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Efficient and Sensitive Method for Quantitative Analysis of Alkaloids in Hardinggrass (*Phalaris aquatica* L.)

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An efficient high-performance thin-layer chromatography (HPTLC) method for the analysis of alkaloids in hardinggrass (*Phalaris aquatica* L.) was developed. The method employed HPTLC glass plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of ethyl acetate/chloroform/7 N NH_4OH in methanol (8:2:1, v/v/v). Using unidimensional double-development, bands were well separated for 10 alkaloid standards as well as alkaloids observed in hardinggrass plant extracts. Identities of compounds observed using HPTLC were validated by high-performance liquid chromatography−mass spectrometry (HPLC-MS). Software was used to quantify individual alkaloids in plant samples based on HPTLC retention factors and intensities relative to standards of known concentration. Correlation coefficients of 0.99 were obtained between estimated and actual concentrations for four standards (methyltyramine, hordenine, gramine, and 5-methoxydimethyltryptamine), with linearity in the range of 120−3840 ng/spot. The HPTLC method is repeatable and specific for β-carboline, tryptamine, gramine, and tyramine type alkaloids in mixed standard and plant extracts. Initial results indicate substantial variation in alkaloid composition among and within hardinggrass populations.

KEYWORDS: ***Phalaris aquatica*; thin-layer chromatography (TLC); high-performance thin-layer chromatography (HPTLC); alkaloids; quantitative analysis; hardinggrass**

INTRODUCTION

Hardinggrass (*Phalaris aquatica* L.) is a cool-season perennial grass that has shown agronomic potential in Oklahoma (*1*) and other southern Great Plains environments. However, a number of alkaloids have been reported in hardinggrass, some of which have been implicated in animal health disorders such as "Phalaris staggers" (*2*, *3*) and a sudden-death syndrome (*4*, *5*). Alkaloid profiles of hardinggrass populations are influenced by genotype (*6*) and environment (*7*). The recent report of alkaloid-induced livestock death from a "low-alkaloid" hardinggrass cultivar (*8*) indicates that greater knowledge of alkaloid composition in hardinggrass is needed.

Several methods have been used to examine alkaloid profiles of hardinggrass or related *Phalaris* species. Methods such as paper chromatography (*6*, *9*), total base saturation (*7*, *9*), a silicotungstic colorimetric test (*10*), and thin-layer chromatography (TLC) (*5*, *11*) have been used. These methods are only able to estimate total alkaloids, cannot distinguish between all of the alkaloid compounds reported in hardinggrass, and/or have limited throughput capacity. Methods such as high-performance liquid chromatography (HPLC) (*12*), gas chromatography−mass spectrometry (GC-MS) (*11*), and GC (*13*) require expensive equipment and have limitations for large-scale screening of samples. An immunoassay (ELISA) method was developed for the analysis of *Phalaris* alkaloids (*14*), although this test was not specific for individual alkaloid compounds and may have been cross-reacting with unknown tryptamines present in hardinggrass. The separation of alkaloids in *Phalaris* species is an interesting methodological problem because these compounds can have different polarities and chromatographic properties as well as closely related structures. Moreover, a relatively accurate quantification method for each individual alkaloid is necessary because the toxic effect appears to be variable between different compounds (*15*).

TLC is a simple and widely used analytic method for mixtures of plant compounds and drugs (*16*). HPTLC has much smaller particle and pore sizes of adsorbents than standard TLC. Advantages of HPTLC may include high efficiency, shorter migration distance, decreased analysis time, and a smaller volume of mobile phase compared to TLC or other methods that have been used in the analysis of plant natural products and drugs. The objective of this study was to develop a high-

