JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Pyrrolizidine Alkaloids in *Crotalaria* Taxa from Northern Australia: Risk to Grazing Livestock

MARY T. FLETCHER,* ROSS A. MCKENZIE, BARRY J. BLANEY, AND KEITH G. REICHMANN

Department of Primary Industries and Fisheries Queensland, Animal Research Institute, Locked Mail Bag No. 4, Moorooka, Queensland 4105, Australia

Crotalaria species containing hepatotoxic pyrrolizidine alkaloids grow widely in pastures in northern Australia and have sporadically poisoned grazing livestock. The diverse *Crotalaria* taxa present in these pastures include varieties, subspecies, and chemotypes not previously chemically examined. This paper reports the pyrrolizidine alkaloid composition and content of 24 *Crotalaria* taxa from this region and assesses the risk of poisoning in livestock consuming them. Alkaloids present in *C. goreensis*, *C. aridicola* subsp. *densifolia*, and *C. medicaginea* var. *neglecta* lack the esterified 1,2-unsaturated functionality required for pyrrole adduct formation, and these taxa are not hepatotoxic. Taxa with high levels of hepatotoxic alkaloids, abundance, and biomass pose the greatest risk to livestock health, particularly *C. novae-hollandiae* subsp. *novae-hollandiae*, *C. ramosissima*, *C. retusa* var. *retusa*, and *C. crispata*. Other species containing moderate alkaloid levels, *C. spectabilis* and *C. mitchellii*, also pose significant risk when locally abundant.

KEYWORDS: Gas chromatography−mass spectrometry, GC-MS; pyrrolizidine alkaloids; Crotalaria

INTRODUCTION

There are about 600 species of *Crotalaria* L. (family Fabaceae) (rattlepods) in the tropical and subtropical regions of the world, 36 of which are native or naturalized in Australia (*1*). Many are common plants in the pastoral regions of northern Australia, where several species are known as sources of hepatotoxic pyrrolizidine alkaloids that sporadically poison grazing horses, cattle and sheep or pigs and poultry fed grain contaminated with their seeds (*2, 3*). Pyrrolizidine alkaloidosis has been attributed to liver transformation of pyrrolizidine alkaloids into reactive alkylating agents and requires the presence of a 1,2-double bond in the pyrrolizidine ring and the esterification of hydroxyl groups at C9 and/or C7 (*4*). The pyrrolizidine alkaloids are metabolized soon after absorption from the alimentary tract, but pyrrolic metabolites form adducts with macromolecules in the liver and sometimes other tissues, persisting as long-lasting residues (*5*). Pyrrolizidine alkaloids are regarded as potential carcinogens (*6*), but the toxicological significance of pyrrolic adducts in food animal tissues is unknown. Nevertheless, the presence of residues of these alkaloids or their metabolites in animal tissues is of interest in the context of human health and trade in animal products and is the subject of research in Australia and elsewhere (*4*). This study was undertaken to identify and quantitate pyrrolizidine alkaloids from 24 *Crotalaria* taxa in northern Australia and assess the potential toxicity of these taxa to cattle. This work was part of the hazard identification phase of risk assessment studies of natural toxins from northern Australia with potential to impact directly on livestock production and potentially on food safety and trade in animal products.

* Author to whom correspondence should be addressed (telephone 61 7 3362 9426; fax 61 7 3362 9429; e-mail mary.fletcher@dpi.qld.gov.au).

MATERIALS AND METHODS

Plant Material. A number of major field trips traversing all of the northern Australian grazing regions were undertaken at various times from 2002 to 2005 to locate and collect *Crotalaria* taxa. When possible, *Crotalaria* collections for analysis were of new growth and flowering tops (those parts of plants considered most likely to be eaten by grazing livestock) and were a composite of 10 or more plants collected at a single site. The nature of collection sites and GPS coordinates were recorded. Samples (200 g−1 kg) were placed in paper bags and kept in a mesh container on the roof of the vehicle, where they dried within 24−72 h. A separate sample was pressed between absorbent paper in a plant press for botanical identification. Plant identifications made in the field were confirmed by the Queensland Herbarium, and botanical specimens from all batches collected were incorporated into their permanent collection as vouchers against any future taxonomic changes. Field-collected plant material was transported to the laboratory, air-drying was completed if necessary, and material was milled and stored frozen prior to analysis.

Geographical regions across Queensland, Northern Territory, and Western Australia for our collections were derived from accepted Botanical Districts (*7*) shown in italics. Southeastern Queensland (SEQ = *Moreton*, *Burnett*, and *Wide Bay*); Southern Queensland (SQ = *Darling Downs* and *Maranoa*); Central Queensland (CQ = *Port Curtis*, *Leichhardt*, and *South Kennedy*); Southwestern Queensland (SWQ = *Mitchell*, *Warrego*, *Gregory North*, and *Gregory South*); Northeastern Queensland (NEQ = *Cook* and *North Kennedy*); Northwestern Queen-

10.1021/jf8026099 Published 2009 by the American Chemical Society
Published on Web 12/05/2008

