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TOXIC AMINES AND ALKALOIDS FROM *ACACIA BERLANDIERI*

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Abstract—Consumption of *Acacia berlandieri* Benth. by domestic livestock during periods of drought may result in a locomotor ataxia, as well as having negative effects on intake and male fertility. Four phenolic amines (*N*-methyl-β-phenethylamine, tyramine, *N*-methyltyramine, and hordenine), had previously been extracted from the plant, and *N*-methyl-β-phenethylamine has been shown to negatively impact fertility in female Angora goats. In order to clarify the possible role of other secondary compounds from *Acacia berlandieri* on non-lethal toxicities in domestic livestock, leaf samples collected in the spring and autumn were subjected to rigorous chemical analysis. In addition to the four previously detected amines, 29 other alkaloids and amines were isolated and identified by GC-MS, these including nicotine, nornicotine, mescaline, mimosine, and four amphetamines. A significant increase in the number and relative quantities of these compounds was observed in late season foliage. © 1997 Elsevier Science Ltd

INTRODUCTION

Sheep and goats grazing on *Acacia berlandieri* Benth. during periods of drought in the Rio Grande Plains of Texas develop a locomotor ataxia referred to as 'guajillo wobbles' or 'limberleg' [1]. Previous analysis of *A. berlandieri* by paper chromatography [2], TLC [3], GC [4], and most recently HPLC [5] has detected and identified four amines, *N*-methyl-β-phenethylamine (NMPEA), tyramine, *N*-methyltyramine, and hordenine. Research has shown that NMPEA has negative effects on progesterone concentrations in pregnant Angora nannies, reduces the numbers of animals becoming pregnant [6], and reduces luteinizing hormone production in response to gonadotrophin-releasing hormone stimulation in castrated male sheep [7]. In addition, male Angora goats consuming high proportions of *Acacia berlandieri* and *A. rigidula* have been found to have reduced sperm production and elevated levels of cortisol in the peripheral circulation [8]. Intake and digestibility of *A. berlandieri* fed to sheep, goats and white-tailed deer in a metabolism trial were low (< 2.5% body weight and < 35% dry matter digestibility), and all animals were in negative nitrogen balance, despite the forage having Kjeldahl nitrogen values in excess of 2.5% (Forbes, pers. comm.).

The locomotor ataxia developed in the early *A. berlandieri* feeding trials [9] was not observed in adequately fed animals injected with NMPEA or tyramine [6]. Previous work [10] has shown that many plant species endemic to southwest Texas and northern Mexico contain a wide variety of phenolic monoamines. A need to explain more fully the results of earlier studies, together with advances in GC-MS technology, led to an intensive chemical analysis of *A. berlandieri* to identify other amines and alkaloids present in the leaves.

RESULTS AND DISCUSSION

Leaves and unlignified stems that would comprise browse were packed into an extraction thimble, placed in a Soxhlet apparatus and extracted with methanol followed by extraction with chloroform. This extraction procedure was compared with the more traditional methods of extraction by soaking the plant material in acid solution [2, 5] and found to produce a cleaner, more complete isolation of the amines and alkaloids present in the sample. The initial extract was fractionated by extraction with aq. acid, the pH of the acid extract was adjusted to *ca* pH 10.3 prior to re-isolation of the alkaloids by extraction with organic solvents. The pH of the aq. fraction was routinely checked between extractions and adjusted (dil. NaOH added) as necessary. It was found that when the pH exceeded 11, a large portion of the phenol's alkaloids remained in the aqueous solution as their corresponding phenolate salts. Particular care was taken

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to maintain the extracts and isolates under an inert atmosphere. Left unprotected, the isolated amines and alkaloids readily absorbed atmospheric CO_2 and decomposed with significant polymerization being detected.

Thirty-three amines and alkaloids, including the four previously encountered amines, NMPEA, tyramine, *N*-methyltyramine, and hordenine, were identified by GC-MS. Both splitless injection and dedicated on-column injection systems were employed for GC-MS analysis. The on-column injection is a much milder method of sample introduction. This technique, although it produced the most complex chromatograms, was best for the detection of the phenol containing components of the mixture which tended to be more thermally labile. No derivatization was performed on the analytes, therefore GC-MS analysis would only be expected to detect the volatile amines and alkaloids present in the sample.