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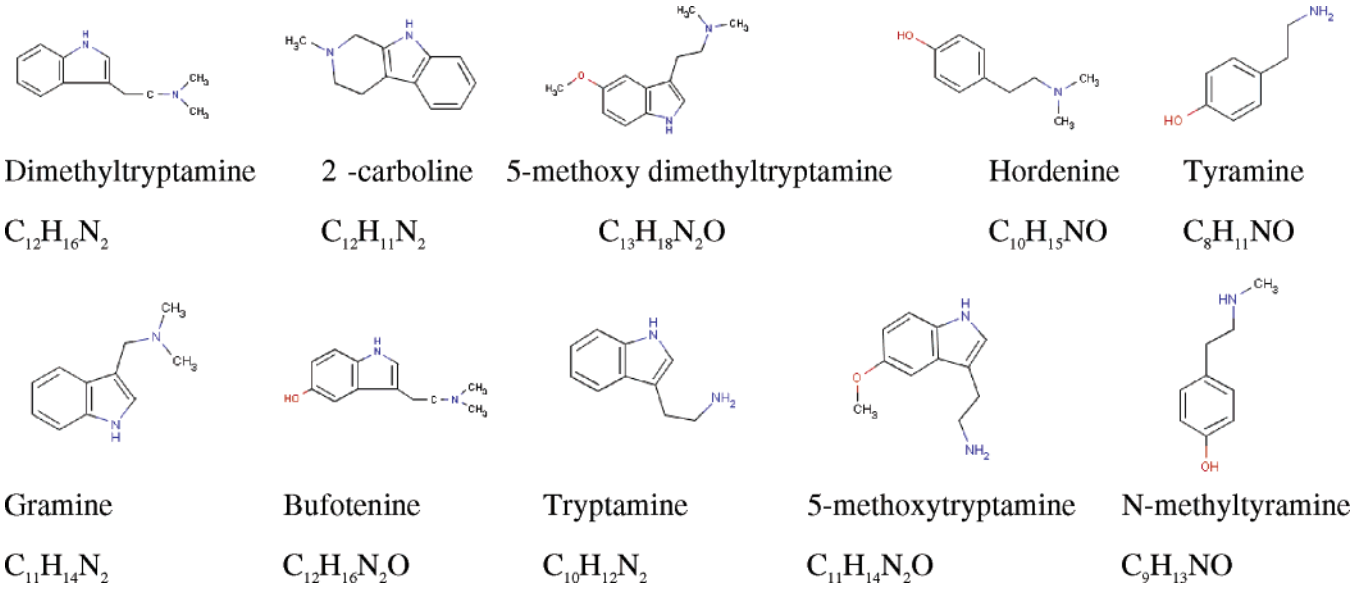

Figure 1. Chemical structures and formulas for alkaloid compounds examined using HPTLC.

throughput HPTLC method for the qualitative and quantitative analysis of alkaloids in hardinggrass that could be used to develop hardinggrass cultivars with decreased risk of alkaloid toxicity.

MATERIALS AND METHODS

Standards and Chemicals. Ten alkaloids were examined in this research (**Figure 1**). Dimethyltryptamine (Sigma-Aldrich, St. Louis, MO) and bufotenine (Cambridge Isotope Labs, Andover, MA) are both controlled substances. The appropriate permits were obtained from the U.S. Drug Enforcement Agency and Oklahoma Bureau of Narcotics for purchasing and possessing these compounds. Controlled substances were stored and disposed of in accordance with federal regulations. Tyramine, 5-methoxytryptamine, tryptamine, gramine, and hordenine sulfate were purchased from VWR International (West Chester, PA); 5-methoxydimethyltryptamine was purchased from Sigma-Aldrich. Small quantities of methyltyramine and 2β-carboline were gifts of Dr. S. M. Colegate's lab (CSIRO, Australia). All standards were dissolved in methanol at a known concentration of 10−20 mg/mL as standard reference stock solutions. A 7 N NH_4OH in methanol solution (product 499145) was purchased from Sigma-Aldrich.