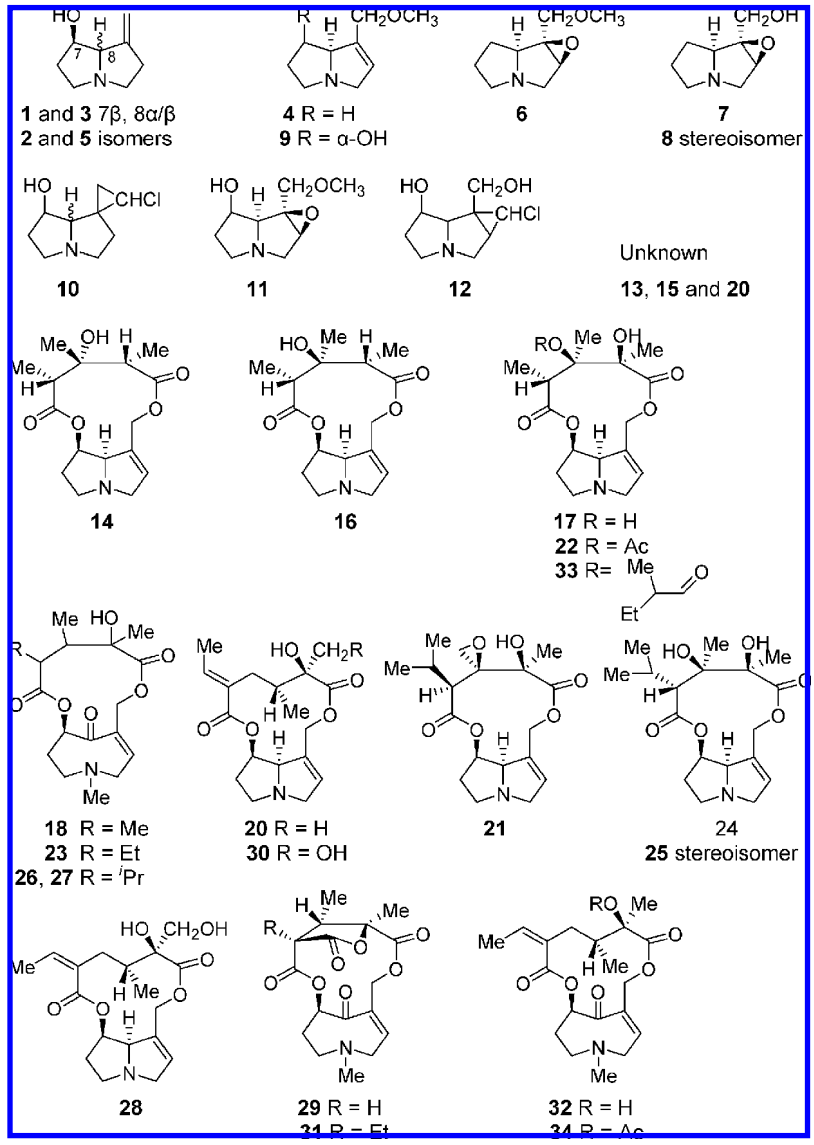

Figure 1. Structures of pyrrolizidine alkaloids detected and characterized in *Crotalaria* taxa, with the internal standard retrorsine (**28**).

sland (NWQ = *Burke*); Darwin and Gulf (DG = *Darwin* and *Gulf*); Victoria River (VR = *Victoria River*); Barkly Tableland (BT = *Barkly Tablelands*); Central Northern Territory (CNT = *Central Australia North* and *Central Australia South*); Kimberley (KIM = *Gardner*, *Fitzgerald*, *Dampier*, and *Hall*).

Alkaloid Extraction. Dried and milled plant material (2 g) was extracted with methanol in an ultrasonic bath (2 × 50 mL) for 15 min. After evaporation of the solvent, the residue was suspended in 2.5% hydrochloric acid (20 mL) and washed with ether (20 mL) and dichloromethane (20 mL). The aqueous layer was divided into two equal portions. The first portion (A) was made alkaline with ammonia solution (28%) and extracted with dichloromethane (2 × 20 mL). This portion provided free pyrrolizidine alkaloids for gas chromatography−mass spectrometry (GC-MS) analysis. The second portion (B) was reduced by shaking overnight with zinc dust (200 mg) and then made alkaline with 28% ammonia solution and extracted with dichloromethane (2 × 20 mL). In this portion, *N*-oxides were reduced to their respective free base forms **1**−**34** (**Figure 1**), and total alkaloids were measured by GC-MS analysis.

GC-MS Analysis. The dichloromethane extracts were concentrated to 10 mL and analyzed by GC-MS, with retrorsine (**28**) obtained from Sigma (Sydney, NSW, Australia) as a co-injected internal standard for quantifications. GC-MS analysis was carried out on a Shimadzu GC-17A and QP5050 MS instrument equipped with a 30 m × 0.25 mm i.d. DB-5ms column (J&W Agilent Technologies, Melbourne, VIC, Australia). Analysis conditions were as follows: injector, 300 °C; temperature program from 50 °C (2 min), raised at 20 °C/min to 300 °C; carrier gas, He; column flow, 1.5 mL/min; interface, 300 °C; and electron impact ionization at 70 eV. Chemical ionization mass spectra were collected using methane as the reagent gas to confirm molecular ions.

Retention Indices (RI). Retention indices (*8*) were calculated with respect to a set of standard hydrocarbons (C_{10}−C_{28}) and compared with literature values (*9*, *10*).

Alkaloid Identification by GC-MS. Each component was subjected to a library search by comparison with reference RI and mass spectra stored in our laboratory database or obtained from the literature. When literature mass spectrometric data were not available, MS interpretation was used to identify compounds, supported by previous alkaloid identifications in the relevant species. Molecular ions were confirmed by positive chemical ionization mass spectra when necessary. Monocrotaline (**17**) was obtained from Sigma.

Alkaloid Quantitation by GC-MS. Pyrrolizidine alkaloid content was measured in terms of retrorsine equivalents based on integrated total ion chromatogram (TIC) peak areas compared to peak area of retrorsine (**28**) added as an internal standard.

RESULTS AND DISCUSSION

Alkaloid Identification and Quantitation. A total of 33 different alkaloids (**Figure 1**) were identified by GC-MS across the 24 *Crotalaria* taxa studied and are ranked in RI order (**Table 1**) with taxa composition as detailed in **Table 2**. Many of these

Table 1. Mass Spectrometric Data for Pyrrolizidine Alkaloids in *Crotalaria* Taxa in Retention Index (RI) Order Compared with the Internal Standard Retrorsine (**28**)