The majority of the isolated alkaloids, twenty-four of the thirty-three identified, were related to the parent compound β-phenethylamine (Table 1). These compounds generally varied in the degree of *N*-methylation, α-methylation (amphetamine family), and in hydroxylation of the aromatic ring (tyramine, dopamine, and mescaline families). Other noteworthy alkaloids found in the extract include nicotine, nornicotine, and three tetrahydroisoquinoline alkaloids: anhalamine, anhalidine, and peyophorine. The presence of the amphetamines may help explain the low intakes observed when fresh *A. berlandieri* forage is fed to livestock. To our knowledge this is the first report of amphetamines in the genus *Acacia*. Smith [10] in his review of the phenethylamines does not report the presence of amphetamines in any plant family.

The methyl ester of the amino acid, mimosine, was detected. It is not known if this ester occurs naturally in the plant or if it is an artifact of the extraction procedure. It should be noted, however, that no other amino acid esters were detected in the extract. The presence of three non-volatile quaternary ammonium salts was indirectly detected in the late season foliage. Styrene, *p*-hydroxystyrene, and 3,4,5-trimethoxystyrene were detected in the chromatograms from the samples introduced via the heated injection port. The peaks corresponding to these compounds were absent when the samples were introduced by on-column injection. These compounds would be expected from the Hoffmann elimination of the trimethylammonium analogues of β-phenethylamine, tyramine and mescaline [11]. In an effort to prove this, the *N,N,N*-trimethylammonium salts were created by exhaustive methylation of β-phenethylamine, tyramine, and authentic mescaline. Solutions of these salts were injected onto the heated injection port and styrene, *p*-hydroxystyrene, and 3,4,5-trimethoxystyrene were detected by GC-MS. This was used as indirect proof of the presence of these three salts. The values reported for these three quaternary ammonium salts are based upon the quantity of the corresponding styrene detected.

NMPEA has previously been isolated and identified as the major amine constituent present in *A. berlandieri* [12]. Because seasonal variation in the quantities of phenolic amines has been reported in *A. berlandieri* [13], the amount of NMPEA in early and late season foliage was carefully determined by HPLC following the procedures of Nair *et al.* [14]. These values are compared to those from GC-MS quantification. Results are shown in Table 2. The quantities of amines and alkaloids detected in *A. berlandieri* were based upon their relative abundance compared to the amount of NMPEA present in the sample.

Initial identification of the alkaloids present within the plant extracts was based upon library comparison of their mass spectral fragmentation patterns with the final confirmation of identification made by direct comparison with spectra of authentic samples. Leaves, petioles and unlignified stems were collected twice, a first growth sample collected early in the spring and a late season sample collected before frost in the fall. As was found with NMPEA the foliage collected in the fall contained higher quantities of amines and alkaloids. There was also a distinct increase in the number and quantity of methylated analogues present (Table 3).

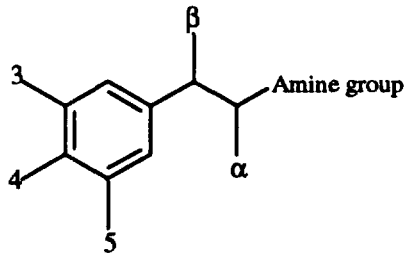
Several other nitrogen-containing compounds have been detected but have not yet been identified. Because no derivatization was performed, only the volatile amines and alkaloids were detected. By use of the on-column injection system, loss of thermally labile compounds was minimized.

Phenolic amines, as a group, impact the hypothalamic–pituitary–adrenal axis [15]. The consequent release of ACTH and cortisol results in sympathomimetic action. The number of phenolic amines reported in Table 3 and their concentrations in the plant indicate a substantial toxic load to animals consuming *A. berlandieri*. The toxicity of nicotine and nornicotine has been well established [16], as has the psychoactivity of mescaline and its derivatives. None of the compounds identified appear to have been implicated in locomotor ataxic. Nevertheless, the presence of the amphetamines suggests the possibility for a reduction of monoamine oxidase activity [17].