Extraction and Purification of Alkaloids from *P. aquatica*. To determine variations in alkaloid profiles between hardinggrass populations, plots were seeded in the fall of 2004 at Ardmore, in south central Oklahoma; near Iowa Park, in north central Texas; and in Lubbock, in far west Texas. A randomized complete block design, with three or four replications, was used at each location. Hardinggrass plots were sampled in May 2005 from several locations within each plot by hand clipping at approximately 10 cm of height. Samples were placed immediately on ice and subsequently freeze-dried before grinding in a UDY cyclone mill (Udy Corp., Fort Collins, CO) to pass a 1 mm screen. Extraction and purification of alkaloids from hardinggrass was performed using a modification of the procedure of Anderton et al. (*11*). Dry powder (0.2 g) was immersed in a 15 mL centrifuge tube containing 10 mL of 1% HCl and kept at room temperature with occasional shaking. After 3−4 days of extraction, the sample was centrifuged at 3000 rpm for 15 min at 4 °C. A 3 mL solid-phase extraction (SPE) aromatic sulfonic acid column (J. T. Baker Inc., Phillipsburg, NJ; product 7090-03), containing 500 mg of packing material, was prepared by washing with 3 mL of 80% methanol and 3 mL of distilled water under a moderate vacuum, after which the sample supernatant was loaded onto the column to adsorb alkaloids. The column was rinsed with 2−3 mL each of 1% HCl, distilled water, and methanol. Alkaloids were eluted from the column using 2 mL of an ethanol/7 N NH_4OH in methanol (1:1, v/v) solution. The solvent was evaporated immediately under N_2 gas flow and the residue dissolved in 50 μL of methanol.

Variation of alkaloid profile within a population was examined by collecting samples from the parent plants of MIP C2, which were derived from the cultivar Maru following selection for persistence (*17*). Samples were collected in the fall of 2003 from individual plants grown in a space planted nursery at Ardmore, OK, that was planted as a randomized complete block design with two replications. Alkaloids were extracted with HCl from 4 g of fresh leaf tissue following the procedure of Anderton et al. (*11*). Purification methods were as described above.

Chromatographic Materials and Methods. Whatman LK6DF TLC silica gel 60 F254 glass plates (20 × 20 cm) were purchased from VWR International (Bristol, CT). Merck silica gel 60 F254 preconcentration zone HPTLC glass plates were purchased from Alltech (Deerfield, IL). Plant extract (2 μL) was loaded to the edge of the layer. Standard stock solutions were loaded at 1−2 μL for a known amount of 1−2 μg per standard per lane. After the screening of several mobile phase solvent systems, a preferred mobile phase solvent system was developed that consisted of ethyl acetate/chloroform/7 N NH_4OH in methanol (8:2:1, v/v/v). Plates were developed until the mobile phase solvent front was within 2 cm of the top of the plate, which took approximately 20 min. For double-development, plates were allowed to dry for 15−20 min before being placed a second time in mobile phase solvent. Plates were dried at room temperature for 15 min, heated in an oven for about 5 min at 70 °C, and documented by photography using a GDS 8000 gel documentation system (UVP, Inc., Upland, CA) under UV 254 and UV 365 irradiation. After spraying with acidified anisaldehyde reagent consisting of ethanol/H_2SO_4/acetic acid/anisaldehyde (36:2:0.4:2, v/v/v/v), plates were heated with a Sunbeam 1600 hair dryer (Jarden Consumer Solutions, Inc., Boca Raton, FL) for 1−2 min on low followed by 4−5 min on high and then placed for 30 min in an oven at 70 °C. Color images of plates were then scanned with a UMAX PowerLook 1100 flatbed digital scanner (UMAX Technologies, Inc., Dallas, TX).

Quantitative Analysis. The quantification method was evaluated via linear regression analysis of a series of known standards on a HPTLC plate. Alkaloid standards gramine, 5-methoxydimethyltryptamine, methyltyramine, and hordenine (sulfate salt), the four major alkaloids in hardinggrass, from a stock solution of 20 mg/mL were mixed and diluted to 15, 30, 60, 120, 240, 480, 960, 1920, and 3840 ng/μL for each standard. One microliter each of the mixed dilutions was applied to the HPTLC plate. The plate was developed, sprayed, dried, and scanned as described above. The image was imported into DNA Quantity One software (Bio-Rad Laboratories, Hercules, CA), the band intensity was measured, and data were exported to an Excel spreadsheet file.