alkaloid	RI	(M^+)	mass spectrometric data
7β-hydroxy-1-methylene-8α/β-pyrrolizidine (**1**)	1170	139	139 (6), 120 (1), 108 (1), 96 (8), 95 (100), 94 (26), 82 (4), 81 (2), 80 (8), 68 (5), 67 (17), 65 (2), 56 (2), 55 (6), 54 (6), 53 (6), 52 (2), 51 (2), 43 (2)
isomer of **1** and **3** (**2**)	1179	139	139 (6), 120 (4), 96 (5), 95 (100), 94 (18), 82 (8), 80 (6), 68 (13), 67 (11), 57 (9), 56 (22), 55 (13)
7β-hydroxy-1-methylene-8α/β-pyrrolizidine (**3**)	1227	139	139 (3), 120 (2), 96 (8), 95 (100), 94 (26), 82 (3), 81 (2), 80 (8), 68 (5), 67 (16), 66 (2), 65 (2), 55 (8), 54 (5), 53 (7), 52 (2), 51 (2), 44 (2), 43 (3)
1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**4**)	1247	153	153 (19), 152 (10), 124 (7), 123 (34), 122 (100), 121 (8), 120 (46), 110 (10), 108 (23), 94 (20), 93 (23), 81 (9), 80 (81), 68 (10), 67 (13), 55 (8), 53 (21), 52 (11), 45 (41)
isomer of **1** and **3** (**5**)	1293	139	139 (2), 120 (4), 96 (8), 95 (100), 94 (29), 82 (4), 81 (3), 80 (10), 68 (8), 67 (17), 66 (3), 65 (2), 55 (6), 54 (7), 53 (6), 52 (3), 51 (2), 44 (3), 43 (2)
1β,2β-epoxy-1α-methoxymethyl-8α-pyrrolizidine (**6**)	1313	169	169 (10), 138 (2), 124 (13), 110 (2), 108 (3), 96 (8), 80 (4), 71 (6), 70 (100), 69 (3), 68 (5), 67 (3), 55 (34), 54 (6), 53 (4), 45 (15), 43 (5)
1β,2β-epoxy-1α-hydroxymethyl-8α-pyrrolizidine (**7**)	1342	155	155 (21), 126 (5), 124 (14), 110 (2), 108 (4), 106 (3), 96 (15), 80 (6), 71 (8), 70 (100), 68 (10), 67 (4), 56 (4), 55 (55), 54 (10), 53 (5), 43 (10)
1β,2β-epoxy-1α-hydroxymethyl-8α-pyrrolizidine (isomer) (**8**)	1360	155	155 (13), 126 (2), 124 (11), 106 (3), 96 (11), 80 (4), 71 (3), 70 (100), 68 (7), 56 (3), 55 (36), 54 (7), 53 (4), 43 (3)
7α-hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**9**)	1422	169	169 (7), 139 (9), 138 (19), 137 (13), 125 (33), 110 (7), 95 (18), 94 (44), 93 (36), 82 (9), 81 (8), 80 (100), 68 (8), 67 (12), 53 (20), 45 (20)
chlorocarbene artifact of **1**or **3** (tentative) (**10**)	1426	187/189	187 (0.2), 138 (2), 96 (11), 95 (100), 94 (22), 80 (5), 68 (4), 67 (15), 66 (2), 65 (2), 57 (2), 55 (6), 54 (4), 53 (6), 52 (2), 51 (2), 49 (2)
1β,2β-epoxy-7-hydroxy-1α-methoxymethyl-8α-pyrrolizidine (tentative) (**11**)	1460	185	185 (23), 154 (58), 140 (37), 112 (20), 111 (100), 110 (62), 96 (42), 86 (76), 82 (34), 81 (22), 80 (33), 68 (40), 57 (22), 56 (23), 55 (88), 54 (26), 53 (30), 45 (82), 43 (30)
chlorocarbene artifact of **9** (tentative) (**12**)	1637	217/219	217 (0.2), 126 (5), 125 (67), 110 (6), 95 (18), 94 (30), 93 (41), 82 (7), 81 (8), 80 (100), 67 (11), 65 (6), 58 (6), 53 (13), 45 (28)
C. verrucosa unknown (**13**)	2130	283	283 (0.1), 268 (0.4), 239 (1), 224 (2), 155 (21), 139 (8), 138 (54), 137 (37), 136 (19), 95 (7), 94 (55), 93 (100), 80 (22), 69 (7), 67 (10), 53 (11), 45 (13), 43 (10)
crispatine (**14**)	2231	309	309 (0.9), 264 (0.4), 222 (4), 138 (16), 137 (19), 136 (75), 121 (26), 120 (75), 119 (100), 118 (14), 108 (17), 106 (15), 95 (48), 94 (45), 93 (70), 80 (30), 67 (11), 53 (17), 43 (46)
C. montana unknown 1 (**15**)	2283	307	307 (4), 262 (1), 232 (2), 137 (11), 136 (46), 122 (10), 121 (12), 120 (64), 119 (100), 118 (17), 108 (12), 106 (14), 95 (20), 94 (48), 93 (82), 80 (30), 67 (15), 53 (22)
fulvine (**16**)	2306	309	309 (3), 237 (4), 236 (22), 193 (4), 138 (17), 137 (21), 136 (92), 123 (10), 121 (15), 120 (86), 119 (100), 108 (19), 106 (19), 95 (17), 94 (50), 93 (88), 80 (35), 53 (21), 43 (43)
monocrotaline (**17**)	2388	325	325 (0.4), 254 (2), 237 (10), 236 (34), 138 (10), 137 (15), 136 (68), 121 (26), 120 (100), 119 (91), 118 (16), 108 (19), 106 (15), 95 (19), 94 (47), 93 (73), 80 (36), 53 (20), 43 (58)
croaegyptine (**18**)	2424	339	339 (0.4), 223 (9), 150 (19), 127 (22), 123 (24), 122 (29), 110 (37), 96 (28), 94 (21), 83 (19), 82 (28), 81 (24), 70 (31), 58 (62), 57 (31), 55 (38), 53 (48), 44 (58), 43 (100)
C. montana unknown 2 (**19**)	2438	323	323 (5), 250 (3), 206 (15), 138 (21), 137 (23), 136 (94), 121 (17), 120 (54), 119 (100), 118 (14), 108 (13), 95 (31), 94 (54), 93 (93), 80 (28), 69 (15), 53 (14), 45 (19), 43 (16)
integerrimine (**20**)	2451	335	335 (2), 291 (4), 248 (5), 220 (12), 138 (36), 136 (80), 121 (59), 120 (88), 119 (85), 118 (18), 109 (22), 108 (19), 106 (19), 95 (65), 94 (70), 93 (100), 80 (35), 53 (29), 43 (54)
pumiline A (tentative) (**21**)	2473	351	351 (3), 264 (7), 236 (7), 138 (19), 137 (18), 136 (78), 122 (25), 121 (27), 120 (96), 119 (100), 118 (18), 108 (15), 95 (30), 94 (56), 93 (75), 80 (32), 67 (13), 53 (15), 43 (49)
spectabiline (**22**)	2485	367	367 (2), 324 (2), 280 (6), 237 (9), 236 (25), 137 (13), 136 (66), 121 (11), 120 (78), 119 (100), 118 (18), 108 (10), 106 (10), 94 (39), 93 (61), 80 (25), 67 (12), 53 (15), 43 (78)
crosemperine ethyl analogue (tentative) (**23**)	2487	353	353 (1), 324 (1), 282 (2), 254 (2), 238 (5), 237 (9), 222 (9), 168 (18), 123 (25), 122 (25), 111 (26), 110 (35), 96 (30), 94 (21), 82 (32), 81 (25), 70 (32), 69 (22), 58 (55), 57 (29), 55 (37), 53 (46), 44 (53), 43 (100)
trichodesmine (**24**)	2510	353	353 (0.3), 282 (4), 265 (14), 264 (44), 222 (11), 138 (18), 137 (32), 136 (63), 121 (30), 120 (99), 119 (81), 118 (20), 95 (37), 94 (51), 93 (59), 83 (19), 80 (35), 69 (18), 43 (100)
trichodesmine isomer (tentative) (**25**)	2517	353	353 (0.8), 282 (4), 265 (9), 264 (39), 222 (9), 138 (16), 137 (20), 136 (53), 121 (23), 120 (100), 119 (31), 95 (10), 94 (29), 93 (48), 80 (23), 69 (10), 67 (13), 43 (61)
crosemperine (**26**)	2538	367	367 (0.7), 352 (1), 324 (4), 251 (7), 236 (29), 168 (26), 123 (24), 122 (22), 111 (32), 110 (33), 96 (27), 82 (32), 81 (22), 70 (30), 69 (58), 58 (42), 57 (29), 55 (33), 53 (42), 44 (45), 43 (100)
crosemperine stereoisomer (tentative) (**27**)	2615	367	367 (1), 352 (2), 338 (3), 251 (35), 236 (34), 167 (27), 123 (21), 122 (25), 111 (22), 110 (29), 96 (24), 82 (29), 81 (23), 70 (36), 69 (42), 58 (66), 57 (25), 55 (52), 53 (43), 44 (45), 43 (100)
retrorsine (**28**)	2633	351	351 (3), 246 (9), 220 (19), 218 (5), 138 (36), 137 (20), 136 (100), 121 (48), 120 (96), 119 (94), 118 (23), 109 (20), 95 (48), 94 (83), 93 (96), 80 (43), 67 (20), 54 (21), 53 (36)
de-ethylretusamine (tentative) (**29**)	2669	351	351 (2), 236 (39), 208 (5), 168 (27), 139 (15), 122 (31), 110 (46), 107 (20), 94 (32), 81 (20), 70 (28), 69 (100), 67 (25), 58 (25), 57 (44), 55 (23), 53 (63), 44 (48), 43 (54)
usaramine (**30**)	2695	351	351 (2), 307 (2), 246 (3), 248 (3), 220 (7), 138 (35), 137 (18), 136 (85), 121 (60), 120 (95), 119 (98), 118 (17), 106 (19), 95 (68), 94 (72), 93 (100), 80 (38), 67 (18), 54 (19), 53 (32)
retusamine (**31**)	2709	379	379 (5), 320 (7), 264 (32), 150 (16), 122 (26), 110 (48), 107 (21), 97 (100), 96 (24), 94 (22), 81 (20), 70 (23), 69 (32), 58 (22), 57 (26), 55 (25), 53 (48), 44 (47), 43 (60)
O-crotaverrine (**32**)	2710	365	365 (0.4), 337(1), 294 (5), 266 (11), 250 (10), 168 (29), 153 (39), 151 (34), 123 (40), 122 (42), 110 (59), 96 (35), 94 (30), 83 (31), 82 (55), 81 (51), 70 (46), 58 (51), 57 (35), 55 (51), 54 (32), 53 (76), 44 (69), 43 (100)
grahamine (**33**)	2711	409	409 (0.3), 324 (3), 280 (7), 237 (8), 236 (31), 194 (2), 157 (4), 137 (13), 136 (50), 121 (12), 120 (77), 119 (100), 118 (14), 94 (33), 93 (54), 80 (18), 57 (64), 44 (17), 43 (48)
O-acetylcrotaverrine (**34**)	2830	407	407 (1), 364 (2), 347 (17), 266 (10), 166 (26), 153 (58), 136 (26), 122 (65), 110 (74), 109 (28), 108 (27), 107 (49), 100 (33), 96 (35), 94 (37), 83 (65), 82 (55), 81 (58), 70 (38), 58 (54), 57 (32), 55 (66), 54 (31), 53 (77), 44 (69), 43 (100)

