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Technical Note: An Improved Method for Extraction and Quantification of Toxic Phenethylamines from *Acacia berlandieri*[1]

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ABSTRACT: N-methyl-β-phenethylamine (NMPEA) has been previously identified as the toxin causing locomotor ataxia in sheep and goats grazing the browse plant, *Acacia berlandieri*. We describe a simplified procedure for extraction and quantification of naturally occurring β-phenethylamines from this *Acacia* species. Dried, ground plant tissue was extracted (1:20 wt/vol) with 1% glacial acetic acid and filtered. The filtrate was passed through a high-sulfonated polymeric solid-phase extraction (SPE) tube, which retained the compounds of interest (tyramine, hordenine, NMPEA) but allowed many impurities co-extracted from the plant tissue to be washed through. Amines were eluted from the tube, then separated and detected by reversed-phase HPLC. Extracted amines were resolved by HPLC in < 15 min, and UV-absorbance spectra matched those of authentic standards. Recovery efficiency of amine standards (125 μg/mL) from SPE tubes averaged 97, 101, and 98% for tyramine, hordenine, and NMPEA, respectively. Excess sample loss was prevented and the large volumes of solvents required for liquid-liquid extraction eliminated by use of solid-phase extraction techniques.

Key Words: *Acacia berlandieri*, Phenethylamines, Extraction, HPLC

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Introduction

β-phenethylamine and its derivatives commonly occur throughout the plant kingdom (see review by Smith, 1977). These compounds are important due to their physiological activity in higher animals and humans (Camp and Norvell, 1966; Strong, 1966). During periods of drought in west Texas, sheep and goats grazing on *Acacia berlandieri* Benth. have developed locomotor ataxia, also referred to as the "guajillo wobbles" or "limberleg" (Price and Hardy, 1953). N-methyl-β-phenethylamine (**NMPEA**) was isolated and identified as the toxic compound inducing this condition (Camp and Lyman, 1956). Later studies revealed the presence of three additional β-phenethylamines in *A. berlandieri*: tyramine, N-methyltyramine, and hordenine (Adams and Camp, 1966).

A rapid, easy method of extraction and quantification of potentially toxic amines in forage or browse plants is needed. In the past, researchers have relied on liquid-liquid partitioning, spectroscopy, and paper, thin-layer, and gas-liquid chromatography (Camp and Lyman, 1956; Camp and Moore, 1960; Adams and Camp, 1966). More recently, HPLC has been employed to analyze naturally occurring toxic amines as well as follow the course of these compounds in physiological studies (Knox et al., 1983; Kawasaki et al., 1989; Mower et al., 1989; Slocum et al., 1989). Solid-phase extraction procedures have been developed in recent years for isolation of drugs, such as amphetamine, from urine (Patel et al., 1990). Our objective was to develop a simple, rapid procedure with high recovery rates to extract and quantify toxic β-phenethylamines from the shrub, *A. berlandieri*, using a combination of solid-phase extraction and HPLC methods.

Materials and Methods

Collection of Plant Samples. Samples of *Acacia berlandieri* Benth. were collected from plants growing on a southwest-facing slope in Zavala County, TX. All leaves and petioles were removed from adjoining stems until approximately 500 g of fresh weight had been gathered. The fresh material was sealed in waterproof bags and placed on ice immediately. Samples were then frozen at −20°C, lyophilized, ground through a 1-mm screen in a cyclone mill, and stored at 7°C until extraction.

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Table 1. Recovery efficiency of β-phenethylamine standards from solid-phase extraction tubes

Tube	Recovery, %		
	Tyramine	Hordenine	N-methyl-β-phenethylamine
1	96	97	101
2	103	92	103
3	96	106	100
4	94	106	95
5	96	104	91
$\bar{x}$ ± SE	97 ± 3.5	101 ± 6.2	98 ± 4.9

[a]Standards were prepared in .1% phosphoric acid at a concentration of 125 μg/mL.