EXPERIMENTAL

GC/quadrupole MS (EIMS at 70 eV) were obtained on Hewlett Packard Model 5988A and Model 5970C systems each having both splitless heated injection port and dedicated on-column injection capabilities. Grade 0 He was used as the carrier gas and the transfer line was maintained at 280°. Heated injection conditions include: head pressure, 5 psi; injection volume 1 μl; injection port 200°; splitless time 1 min; purge flow, 60 ml min^{-1}; initial time 1 min; ramp 3° min^{-1}; final temp 270°; final time 20 min. On-column injection conditions include: head pressure 5 psi; injection

Table 1. Structures of β-phenethylamines found in *A. berlandieri*



Compound	3	4	5	β	α	Amine group
β-Phenethylamine	H	H	H	H	H	$-NH_2$
N-Methyl-β-phenethylamine	H	H	H	H	H	$-NHMe_2$
N,*N*-Dimethyl-β-phenethylamine	H	H	H	H	H	$-NMe_2$
N,*N*,*N*-Trimethyl-β-phenethyl-ammonium hydroxide	H	H	H	H	H	$-NMe_3^+$
Amphetamine	H	H	H	H	Me	$-NH_2$
Methamphetamine	H	H	H	H	Me	–NHMe
N,*N*-Dimethyl-α-methyl-β-phenethylamine	H	H	H	H	Me	$-NMe_2$
p-Hydroxyamphetamine	H	OH	H	H	Me	$-NH_2$
p-Methoxyamphetamine	H	OMe	H	H	Me	$-NH_2$
Tyramine	H	OH	H	H	H	$-NH_2$
N-Methyltryamine	H	OH	H	H	H	–NHMe
Hordenine (anhaline)	H	OH	H	H	H	$-NMe_2$
Candicine	H	OH	H	H	H	$-NMe_3^+$
Dopamine	OH	OH	H	H	H	$-NH_2$
N-Methyldopamine	OH	OH	H	H	H	–NHMe
3-Methoxytyramine	OMe	OH	H	H	H	$-NH_2$
Mescaline	OMe	OMe	OMe	H	H	$-NH_2$
N-Methylmescaline	OMe	OMe	OMe	H	H	–NHMe
Trichocereine	OMe	OMe	OMe	H	H	$-NMe_2$
3,4,5-Trimethoxy-β-phenethyl-*N*,*N*,*N*-trimethylammonium hydroxide	OMe	OMe	OMe	H	H	$-NMe_3^+$
3,5-Dimethoxytyramine	OMe	OH	OMe	H	H	$-NH_2$
3,4-Dimethoxy-5-hydroxy-β-phenthylamine	OMe	OMe	OH	H	H	$-NH_2$
β-Methoxy-3,4-dihydroxy-5-Methoxy-β-phenethylamine	OH	OH	OMe	OMe	H	$-NH_2$
3,4-Dimethoxy-α-methyl-5-hydroxy-β-phenethylamine	OMe	OMe	OH	H	Me	$-NH_2$

Table 2. Concentration of *N*-methyl-β-phenethylamine (mg g^{-1} dry wt)

	Early season					Late season			
	HPLC	S.D.	GC-MS	S.D.		HPLC	S.D.	GC-MS	S.D.
Rep 1	1.67	0.11	1.68	0.13	Rep 1	3.71	0.15	3.72	0.14
Rep 2	1.73	0.14	1.72	0.14	Rep 2	3.77	0.19	3.76	0.16
Rep 3	1.70	0.13	1.71	0.11	Rep 3	3.73	0.13	3.74	0.13
Mean	1.70		1.70		Mean	3.74		3.74	

volume 1 μl; initial temp 60°; initial time 1 min; ramp 1.5° min^{-1}; final temp 270°; final time 35 min. WCOT cross-linked methyl silicone 12 (only used on HP 5970 system) and 36 m, 0.2 mm I.D., 33 μ coating thickness capillary columns were used. Mass spectral data was collected as the total ion chromatograms in the operating range of 35 to 800 amu. The GC-MS operating system included the Wiley mass spectral library and the NBS mass spectral library of standards. Preliminary identification was made by library compari-