Table 2. Pyrrolizidine Alkaloid Content and Composition of *Crotalaria* Taxa

Crotalaria taxon[a]	pyrrolizidine alkaloid range[b]	mean alkaloid content[b]	type: pyrrolizidine alkaloids present [c,d]	Queensland Herbarium voucher no. (region)[e,f]
C. alata Buch.-Ham. ex D.Don	0−0.06	0.03	RC: **17**, **16**	AQ734565 (KIM), AQ734568 (KIM), AQ734574 (KIM), AQ734578 (KIM), AQ734571 (KIM)
C. aridicola subsp. *densifolia* A.E.Holland	7−22	13.6	nonester: **7**	AQ734595 (BT), AQ734510 (KIM), AQ734526 (VR), AQ734587 (DG), AQ734583 (DG), AQ734515 (DG), AQ734588 (BT), AQ734581 (DG)
C. brevis Domin	0−0.15	0.03	RC (free): **17**, **16**	AQ734520 (DG), AQ734522 (DG), AQ734524 (DG), AQ734577 (KIM), AQ734582 (DG), AQ734590 (DG), AQ734593 (DG), AQ734564 (KIM), AQ734513 (KIM), AQ734566 (KIM), AQ734521 (DG), AQ734518 (DG), AQ734602 (DG), AQ734592 (DG)
C. crispata F.Muell. ex Benth.	3−21	7.7	RC: **16**, **17**, **14**	AQ734514 (DG), AQ734516 (VR), AQ734523 (DG), AQ734576 (KIM), AQ734585 (DG), AQ734517 (VR), AQ734575 (KIM), AQ734509 (KIM)
C. cunninghamii R.Br. subsp. *cunninghamii*	0.04−0.08	0.06	OC: **31**	AQ734557 (KIM), AQ734547 (KIM)
C. dissitiflora Benth. subsp. *dissitiflora*	<LOD	<LOD		AQ734609 (SQ), AQ762189 (SWQ), AQ762192 (SWQ), AQ775296 (NWQ), AQ775298 (NWQ)
**C. goreensis* Guill. & Perr.	0.3−14	4.5	nonester: **1**, **3**, **10**, **2**, **5**	AQ776731 (NEQ), AQ741264 (NEQ), AQ776737 (NEQ), AQ778649 (NEQ), AQ734580 (DG), AQ734519 (DG), AQ734530 (DG), AQ734531 (DG)
**C. grahamiana* Wight & Arn.	1.3	1.3	RC: **17**, **33**	AQ751287 (SEQ)
**C. incana* L. subsp. *incana*	0.04	0.04	RC (free): **20**	AQ734533 (NEQ)
**C. incana* subsp. *purpurascens* (Lam.) Milne-Redh.	0.03	0.03	RC: **20**	AQ741887 (SEQ)
**C. lanceolata* E.Mey. subsp. *lanceolata*	<LOD	<LOD		AQ741889 (SEQ), AQ762201 (SEQ), AQ778647 (NEQ)
C. medicaginea var. *neglecta* (Wight & Arn.) Baker (chemotype 1)	0.7−4	2.2	nonester: **9**, **12**, **4**	AQ741277 (NWQ), AQ775300 (NWQ), AQ734507 (VR)
C. medicaginea var. *neglecta* (Wight & Arn.) Baker (chemotype 2)	0−11	4.6	nonester: **7**, **8**	AQ734586 (DG), AQ734554 (KIM), AQ734541 (KIM), AQ734548 (KIM), AQ734536 (KIM), AQ734551 (KIM)
C. medicaginea var. *neglecta* (Wight & Arn.) Baker (chemotype 3)	6.8	6.8	nonester: **6**, **4**, **11**	AQ751147 (NWQ)
C. mitchellii Benth. subsp. *mitchellii*	0.3−0.7	0.5	OC: **31**, **26**, **29**	AQ741884 (SEQ), AQ751278 (SQ), AQ741893 (SEQ)
C. montana var. *angustifolia* (Gagnep.) Niyomdham	0−0.3	0.1	RC (free): **15**, **19**, **16**	AQ734553 (KIM), AQ734559 (KIM), AQ734562 (KIM), AQ734603 (DG), AQ734599 (DG), AQ734597 (DG)
C. montana var. *exserta* (Domin) A.E.Holland	<LOD	<LOD		AQ775275 (NWQ)
C. novae-hollandiae subsp. *crassipes* (Hook.) A.E.Holland	2.3	2.3	OC: **31**, **26**, **27**, **23**, **18** RC: **17**	AQ734573 (KIM), AQ734558 (KIM)
C. novae-hollandiae subsp. *lasiophylla* (Benth.) A.T.Lee	0−0.6	0.2	OC: **31**, **26**	AQ734589 (DG), AQ734600 (BT), AQ734596 (CNT), AQ734584 (DG)
C. novae-hollandiae DC. subsp. *novae-hollandiae* (chemotype 1)	0.1−1.4	0.6	OC: **31**, **26** RC: **17**	AQ734549 (KIM), AQ734508 (VR), AQ734528 (VR), AQ734529 (DG), AQ734512 (KIM), AQ734601 (DG)
C. novae-hollandiae DC. subsp. *novae-hollandiae* (chemotype 2)	0.3−23	6.0	OC: **26** RC (free): **17**, **21**, **14**, **24**, **25**	AQ734598 (DG), AQ751145 (NWQ), AQ734532 (NEQ), AQ776272 (NWQ), AQ741276 (NWQ), AQ751148 (NWQ), AQ741246 (NEQ), AQ751181 (NWQ), AQ751163 (NWQ), AQ751185 (NWQ)
C. novae-hollandiae DC subsp. *novae-hollandiae* (chemotype 3)	0.2−0.7	0.4	OC: **31**, **26** RC (free): **17**, **21**	AQ734538 (KIM), AQ734540 (KIM), AQ734534 (KIM)
**C. pallida* var. *obovata* (G.Don) Polhill	0−0.2	0.1	RC: **30, 20**	AQ776736 (NEQ), AQ741241 (NEQ), AQ778648 (NEQ)
C. ramosissima Roxb.	11−71	22.3	RC: **16**, **17**, **14**	AQ734591 (DG), AQ734542 (KIM), AQ734511 (KIM), AQ734545 (KIM), AQ610814 (KIM), AQ734525 (KIM), AQ734572 (KIM), AQ734544 (KIM), AQ734552 (KIM), AQ734594 (DG), AQ734567 (KIM), AQ734556 (KIM), AQ734550 (KIM), AQ734560 (KIM)
**C. retusa* L. var. *retusa*	0.5−31	12.2	RC: **17**, **22**	AQ776740 (NWQ), AQ776732 (NWQ), AQ734537 (KIM), AQ734579 (KIM), AQ734563 (KIM), AQ734561 (KIM), AQ734543 (KIM)
**C. spectabilis* Roth	0.6	0.6	RC (free): **17**, **22**	AQ762203 (SEQ)
C. verrucosa L.	0−0.05	0.01	OC: **32**, **34** RC: **16**, **21** RM: **13**	AQ734535 (KIM), AQ734539 (KIM), AQ734546 (KIM), AQ734555 (KIM)
**C. zanzibarica* Benth.	0.3	0.3	RC: **30**, **20**	AQ751328 (SEQ)