The alkaloid amount for each individual band with the same retention factor (R_f) and color as a corresponding standard was

calculated via the equation

alkaloid (μg) = (intensity from sample/intensity from standard) × μg of standard loaded

High-Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS). Burdick and Jackson HPLC grade acetonitrile and water were obtained from VWR International (West Chester, PA). Ammonium acetate was obtained from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Samples were extracted as for HPTLC, and HPLC separation was achieved using a 250 × 4.6 mm i.d., 5 μm, reverse-phase, C18 column (J. T. Baker, Inc.). Samples were eluted with a gradient of 90:10 mobile phases A/B to 10:90 in 40 min, at a flow rate of 0.8 mL/min. Mobile phase A consisted of 0.1 M ammonium acetate and 0.05% TFA in water. Mobile phase B was 100% acetonitrile. The ammonium acetate and TFA were added to aid in positive-ion generation by atmospheric pressure chemical ionization (APCI).

All mass spectra were acquired using a Bruker Esquire LC equipped with an APCI source. Postitive-ion APCI was performed using a source voltage of 4000 V and a capillary offset voltage of 45 V. The corona voltage was set at 2000 V. Nebulization was achieved using nitrogen gas at a pressure of 0.483 MPa. Desolvation was aided by the use of a nitrogen counter current gas at a pressure of 0.076 MPa. The capillary temperature was set at 350 °C and the APCI temperature at 400 °C. Mass spectra were recorded over the range of *m*/*z* 50−1500. The Bruker ion-trap was operated under an ion current control of 20000, a maximum acquire time of 100 ms, and a trap drive setting of 35. The identity of compounds from HPTLC analysis was examined by scraping bands from the HPTLC plate, dissolving in methanol, analyzing by HPLC-APCI-MS as described above, and cocharacterization of authentic compounds.

RESULTS AND DISCUSSION

Solvent System Selection and Developing Method. The mixtures obtained from hardinggrass plant extracts contain four groups of alkaloids (tryptamines, gramines, tyramines, and β-carbolines) with similar molecular structures and chemical properties, which can be very difficult to separate (*11*). Of the wide range in mobile phase solvents examined, an ethyl acetate/chloroform/7 N NH_4OH in methanol (8:2:1) solution generally gave the best separation of standards, with specific alkaloids being more clearly separated by HPTLC compared to TLC (data not shown). In addition, HPTLC required less sample and standards and had faster development times than TLC. Double-development in the unidimensional direction resulted in clear separation of 10 alkaloid standards on a 20 × 10 cm preconcentration zone HPTLC plate (**Figure 2** and **Table 1**). This system allowed detection and separation of 5-methoxydimethyltryptamine, hordenine, gramine, and methyltyramine standards at concentrations as low as 60−120 ng/μL (**Figure 3**). Slight modification of chloroform or NH_4OH concentration in this solvent system resulted in reduced separation resolution (data not shown). In particular, ammonia concentration was critical in the separation of hordenine from 5-methoxydimethyltryptamine and tyramine from 5-methoxytryptamine. Precautions used to maintain proper NH_4OH concentration included use of fresh solutions for each series of analyses, sealing containers holding NH_4OH solutions, refrigeration of 7 N NH_4OH in methanol, and storage inside sealed plastic.

HPTLC plates were visualized following development with anisaldehyde and then imaged using multiple ultraviolet frequencies, which provided additional information for the identification of hardinggrass alkaloids. For example, dimethyltryptamine, tryptamine, 5-methoxydimethlytrypamine, and 5-methoxytryptamine appeared as dark bands under UV 254, whereas only the 5-methoxy derivatives were visible, as bright bands, under UV 365 (**Figure 2**). If these compounds are of specific interest, they can be identified without subsequent spray reagent steps.