Extraction of Phenethylamines from Plant Tissue. The dried, ground *Acacia* leaves were extracted overnight on a rotary shaker at 150 rpm, using a ratio of 1 g of tissue:20 mL of 1% glacial acetic acid. All glassware used for this study was silanized (surface deactivated) with Aquasil (Pierce, Rockford, IL) as per label directions. The extract was filtered through a Whatman No. 1 filter (Fisher Scientific, Raleigh, NC) to remove plant tissue.

Further sample extraction was performed using a Polysorb MP-3 high-sulfonated C-18 polystyrene solid-phase extraction (**SPE**) tube (Interaction Chemical, Mountain View, CA). Tubes were conditioned with 2 mL of HPLC-grade methanol, followed by 2 mL of deionized water, then 2 mL of 1% glacial acetic acid, taking care to leave enough acid solution in the tube to ensure a continuous flow of liquid through the tube when the sample was added. Four milliliters (2 × 2.0-mL aliquots) of the *Acacia* filtrate were passed through the tube, again maintaining a continuous flow of liquid between aliquots. (Two aliquots were necessary because tube capacity was only 3 mL). Most unwanted compounds were washed from the tube with 2 mL of deionized water followed by 2 mL of 50% methanol (acidified with one drop of glacial acetic acid per 100 mL). Amines were eluted into autosampler vials with 2.0 mL of 10% ammonium hydroxide in methanol. The eluent was acidified by addition of 1.0 mL of 10% glacial acetic acid first, followed by 1.0 mL of 4% phosphoric acid. No further sample preparation was necessary before HPLC. The SPE tubes were regenerated with 3 mL of methanol, followed by 3 mL of deionized water, and 3 mL of methanol again, then reconditioned as above. Tubes were reused three times to determine whether reuse caused any degradation in performance. Aliquots of the same *Acacia* sample were used for this procedure. Performance data were subjected to the GLM procedure (SAS, 1987) and means were compared using Fisher's protected LSD test (Steel and Torrie, 1980).

High Pressure Liquid Chromatography of Amine Standards and Acacia Extract. N-methyl-β-phenethylamine and tyramine (4-hydroxyphenethylamine) were purchased from Aldrich Chemical (Milwaukee, WI), hordenine (N,N-dimethyl-2-[4-hydroxyphenyl]ethylamine) was purchased from Lancaster Synthesis (Windham, NH), and phenethylamine was purchased from Spectrum Chemical (Gardena, CA). Standards (140 μg/mL) were prepared in .1% of phosphoric acid and stored at 7°C until use.

Amine standards and *Acacia* extracts were separated at ambient temperature on a 10-μm poly (styrene-divinylbenzene) Hamilton PRP-1 column, 150-mm × 4.1-mm i.d. (Hamilton, Reno, NV), using gradient elution and detected at 258 nm. Solvent A was .1% phosphoric acid (pH 2.2), solvent B was 35% HPLC-grade acetonitrile in .1% phosphoric acid (pH

Table 2. Multiple-use performance of regenerated solid-phase extraction tubes for extraction of β-phenethylamines from *Acacia berlandieri*

Tube use	Tyramine			Hordenine			N-methyl-β-phenethylamine		
	n	mg/g of DW	CV	n	mg/g of DW	CV	n	mg/g of DW	CV
1st	5	1.94[a]	5.9	6	.24[a]	24.3	6	3.01[a]	7.5
2nd	7	1.78[b]	5.7	6	.27[a]	23.2	6	3.28[a]	8.9
3rd	7	1.78[b]	2.7	7	.25[a]	27.3	6	3.21[a]	8.3
4th	8	1.71[b]	6.4	8	.25[a]	15.8	8	3.27[a]	7.8
LSD, .05	—	.11	—	—	.06	—	—	.30	—
Overall CV	—	—	6.8	—	—	22.0	—	—	8.4

[a,b]Means in the same column with different superscripts differ significantly ($P < .05$) according to Fisher's protected lsd.