[a] An asterisk before the botanical name denotes a naturalized exotic taxon. [b] Measured in retrorsine equivalents, mg/g of dried plant. [c] RC, retronecine macrocyclic diester; RM, retronecine monoester; OC, otonecine macrocyclic diester; nonester, lacking functionality for adduct formation; (free), alkaloids predominantly present as free alkaloid rather than *N*-oxide. [d] Major (high level) alkaloids are underlined; other alkaloids are at low level or trace. [e] Samples with alkaloid content <LOQ are underlined (listed in numeric order only); other samples within species are listed in increasing pyrrolizidine alkaloid content. [f] Geographical regions from which collections were made as described in the text.

compounds represent alkaloids that have previously been identified in these *Crotalaria* taxa (*11*), but many of the early literature references lack mass spectrometric and RI data. Mass spectra for these alkaloids are reported here for the first time (**Table 1**) and have been included in a pyrrolizidine alkaloid mass spectra database maintained at this institute. RI values were determined on a DB-5ms column (5% phenyl, 95% dimethylarylene siloxane) and occur in the same retention order, although proportionally slightly higher than literature RI values obtained on DB-5 columns (5% phenyl, 95% dimethylpolysiloxane). For example, the RI of internal standard retrorsine is 2638 (DB-5ms) compared to 2580 (DB-5) (*9*), and RI values obtained for other alkaloids compare similarly to DB-5 literature values (factor ~1.02).

Identified pyrrolizidine alkaloids in *Crotalaria* taxa were characterized as belonging to general types determined by

identity of necine base and kind of ester: retronecine base macrocyclic diesters, retronecine base monoesters, and otonecine base macrocyclic diesters (**Table 2**). Pyrrolizidine alkaloids of different types contain characteristic mass fragmentation patterns (*9, 12*), enabling the alkaloid type to be determined even for alkaloids that could not be identified (**Table 2**). For example, macrocylic diesters of retronecine have three prominent triads in their mass spectra at *m*/*z* 138, 137, and 136; 121, 120, and 119; and 95, 94, and 93, whereas mass spectra of C9-monoesters of retronecine contain a triad at *m*/*z* 137, 138, and 139 and a strong *m*/*z* 93 with lesser *m*/*z* 94 and 95. By comparison, otonecine alkaloids are identified by the presence of characteristic peaks at *m*/*z* 168, 151, 150, 149, 123, 122, 110, 96, and 94. Several nonesterified pyrrolizidine alkaloids were also identified in three taxa (**Table 2**). These alkaloids **1**−**12** lack the esterified 1,2-unsaturated functionality required for adduct formation (*4*) and are not hepatotoxic.

All alkaloids were tentatively identified by mass spectra of the free alkaloids but were predominantly present in *Crotalaria* plant material as *N*-oxides, which were reduced by zinc dust prior to analysis. Exceptions were the retronecine-base alkaloids of *C. brevis*, *C. incana* subsp. *incana*, *C. montana* var. *angustifolia*, *C. novae-hollandiae*, and *C. spectabilis*, which were largely present as free alkaloids, and the various otonecine-base alkaloids, which generally do not occur as *N*-oxides.

Pyrrolizidine alkaloid content was measured in terms of retrorsine equivalents (**Table 2**), as standards of the individual alkaloids were not available to determine response factors. Total ion chromatogram (TIC) responses for monocrotaline and retrorsine showed a linear calibration curve with a 1:1 correlation ($R = 0.96$) for standard solutions in the range from 0.02 to 0.2 mg/mL. Retrorsine was chosen as the preferred internal standard due to the occurrence of monocrotaline as a constituent of several *Crotalaria* species.

It has been reported that enzymatic decomposition of pyrrolizidine alkaloids may occur during plant drying with significant losses reported in some species (*2*). Consequently, we anticipate that pyrrolizidine alkaloid content calculated for dried milled material in this study may be less than is present in fresh, growing material when expressed on a dry weight basis. The practical difficulties of collection of numerous batches of plants and their transport over long distances prevented the analysis of fresh material or material preserved in ethanol in this study. Culvenor et al. (*13*) reported, for example, that *C. aridicola* plants from the same batch (SN7554) that were air-dried or immersed in ethanol immediately after collection contained 0.19 and 0.6% total alkaloids, respectively, on a dry matter basis and that similar 3-fold differences occurred in paired air-dried and ethanol-immersed samples of *C. medicaginea* (CC388, IC2130) in the same study. However, as all of our samples have been treated similarly, we believe that a comparison of alkaloid content across taxa and with literature values from air-dried plants is still valid. These air-drying losses of pyrrolizidine alkaloids may account for our failure to detect alkaloids in taxa reputed to cause pyrrolizidine alkaloidosis, such as *C. dissitiflora* subsp. *dissitiflora* (*3*).