Table 3. Amines and alkaloids from *A. berlandieri* Benth

	Early season ppm	Late season ppm
Phenethylamine	991.3	1390.0
N-Methylphenethylamine	1702.7	3742.2
N,N-Dimethylphenethylamine	99.1	604.4
N,N,N-Trimethyl-β-phenethylammonium hydroxide*	nd†	23.6†
Amphetamine	3.1	10.1
Methamphetamine	20.1	11.5
N,N-Dimethyl-α-methylphenethylamine	45.6	229.7
p-Hydroxyamphetamine	8.0	7.3
p-Methoxyamphetamine	nd	35.7
Tyramine	367.2	1263.4
N-Methyltyramine	188.5	745.7
Hordenine (anhaline)	9.2	333.1
Candicine‡	nd†	35.1†
Dopamine	3.6	25.3
N-Methyldopamine	1.9	10.8
3-Methoxytyramine	2.6	15.3
Mescaline	4.9	35.7
N-Methylmescaline	3.2	30.2
Trichocereine	nd	28.1
3,4,5-Trimethoxy-β-phenethyl-*N,N,N*-trimethyl-ammonium hydroxide§	nd†	13.2†
3,5-Dimethoxytyramine	2.7	34.4
3,4-Dimethoxy-5-hydroxy-β-phenethylamine	11.4	40.9
β-Methoxy-3,4-dihydroxy-5-methoxy-β-phenethylamine	nd	30.2
3,4-Dimethoxy-α-methyl-5-hydroxy-β-phenethylamine	2.0	47.2
Nicotine	39.6	108.3
Nornicotine	19.2	72.5
Anhalamine	4.9	39.6
Anhalidine (*N*-methylanhalamine)	2.9	40.9
Peyophorine	2.7	46.8
Mimosine, methyl ester	10.6	24.2
3α-Cumyl-1,3,4-oxadiazolidine-2,5-dione	308.4	420.9
Nortriptyline	19.8	71.5
Musk ambrette	26.5	27.3

* The identity of this compound is inferred from the presence of styrene.

† These values reflect the relative amount of styrene detected and may not accurately reflect the quantity of this material present in the plant.

‡ The identity of this compound is inferred from the presence of *p*-hydroxystyrene.

§ The identity of this compound is inferred from the presence of 3,4,5-trimethoxystyrene.

son, final identification was made by direct spectral comparison with the spectra of an authentic sample obtained from the GC-MS. Authentic samples were either purchased or prepd by known chemical procedures.

GC-MS quantification was made by using the area under a selected *m/z* peak for each compound and comparing this to a standard curve generated by injection of a series of known standards. Care was taken to make sure that the detector response was linear in the concn range being run.

HPLC quantification was obtained using Waters Model 510 pumping system and a C18, 8 μ, 8 mm I.D. × 10 cm, radial compression cartridge. Mobile phase was MeOH–H_2O (1:9), flow rate was 1 ml min^{-1}. Detection was by a Waters Model 486 variable wavelength fluorescence detector with excitation at 383 nm and detection at 478 nm.

Collection of leaves and stems. Samples of *Acacia berlandieri* Benth. were collected from plants growing on a southwest-facing slope in Zavala County, TX. Early season collection was performed in the spring after vigorous new growth appeared. Late season collection was performed in the late fall prior to the first frost and before colour change was detected. Leaves, petioles, and attached tender stems were collected until *ca* 500 g of fr. wt had been gathered. The material was sealed in waterproof bags and placed on ice immediately. Samples were then frozen at −20° and stored until extraction. Voucher specimens were collected and stored at the Texas A&M University Agricultural Research and Extension Center, Uvalde, TX.