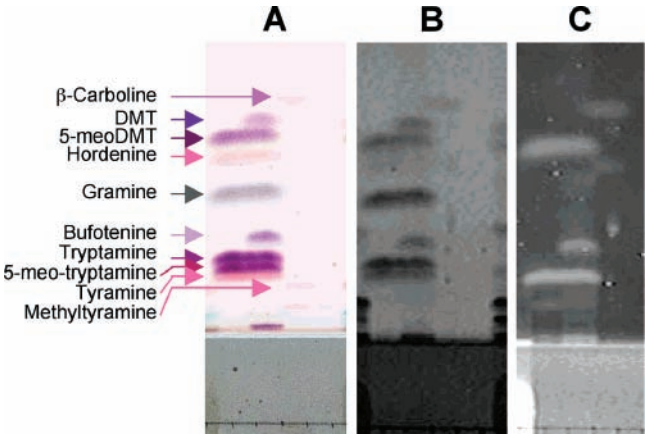

Figure 2. Chromatography for separation of 10 standards in HPTLC 20 × 10 preconcentration zone plate: (**A**) anisaldehyde reagent spray; (**B**) UV 254 μm imaging; (**C**) UV 365 μm imaging. (lane 1) six mixed standards; (lane 2) eight mixed standards; (lane 3) standard for β-carboline and methyltyramine. Double development was performed using a mobile phase consisting of ethyl acetate/chloroform/7 N NH_4OH in methanol (8:2:1). DMT, dimethyltryptamine; 5-meoDMT, 5-methoxydimethyltryptamine; 5-meo-tryptamine, 5-methoxytryptamine.

Table 1. Retention Factor (R_f) and Color for Alkaloid Standard Compounds Separated Using High-Performance Thin-Layer Liquid Chromatography[a]

standard	R_{xc4}	color
β-carboline	0.64	pink
dimethyltryptamine	0.59	light purple
5-methoxydimethyltryptamine	0.55	purple
hordenine	0.51	orangish red
gramine	0.4	grayish purple
bufotenine	0.29	purple
tryptamine	0.24	dark purple
5-methoxytryptamine	0.21	purple
tyramine	0.19	red
methyltyramine	0.16	red

[a] Preconcentration zone 20 × 10 plates were double developed using a mobile phase solvent consisting of ethyl acetate/chloroform/7 N NH_4OH in methanol (8:2:1) followed by spraying with anisaldehyde.

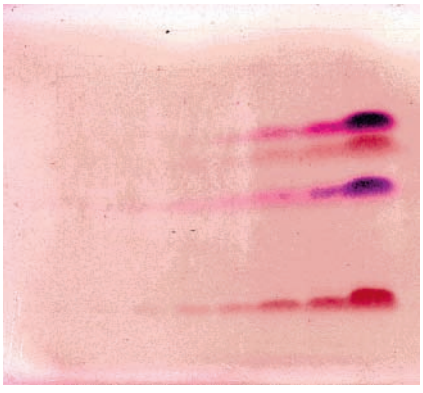

Figure 3. HPTLC of four selected standards, from top to bottom, 5-methoxydimethyltryptamine, hordenine, gramine, and methyltyramine with variable concentration in preconcentration zone 20 × 10 HPTLC plate. From right to left, standard concentration was reduced by half in each lane from 3820 ng/μL to a final concentration of 15 ng/μL.