Alkaloid Profiles of *Crotalaria* Taxa. Collections of the 24 *Crotalaria* taxa were sourced across 11 regions of northern Australia (**Table 2**). Between 1 and 18 specimen batches were obtained for each taxon and analyzed for pyrrolizidine alkaloids (**Table 2**). Most *Crotalaria* taxa examined in this work provided GC-MS alkaloid profiles consistent with previously reported alkaloid composition, which enabled mass spectra to be readily assigned.

Most taxa had a consistent pyrrolizidine alkaloid profile across all samples regardless of source location, albeit with varying total alkaloid contents (**Table 2**). There were two notable exceptions, *C. medicaginea* var. *neglecta* and *C. novae-hollandiae* subsp. *novae-hollandiae*, for which distinctly different chemotypes were recorded, and these are discussed further.

Alkaloidal Composition of Taxa with a Single Chemotype. Alkaloidal extracts of *C. goreensis* have previously been reported to contain 7β-hydroxy-1-methylene-8α- and 7β-hydroxy-1-methylene-8β-pyrrolizidine (**1**)/(**3**) (*14*) and an unusual dichlorocyclopropyl artifact from reaction of **1** and/or **3** with chloroform (*15*) with no mass spectra reported. Our GC-MS analysis of this species showed three major components with mass spectra consistent with structures **1** and **3** and a chlorocyclopropyl artifact (**10**) (presumably from the reaction of **1** and/or **3** with dichloromethane) and two additional minor components with mass spectra identical to that of **1** and **3**, which are presumed to be further isomers **2** and **5**.

Seed of *C. incana* has previously been reported to contain integerrimine (**20**) (*16*) together with usaramine (**30**) (*17*), whereas stem, leaf, and flowers contained anacrotine (*18*). More recently, *C. incana* subsp. *purpurascens* has been reported to contain dihydrosenecionine, integerrimine, anacrotine, and a nemorensine isomer (*19*). Our analysis of *C. incana* subsp. *purpurascens* also revealed low levels of integerrimine (**20**) and traces of a further alkaloid thought to be a stereoisomer. Other alkaloids reported in this taxa in Ethiopia (*19*) were not seen in our analysis. Our mass spectrum of integerrimine (**20**) was in agreement with the literature (*19*), and the RI obtained, 2452 (DB-5ms), compares favorably with literature values of 2402 (DB-5) (*9*) and 2448 (DB-5ms) (*10*). Our GC-MS analysis of *C. incana* subsp. *incana* also showed low levels of integerrimine (**20**).

C. pallida has previously been named *C. mucronata* and *C. striata*, and integerrimine (**20**) and usaramine (**30**) have been reported in this plant (*20*). Mucronatinine isolated from *C. mucronata* seed (*21*) is thought to be identical with usaramine (**30**) (*17*). Our GC-MS analysis of *C. pallida* var. *obovata* revealed two low-level components identified as **20** and **30**. Usaramine (**30**) had mass spectra similar to the isomeric retrorsine (**28**) and RI consistent with the literature (*9, 10*). Nilgirine and crostastriatine (acetylnilgirine) have also been reported in *C. mucronata* and *C. striata* (*22*−*25*), but were not seen in our analysis. Alkaloids of the closely related *C. zanzibarica* have not previously been investigated, and our GC-MS analysis demonstrated the presence of low levels of the same two alkaloids, integerrimine (**20**) and usaramine (**30**), in this species.

Previous analyses of *C. spectabilis* have reported monocrotaline (**17**) (*26*−*28*) and also spectabiline (**22**) (*29*), and our GC-MS analysis of a single sample of this species confirmed the same two components. Monocrotaline (**17**) was identified by co-injection with a commercial standard and spectabiline (**22**) by comparison with literature MS data (*30*). Previous analyses of *C. retusa* have also reported monocrotaline (**17**) as the major alkaloid with various minor alkaloids including spectabiline (**22**) (*26*−*28, 31*−*33*). Our analysis of *C. retusa* var. *retusa* revealed predominantly monocrotaline (**17**) with traces of other alkaloids including spectabiline (**22**).

The presence of monocrotaline (**17**), fulvine (**16**), and crispatine (**14**) in roughly equal amounts has previously been reported in *C. crispata* (*34*). However, no voucher specimen was lodged for this sample, and given the site of collection

(Mount Anderson Station, West Kimberley) and known distribution of the related species (*1*), it was considered that the species involved may have been the closely related *C. ramosissima*. Our GC-MS analysis of *C. crispata* revealed the presence of fulvine (**16**) and monocrotaline (**17**) with a lesser amount of crispatine (**14**), with fulvine and crispatine being identified by comparison with literature mass spectra (*35, 36*). By contrast, our samples of *C. ramosissima* contained very high levels of fulvine (**16**) with lesser amounts of monocrotaline (**17**) and only traces of crispatine (**14**). The results reported previously (*34*) are thus consistent with *C. crispata* as presently defined (*1*) rather than *C. ramosissima*. In our study, *C. ramossissima* samples contained the greatest amounts of total alkaloids determined (up to 7% on a dry weight basis). Monocrotaline (**17**) and fulvine (**16**) were also detected in trace amounts in our GC-MS analysis of samples of *C. alata* and *C. brevis*. No previous alkaloid analyses have been reported for either of these species.

Monocrotaline (**17**) and grahamine (**33**) have previously been isolated from the seeds of *C. grahamiana* (*30, 37*). Our GC-MS analysis of this species revealed the presence of monocrotaline (**17**) and a second component with a mass spectrum consistent with that previously reported for grahamine (**33**) (*30*).

A number of pyrrolizidine alkaloids have been reported in previous analyses of *C. verrucosa* seed including crotaverrine (**32**), *O*-acetylcrotaverrine (**34**) (*38*), and crotalaburnine (anacrotine) (*39*). Our GC-MS analysis of this species revealed two components consistent with the MS data provided for crotaverrine (**32**) and *O*-acetylcrotaverrine (**34**) (*38*), with lesser amounts of an unknown pyrrolizidine alkaloid monoester M^+ 283 (**13**), fulvine (**16**), and the tentatively identified pumiline A (**21**), which was also seen in *C. novae-hollandiae* taxa. Our mass spectra for pumiline A (**21**) showed good agreement with literature data (*40*), and the literature RI of 2430 (ZB5) is comparable with our RI of 2473 (DB5-ms).

Previous alkaloid analyses of *C. aridicola* reported the major components as 1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**4**) and 7β-hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**9**) (*13, 41*), with a minor component later identified as 7β-acetoxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (*13*). None of these three components was present in our samples of *C. aridicola* subsp. *densifolia*. Our GC-MS analysis of eight samples of this taxon revealed a single component that had a mass spectrum identical to that of 1β,2β-epoxy-1α-hydroxymethyl-8α-pyrrolizidine (**7**) as previously reported in a form of the closely related *C. trifoliastrum* (now *C. medicaginea*) (*13*) and also in *Heliotropium ternatum*, *Heliotropium molle*, and *Heliotropium angiospermum* (*42*). It is thus likely that the previous analyses of *C. aridicola* from Atherton and Townsville in northern Queensland (*13, 41*) relate to a subspecies different from that examined in this study, consistent with the reported distributions of the three subspecies of *C. aridicola* (*1*).