Soxhlet extraction procedure. Still frozen *A. berlandieri* was packed into Whatman single thickness cellulose extraction thimbles (*ca* 50 g thimble, 200 g total) and extracted continuously for 24 hr with MeOH. The

MeOH was removed and replaced with $CHCl_3$ and the extraction continued an additional 24 hr. A pilot study established that the extracts could be safely concd by rotary evapn (H_2O aspirator). The MeOH extract was concd and the residue dissolved in 100 ml of $CHCl_3$. This soln was extracted $\times 3$ with 50 ml portions of 10% aq. HCl. The acid frs were combined, the pH adjusted to *ca* 10.3 by addition of aq. NaOH, and the resulting soln was first extracted $\times 3$ with 50 ml portions of $CHCl_3$ followed by extraction $\times 3$ with 50 ml portions of EtOAc. These organic extracts were combined, dried with $MgSO_4$, filtered, concd under vacuum, and stored under argon prior to analysis by GC-MS. The $CHCl_3$ fr. from the Soxhlet extraction was handled in an identical fashion to the MeOH extract with the exception that it was not first concd prior to aq. acid extraction. The MeOH and $CHCl_3$ extracts from the Soxhlet extraction were not combined.

Acid extraction of plant material. A. berlandieri, 100 g, was placed into a 1000 ml Erlenmeyer flask and mixed with 500 ml of 10% aq. acid (HCl and AcOH were each used). The suspension was stirred under Ar at 60° overnight. The darkened suspension was filtered through glass wool covered with a 2 cm bed of ashed and washed sand. The filtrate was extracted twice with 100 ml portions of EtOAc followed by $\times 3$ extraction with 100 ml portions of $CHCl_3$. The pH of the filtrate was adjusted to *ca* 10.3 by addition of NaOH pellets. This soln was extracted $\times 3$ with 150 ml portions of $CHCl_3$ followed by extraction $\times 3$ with 150 ml portions of EtOAc. The organic extracts were combined, dried over $MgSO_4$, filtered, concd under vacuum and stored under Ar prior to analysis by GC-MS.

Quantification of N-methyl-β-phenethylamine (NMPEA). Frozen samples of the foliage (*ca* 50 g each) were lyophilized. The dried plant matter, pulverised with mortar and pestle, was carefully weighed into 5 g samples. The powdered material was subjected to a Soxhlet extraction identical to that described above. The MeOH fr. was concd and combined with the $CHCl_3$ extract. The fractionation sequence was identical to that followed for the Soxhlet work-up. The residue was diluted to 5 ml in MeOH and aliquots of each sample was quantified by HPLC [14] and GC-MS.

HPLC quantification. Quantification by HPLC was made by derivatization of NMPEA with fluorescamine. This was accomplished by mixing 10 μl of the diluted residue with 100 μl of 0.05 M sodium borate buffer (pH 9.0), and 50 μl of a 0.5 mg ml^{-1} soln of fluorescamine in Me_2CO. The soln was vortexed for 30 sec and then extracted $\times 3$ with 200 μl portions of EtOAc. The pooled extracts were evapd to dryness under N_2 and redissolved in 100 μl of MeOH. The sample, 25 μl, was injected on the HPLC for analysis using a fluorescence detector. Quantification was made by determining the area under the peak. Previous runs with authentic NMPEA had established that the detector response was linear in the concn range being run. Triplicate samples were run for early and late season foliage and each sample was subjected to triplicate HPLC analysis.

GC-MS quantification. A sample, 1 μl, of each extract was injected via the heated injection port of the GC-MS. Quantification was made by determining the area under the *m/z* 44 peak. The concn was determined by comparing this value to a standard curve generated by injection of a series of standards of known NMPEA concn. Previous runs with NMPEA had established that the detector response was linear in the concn range being run. Triplicate samples were run for early and late season foliage and each sample was subjected to triplicate GC-MS analysis.

Preparation and injection of quaternary ammonium salts. Phenethylamine, 1 mg (8.25 μmol) in 0.5 ml of freshly distilled THF was mixed with 50 μl (114 mg, 0.81 mmol) CH_3I and allowed to stir for 30 min. The reaction mixt. was evapd under vacuum, redissolved in MeOH and evapd to dryness to remove traces of CH_3I (repeated twice), the residue was dissolved in 100 μl of 1 M NaOH in MeOH. When a 1 μl sample of this was injected into the heated injection port of the GC-MS, styrene was detected. When an identical sample was injected on-column, no peak corresponding to styrene was detected. Although, after the oven temp. reached 230°, selected ion monitoring detected an increase in the background level and the presence of an ion with *m/z* 104. This would be expected from the decomposition of the quaternary ammonium salt on the column. The quaternary ammonium salts of tyramine and mescaline were prepd and injected with similar results.

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