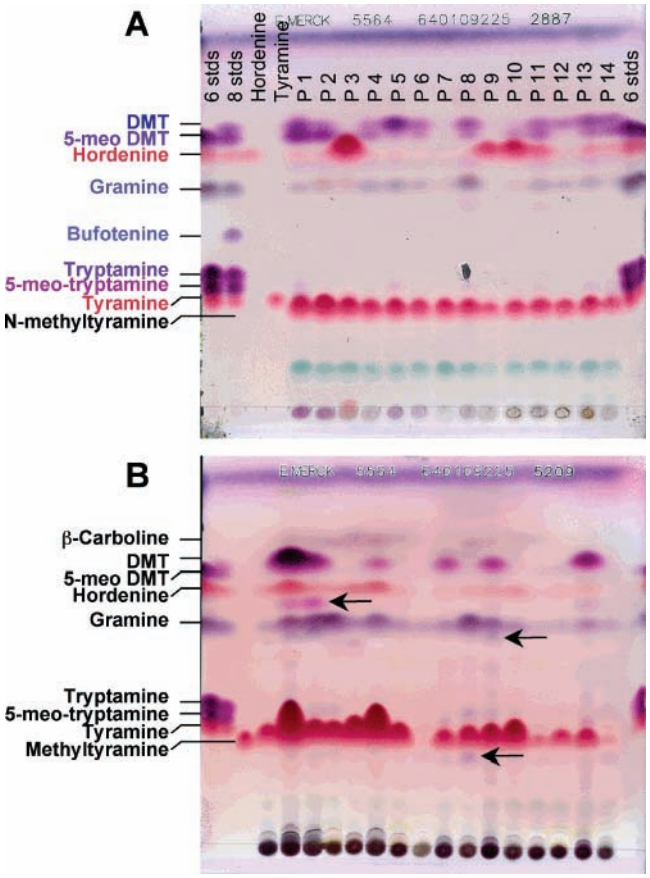

Figure 4. Alkaloid profiles from (**A**) 14 hardinggrass populations sampled in May 2005 (alkaloids extracted from freeze-dried powder of ground plant sample) and (**B**) individual plant leaf samples within a hardinggrass population (alkaloids extracted from 4 g of fresh leaf in 45 mL of 0.1 N HCl). Arrows in (**B**) indicate unknown alkaloids. The green/blue band at the bottom of the HPTLC plate in (**A**) is found in all whole plant samples examined to date including samples from other than hardinggrass and does not appear to be an alkaloid. DMT, dimethyltryptamine; 5-meoDMT, 5-methoxydimethyltryptamine; 5-meo-tryptamine, 5-methoxytryptamine.

Quantification Analysis. Plots of known concentrations of alkaloid standards compared to concentrations predicted on the basis of HPTLC spot intensity, as determined with DNA Quantity One software, revealed linearity in the range of 120−3840 ng/spot. Specifically, correlation coefficients (r^2) were determined to be 0.991 ± 0.006 for methyltyramine, 0.994 ± 0.004 for hordenine, 0.994 ± 0.006 for gramine, and 0.996 ± 0.003 for 5-methoxydimthelytryptamine on the basis of four replications. The method was repeatable and specific for β-carboline, tryptamine, gramine, and tyramine type alkaloids in mixed standard and plant extracts. The method was also repeatable from sample to sample and from plate to plate for plant samples. Quantitative analysis of duplicate plant samples resulted in variation of <5% of the mean (data not shown).

Prescreening of Hardinggrass Alkaloid Profiles. The HPTLC method was applied to samples collected from multiple locations in the southern Great Plains to get an initial indication of variation in alkaloid composition of several hardinggrass populations. **Figure 4** shows alkaloid profiles from (**A**) 14 hardinggrass populations and (**B**) individual plants within a hardinggrass population developed by the Noble Foundation breeding program. To our knowledge, this is the first demonstration that most hardinggrass populations contain five to six known alkaloid compounds (β-carboline, dimethyltryptamine, 5-methoxydimethyltryptamine, gramine, hordenine, and methyltyramine) in addition to some populations containing unidentified compounds (no standards available). Qualitative variation in alkaloids present within a population appears to be greater than that between populations, implying that plant to plant genetic variation is a major factor influencing the alkaloid profile in hardinggrass. This observation is similar to that of Mian et al. (*17*), who found approximately 3 times as much genetic variation within, compared to among, a number of hardinggrass populations. Quantification of alkaloids can be achieved by comparing known standard concentrations with plant samples via conversion of color images to a densitogram using DNA Quantity One software, as was done for five hardinggrass populations analyzed in **Figure 4A (Table 2)**.