Literature searches revealed no previous investigations of alkaloids from *C. dissitiflora*, *C. lanceolata*, or *C. montana* (formerly *C. linifolia* and *C. exserta*). No alkaloids were detected in our GC-MS analysis of *C. dissitiflora* subsp. *dissitiflora* or *C. lanceolata* subsp. *lanceolata*. Analysis of *C. montana* subsp. *angustifolia* revealed small amounts of two unknown alkaloids **15** (M^+ 307) and **19** (M^+ 323) together with fulvine (**16**) in two of the six samples analyzed. Similar analysis of a single sample of *C. montana* subsp. *exserta* revealed no alkaloids.

C. mitchellii has previously been reported to contain retusamine (**31**) (*43*) with no literature mass spectrum available. Secondary literature refers to an unpublished report of monocrotaline in *C. mitchellii* by Culvenor (*2*), but this has not been further substantiated. Our analysis of *C. mitchellii* subsp. *mitchellii* revealed a major component with a mass spectrum consistent with that of retusamine (**31**) and minor components that were tentatively identified as crosemperine (**26**), consistent with literature MS (*44, 45*), and a de-ethyl analogue of retusamine (**29**), on the basis of mass spectrometric interpretation and seen only in one sample from southern Queensland. Retusamine (**31**) was also the major component detected in our GC-MS analysis of *C. cunninghamii* subsp. *cunninghamii*. Literature searches revealed no prior alkaloid analysis of *C. cunninghamii*.

Retusamine (**31**) has previously also been reported from *C. novae-hollandiae* and *C. crassipes* (*43*). Our GC-MS analysis of samples of *C. novae-hollandiae* subsp. *crassipes* [previously *C. crassipes* (*1*)] revealed retusamine (**31**) as the major component with smaller amounts of monocrotaline (**17**) and crosemperine (**26**) and trace amounts of three additional otonecine-base akaloids. One of these appears to be a crosemperine stereoisomer (**27**), and the others by MS interpretation are alkyl analogues of crosemperine in which the isopropyl side chain has been replaced by an ethyl group (**23**) and a methyl group (**18**). This latter alkaloid croaegyptine (**18**) has previously been reported to co-occur with crosemperine (**26**) in *C. aegyptiaca* (*46*). Our analysis of *C. novae-hollandiae* subsp. *lasiophylla* samples revealed small amounts of alkaloids including retusamine (**31**) and probably crosemperine (**26**).

Alkaloidal Composition of Taxa with Multiple Chemotypes. All of the aforementioned taxa had a singular alkaloid profile for all collected samples. Multiple chemotypes were, however, observed for collections of both *C. medicaginea* var. *neglecta* and *C. novae-hollandiae* subsp. *novae-hollandiae*.

Our GC-MS analysis of 19 samples identified as *C. novae-hollandiae* subsp. *novae-hollandiae* revealed two distinct profiles with a third intermediate profile. *C. novae-hollandiae* subsp. *novae-hollandiae* chemotype 1 (six samples largely from Western Australia and the Northern Territory) contained predominantly retusamine (**31**) with low levels of monocrotaline (**17**) and crosemperine (**26**), a profile similar to that of *C. novae-hollandiae* subsp. *crassipes* (above). *C. novae-hollandiae* subsp. *novae-hollandiae* chemotype 2 (10 samples largely from northern Queensland) contained predominantly monocrotaline (**17**) and the tentatively identified pumiline A (**21**) (or stereoisomer) with smaller amounts of crispatine (**14**), crosemperine (**26**), and two stereoisomers of trichodesmine (**24**) and (**25**). Trichodesmine (**24**)/(**25**) demonstrated a MS breakdown similar to that of the structurally similar monocrotaline (**17**) with ions incremented by 28 mass units due to the replacement of a methyl group in **17** by an isopropyl in trichodesmine and is consistent with literature MS (*19*). In addition to the two major profiles, three samples of *C. novae-hollandiae* subsp. *novae-hollandiae* chemotype 3 from the Kimberley region of Western Australia had an alkaloid composition intermediate between these profiles, with both monocrotaline (**17**) and retusamine (**31**) present.

Previous analysis of several morphological forms of *C. trifoliastrum*, now called *C. medicaginea*, in Australia identified a number of different chemical profiles consisting largely of various proportions of 1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**4**), 1β,2β-epoxy-1α-methoxymethyl-8α-pyrrolizidine (**6**), 1β,2β-epoxy-1α-hydroxymethyl-8α-pyrrolizidine (**7**), and 7α-hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**9**) (*13*). Culvenor (*13*) reported the mass spectra of epoxide (**6**) but no MS data for the other compounds. All samples of *C. medicaginea* analyzed in the present study were identified as

C. medicaginea var. *neglecta*, but GC-MS analysis revealed three distinct alkaloid profiles, which bore similarity to those previously reported. *C. medicaginea* var. *neglecta* (chemotype 1) contained predominantly 7α-hydroxy-1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**9**) with a monochloride (**12**) and low levels of 1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**4**). Compounds **9** and **4** were reported in 'form 1' of *C. trifoliastrum* (*13*), and our mass spectra are consistent with these structures. The chloride **12** was not reported by Culvenor (*13*) for this species and is presumed to be a dichloromethane artifact analogous to the artifact **10** seen in *C. goreensis*. Our analysis of a second chemotype, *C. medicaginea* var. *neglecta* (chemotype 2), contained predominantly 1*β*,2*β*-epoxy-1α-hydroxymethyl-8α-pyrrolizidine (**7**) along with traces of a presumed stereoisomer **8** with similar mass spectra. Alkaloid **7** has previously been reported as the major component of *C. trifoliastrum* "form unspecified" (*13*), and our mass spectrum is consistent with that reported. A third chemotype, *C. medicaginea* var. *neglecta* (chemotype 3), contained 1*β*,2*β*-epoxy-1α-methoxymethyl-8α-pyrrolizidine (**6**), 1-methoxymethyl-1,2-dehydro-8α-pyrrolizidine (**4**) (as in chemotype 1), and tentatively identified 1*β*,2*β*-epoxy-7-hydroxy-1α-methoxymethyl-8α-pyrrolizidine (**11**). Culvenor (*13*) reported the presence of alkaloids **4** and **6** together with a comparable proportion of an "unidentified alkaloid" in *C. trifoliastrum* 'form 1*'. Our mass spectrum of alkaloid **6** was identical with that reported by Culvenor, and novel compound **11** demonstrated a MS breakdown analogous to that of the structurally similar alkaloid **6** with the predominant ions incremented 16 units due to the added hydroxyl group.