2.6). The gradient was ramped (changed linearly over time) from 0 to 25% B in .1 min, held until 5 min, then ramped to 50% B in .1 min and held until the end of the run; the total run time was 20 min. Twenty additional minutes were allowed between samples for column reequilibration. Chromatograms and spectra were recorded with an HP 1040A photodiodearray detector coupled with an HP 9000 series 300 computer (Hewlett-Packard, Ft. Collins, CO). The mobile phase was delivered at 1.0 mL/min flow rate by Beckman 110A pumps (Beckman Instruments, Berkeley, CA). Fifty-microliter sample aliquots were injected by a WISP 710B autosampler (Waters Associates, Milford, MA).

Results and Discussion

A simple, rapid procedure for extraction, separation, and quantification of β-phenethylamines from *Acacia berlandieri* is described. Dried, ground plant tissue was extracted overnight, and reextraction of the same sample yielded no detectable amines. The use of solid-phase extraction tubes provided easy sample clean-up without sample breakthrough during tube washing. The polymer packing in these tubes contains both octadecyl and sulfonic acid groups, thus displaying both reversed-phase and cation-exchange properties. Selective retention and release of β-phenethylamines can be achieved by manipulation of pH and percentage of organic solvent during elution. Characteristic pH stability of the packing materials in polymeric-type SPE tubes ($0 < pH < 14$) and HPLC columns ($1 < pH < 13$) allows the use of very acidic/basic washing and eluting solvents. Conventional silica-based materials are limited to $3 < pH < 7$ (Snyder et al., 1988) and would dissolve under the extreme pH conditions of this procedure. Use of a polymeric reversed-phase instead of a silica-based HPLC column also eliminated the possibility of residual silanol interactions and need for mobile phase additives to suppress these secondary retention mechanisms.

β-phenethylamine standards at a concentration of 125 μg/mL were recovered with ≥ 97% efficiency from SPE tubes (Table 1). For analysis of plant samples, the tubes were regenerated for reuse three times to determine whether reuse caused any significant degradation in performance (Table 2). Only in the case of tyramine did tubes exhibit lower ($P < .05$) recovery rates with reuse, but then only between the first use and subsequent reuses. Recovery of NMPEA did not reveal any trends with tube reuse. Hordenine levels were so low in the plant tissue that any changes in tube efficiency would have been obscured by the large variability observed in quantification accuracy. Unidentified compounds coextracted from the plant tissue probably contributed to tube degradation. Some