Table 2. Alkaloid Concentrations, in Milligrams per Kilogram of Dry Matter, Quantified Using High-Performance Thin-Layer Liquid Chromatography of Samples Collected from Five Hardinggrass Populations

population	dimethyl-tryptamine	5-methoxy-dimethyl-tryptamine	hordenine	gramine	methyl-tyramine
P 1	177	176	76	47.5	257.5
P 12	66.3		57.5	45	193.8
P 3			833.8		262.5
P 9			266.3		171.3
P 7				22.5	221.3

Table 3. Molecular Weight (MW), Selected Ion in Atmospheric Pressure Chemical Ionization Mass Spectrometry, and Retention Time (min) in High-Performance Liquid Chromatography for Nine Alkaloid Standard Compounds

compound	MW	selected ion	retention time
tyramine	137.18	121	5.4
methyltyramine	151	151	7
hordenine	165	166	9.3
bufotenine	204.12	204.9	9.3
5-methoxytryptamine	190.24	174	13
tryptamine	160.2	144	14.3
gramine	174.24	130	19
5 methoxydimethyltryptamine	218.3	218.9	19.6
dimethyltryptamine	188	188.9	22.2

Application of HPLC-MS in the Identification of Alkaloids Present in Hardinggrass. An HPLC-MS method was used to confirm the identity of various alkaloids in hardinggrass identified by the HPTLC method. The retention times on HPLC and specific ion for 10 authentic standards in MS are given in **Table 3**. β-Carboline was not detectable using the described HPLC solvent system. Using HPLC-APCI-MS, alkaloids from a plant extract were easily recognizable on the basis of comparison to authentic standards (**Figure 5**). Alkaloid bands scraped from HPTLC plates were similarly identified by HPLC-APCI-MS comparison to authentic peaks. For quantitative analysis of a large number of plant samples, HPLC coupled with MS is relatively expensive, due to equipment costs, and time-consuming because each sample analysis requires approximately 1 h, with additional maintenance time needed for the MS spray source every 10−15 samples. Thus, alternative methods such as the HPTLC protocol reported here provide a lower cost alternative to HPLC-MS.

We are investigating further those bands on HPTLC plates and ion peaks from MS data that do not match available standards. Once these compounds are identified, we will attempt to purchase or synthesize these compounds and apply them to HPTLC analysis as references.

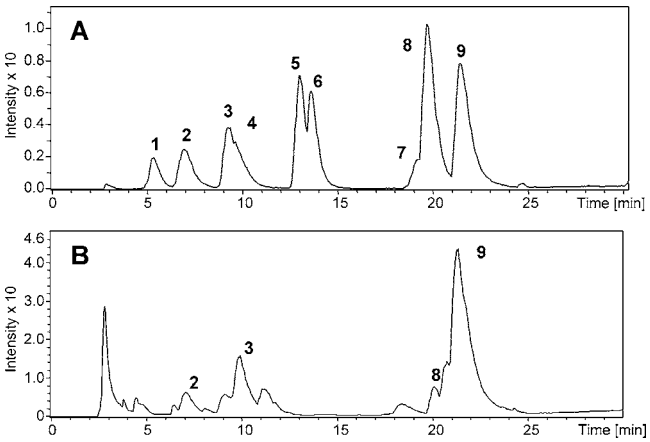

Figure 5. HPLC-MS base peak chromatogram for (**A**) combined standards and (**B**) an individual plant sample: 1, tyramine; 2, methyltyramine; 3, hordenine; 4, bufotenine; 5, 5-methoxytryptamine; 6, tryptamine; 7, gramine; 8, 5-methoxydimethyltryptamine; 9, dimethyltryptamine.

The HPTLC method described here is simple with minimal special equipment required, rapid (analysis of dozens to 100 samples simultaneously within 2−4 h, not including extraction time), low-cost (HPTLC plates, SPE columns, and chemicals cost <$4 per sample), sensitive (detection limits of <100 ng), and accurate compared to previous TLC methods. This HPTLC method is a powerful screening tool that will allow examination of genotype and genotype × environment effects for alkaloid composition in hardinggrass and can be applied in developing hardinggrass cultivars with reduced risk of alkaloid toxicity to livestock. The method could also be directly applied to other *Phalaris* species or in quantifying alkaloids in species such as barley (hordenine and gramine; *18*), toxic mushroom (dimethyltryptamine), sausage (tryptamine and β-carboline; *19*), and other agricultural products (*20*, *21*).

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Supporting Information Available: Supplementary Figure 6 and Tables 4 and 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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