-purple color for oleandrin.

The method was applied to cases presented to the California Veterinary Diagnostic Laboratory System. In one diagnostic case, six goats were hand-fed a branch that was later identified as an azalea. Three of the goats had depression, bloat, and profuse vomiting. Two-dimensional TLC analysis of the feces from the three most affected goats identified grayanotoxin I in all samples and grayanotoxin III in one sample. In another case, a horse was presented to the veterinary hospital with colic, but died during surgery. Necropsy showed coronary artery hemorrhage and cardiac necrosis. Analysis of stomach and colon contents found oleandrin in both samples, with a higher concentration in the stomach content. In a third case, a llama was presented to the hospital with a clinical history of depression, decreased appetite, and gastrointestinal motility. The llama also developed cardiac arrhythmias, was anuric, and had not defecated for 60 h. Foxglove clippings had been found in the paddock. Both gitoxin and digitoxin were identified following analysis of rectal samples.

CONCLUSIONS

This multiresidue cardiotoxin screen has been demonstrated to rapidly extract, clean up, and analyze seven cardiotoxins in alfalfa, bovine feces, and bovine rumen content. The sensitivity of the method enables it to be used as a diagnostic tool in cases of exposure to cardiotoxic plants. The method is rapid, providing a qualitative TLC result within 4 h. The procedure is especially suited to veterinary diagnostic laboratory situations for which rapid diagnosis of exposure to cardiotoxin plants is necessary. Future work will include expanding the screen to include more compounds.

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