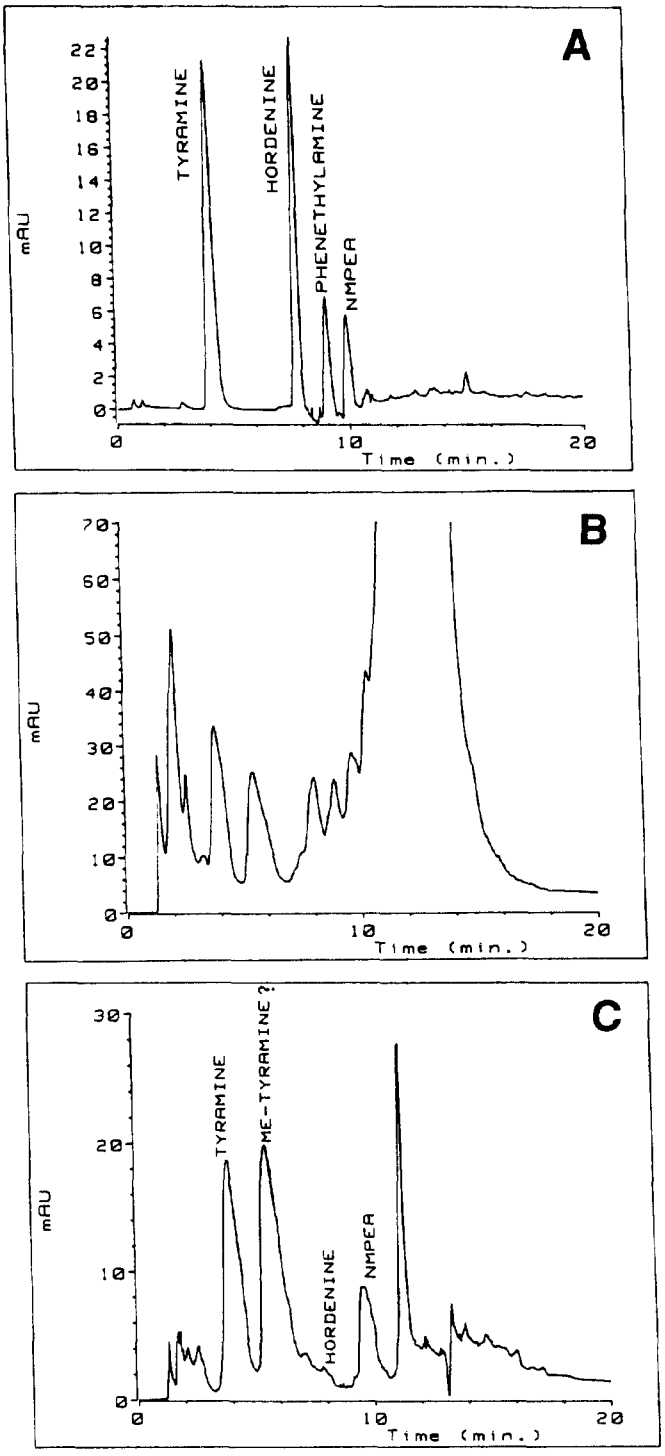

Figure 1. Reversed-phase separation of β-phenethylamines on a 10-μm poly (styrene-divinylbenzene) 150-mm × 4.1-mm column using gradient elution. A) β-phenethylamine standards at 62 μg/mL (tyramine, hordenine, NMPEA) or 50 μg/mL (phenethylamine). NMPEA = N-methyl-β-phenethylamine. B) Crude extract of *Acacia berlandieri*. Note presence of large impurity peak eluting between 8 and 16 min. C) *Acacia berlandieri* extract after clean-up with solid-phase extraction tube.

of these impurities were irreversibly bound to the tube packing and could not be washed off during regeneration. Build-up of impurities was observed as an increasing discoloration of the tube packing. Therefore, for the most accurate quantification of tyramine from *Acacia berlandieri*, SPE tubes should only be used once. If tube reuse is desired, crude plant extracts and other "dirty" samples may require additional clean-up steps to remove impurities before employing SPE tubes as the final extraction step.

Gradient elution procedures employed satisfactorily resolved the β-phenethylamines present in *Acacia berlandieri* in < 15 min (Figure 1). Phenethylamine was included in the standard mixture, but there were no peaks in the sample corresponding to this compound. Phenethylamine has been reported to occur in other *Acacia* species (Camp and Norvell, 1966; Smith, 1977).

No standard was available for N-methyltyramine and thus peak identity could not be confirmed. However, based on the similarity of its chemical structure to that of tyramine, and the fact that its presence has been previously reported in *A. berlandieri* (Adams and Camp, 1966), we speculate that the compound labeled N-methyltyramine in Figure 1 is indeed that. Comparison of its absorption spectra with that of authentic tyramine suggests a high degree of structural similarity.

The extraction and HPLC separation method described here represents a faster and simpler procedure for analysis of β-phenethylamines from *A. berlandieri* using solid-phase extraction tubes. Sample loss is minimized and use of large volumes of hazardous solvents reduced through elimination of liquid-liquid extraction procedures. Our SPE tube procedure may be potentially useful for extraction of other β-phenethylamines present in plant tissues, as well as extraction of catecholamines from biological fluids for metabolic studies.

Implications

The shrub *Acacia berlandieri* contains β-phenethylamine compounds, some of which have proven to be toxic to sheep and goats. A faster procedure with high recovery rates has been developed to extract and measure these toxic compounds. Solid-phase extraction tubes are used to prepare the samples for high performance liquid chromatography. This improved method will enhance research efforts to measure seasonal and environmental effects on levels of toxic phenethylamines in the shrub *Acacia berlandieri*.

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