Relative Risk Associated with Crotalaria Taxa. The primary purpose of this study was to determine which *Crotalaria* taxa presented the greatest risk of pyrrolizidine alkaloid consumption by grazing livestock and of potentially forming pyrrolizidine alkaloid residues in tissues of animals exposed to these plants. There is considerable variation in toxicity of different pyrrolizidine alkaloids relating to molecular structure (*4*) and whether alkaloids occur as free alkaloids or *N*-oxides. Apart from toxin concentration and the form it takes in each plant species, palatability, geographical distribution, abundance (population density), and biomass also determine exposure risk. The most prevalent *Crotalaria* taxa in northern Australia include those analyzed in this study, with the number of specimen batches collected indicative of relative prevalence, at least at the time of collection, which was mostly in June−July, after the wet season. The time and resources available during the collecting trips did not allow accurate measurement of plant population densities, but *Crotalaria* plants generally occurred in scattered discrete populations of from a few to hundreds of plants covering a few to several hundred square meters at densities estimated to be up to about 2−3 plants/m^2, depending on the taxon. The subspecies of *C. novae-hollandiae* had the greatest overall geographical range and abundance; *C. ramosissima* was very prevalent in parts of the Kimberley, whereas *C. medicaginea*, *C. crispata*, *C. aridicola*, *C. montana*, and *C. brevis* were common in their respective ranges. The most prevalent naturalized species were *C. retusa* and *C. goreensis*. *Crotalaria* species with most abundance and biomass during our observations were, in descending order, *C. ramosissima* > *C. novae-hollandiae* > *C. retusa* > *C. crispata* > *C. spectabilis* (limited range) > *C. goreensis*.

It is generally recognized that pyrrolizidine alkaloid-containing plants are less palatable than grasses to grazing ruminants, but livestock that are continually exposed to the plants often consume small amounts. However, this varies widely between herds and individuals. Consumption can be affected by stage of growth; for example, deep-rooted *Crotalaria* spp. are quick to regenerate following fire, and the regrowth is of higher nitrogen content and possibly palatability. Furthermore, the deliberate use of fire for pasture management early in the dry season in parts of northern Australia was observed by the authors to favor localized regrowth of *C. novae-hollandiae*, *C. ramosissima*, *C. crispata*, and other species. Other than this, intake is also likely to increase when general pasture conditions have deteriorated late in the dry season, and hungry animals may be forced to eat plants they would not normally graze. The plants can also regenerate more quickly than grass after early wet season rain. The other high risk of exposure is in fed rather than foraging situations, when hay or feed grains are contaminated with *Crotalaria* spp. components and animals cannot avoid intake.

The highest risk plants if consumed by livestock are also likely to be those of highest alkaloid content. From our analysis (**Table 2**), *Crotalaria* taxa with the highest alkaloid content are *C. ramosissima*, *C. retusa* var. *retusa*, *C. aridicola* subsp. *densifolia*, *C. crispata*, *C. goreensis*, *C. medicaginea* var. *neglecta*, and *C. novae-hollandiae* subsp. *novae-hollandiae* chemotype 2. All of these except *C. goreensis*, *C. aridicola* subsp. *densifolia*, and *C. medicaginea* var. *neglecta* have been associated with pyrrolizidine alkaloid poisoning in both cattle and horses. This is consistent with the latter three taxa containing alkaloids that lack the required chemical functionality for pyrrolizidine alkaloid adduct formation. Although both *C. aridicola* subsp. *aridicola* and *C. medicaginea* var. *neglecta* have been associated with esophageal ulceration of horses in Queensland (Chillagoe horse disease), the toxin responsible for this syndrome is unknown (*47*). *C. goreensis* also contains alkaloids that lacked the required functionality and has been associated with poisoning of chickens, but the effects do not appear to be consistent with the known effects of pyrrolizidine alkaloids (*48*). The current restricted distribution of grazing sheep in northern Australia limits their exposure to the species identified here as having the highest pyrrolizidine alkaloid contents, but sheep are regarded as more resistant to classical pyrrolizidine alkaloid poisoning than cattle and horses (*49*). Sheep have been recorded as suffering acute lung damage from consumption of *C. dissitiflora* subsp. *dissitiflora*, *C. pallida*, and *C. eremea* subsp. *eremea* (bluebush pea) (*3*).

Several taxa, notably *C. alata*, *C. brevis*, *C. cunninghamii* subsp. *cunninghamii*, *C. dissitiflora* subsp. *dissitiflora*, *C. incana*, *C. lanceolata* subsp. *lanceolata*, *C. montana* subsp. *angustifolia*, *C. montana* subsp. *exserta*, *C. pallida*, and *C. verrucosa*, contained very low levels of alkaloids and appear at first sight unlikely to cause significant toxicity. However, they could make a contribution to the overall pyrrolizidine alkaloid exposure of grazing livestock, and *C. dissitiflora*, *C. montana*, and *C. pallida* have been associated with pyrrolizidine alkaloid poisoning in Australia (*3*). Perhaps they are more palatable than those with higher pyrrolizidine alkaloid contents. *C. incana* has been suspected of causing pyrrolizidine alkaloid poisoning of livestock in Ethiopia (*50*).

The differing chemotypes observed within *C. medicaginea* var. *neglecta* and *C. novae-hollandiae* subsp. *novae-hollandiae* are problematic with regard to risk assessment. For instance, risk associated with *C. novae-hollandiae* subsp. *novae-hollandiae* (chemotype 1) containing predominantly the otonecine alkaloid retusamine (**31**) may be significantly less than the risk associated with *C. novae-hollandiae* subsp. *novae-hollandiae* (chemotype 2) in which the retronecine alkaloid monocrotaline

(**17**) predominates as the free alkaloid and is present at considerably higher levels (**Table 2**). Otonecine alkaloids are known to be less hepatotoxic than retronecine alkaloids, as formation of the reactive pyrrolic intermediates from otonecine alkaloids requires oxidative demethylation, ring closure, and dehydration (*4*).

From our investigations, the most abundant *Crotalaria* taxa present in northern Australia with the highest concentrations of the most potent forms of pyrrolizidine alkaloids, and thus with the greatest potential to cause pyrrolizidine alkaloid poisoning if consumed by livestock, would be *C. novae-hollandiae* subsp. *novae-hollandiae* (chemotype 2), *C. ramosissima*, *C. retusa* var. *retusa*, and *C. crispata*. Other species containing moderate amounts of pyrrolizidine alkaloids, such as *C. spectabilis* and *C. mitchellii*, also pose significant risk where they have extensive local abundance. We recommend that risk management for avoiding *Crotalaria* poisoning of livestock should be directed toward the identified high-risk species, in situations when exposure is likely to be greatest.

ACKNOWLEDGMENT

Helen Blaney and Glenyth McKenzie contributed significantly to plant location and collection. Ailsa Holland and Megan Thomas, Queensland Herbarium (Environmental Protection Agency, Queensland), determined plant identifications. Technical assistance was provided by Jason Cole, Denis Webber, and Madeleine Modina. Andrew Craig, Western Australian Department of Agriculture and Food, provided a *C. ramosissima* sample from Bohemia Downs Station, Kimberley region, WA (ABC1722; AQ610814). Tammy Johnson provided a *C. mitchellii* subsp. *mitchellii* sample from Chinchilla, Queensland (AQ751278).

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Received for review August 27, 2008. Revised manuscript received November 4, 2008. Accepted November 6, 2008. This study was partly funded by Meat and Livestock Australia (Project Number AHW.017).

JF8026099