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Note

Quantitative analysis of β-phenethylamines in two *Mammillaria* species *(Cactaceae)*

M. J. KNOX*, W. D. CLARK and S. O. LINK
Department of Botany and Microbiology, Arizona State University, Tempe, AZ 85287 (U.S.A.)
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β-Phenethylamines are a group of biogenic amines or alkaloids commonly found in *Cactaceae*[1]. The occurrence of these compounds is of both taxonomic[2,3] and ecological[4–6] importance.

Currently, there are few chromatographic techniques whereby cactus alkaloids are detected, resolved and quantified. These include thin-layer (TLC), gas and high-performance liquid chromatography (HPLC)[7]. Recent studies on the separation[8–11] and preparative isolation[12] of these compounds by ion-pair reversed-phase HPLC have been successful. However, rapid detection methods with both quantitative accuracy and precision have thus far not been applied to studying the natural variation of cactus alkaloids. The major objective of this study was to develop a system for the rapid screening and quantification of β-phenethylamines in cactus extracts. Tissue extracts of two species of *Mammillaria, M. microcarpa* Engelm. and *M. tetrancistra* Engelm., were analyzed by the method described herein.

EXPERIMENTAL

Plant material

Three plants each of *M. microcarpa* [found on south-facing slopes in the shade of *Larrea tridentata* (DC.) Coville] and *M. Tetrancistra* (found on west-facing slopes in open sunlight) were collected at Tempe Butte, Tempe, AZ, on April 23, 1982. Each plant was longitudinally bisected, one half providing a voucher specimen in Arizona State University. The remaining half of each plant was then divided into four tissue parts for analysis: (1) tubercles (TUBE); (2) cortex (CORT); (3) vascular tissue (VAST) and (4) root (ROOT).

Extraction and purification

The sequence by which extractions and HPLC analyses were made was randomized. Tissue parts were freeze-dried and then pulverized to a fine powder with a mortar and pestle. Sub-samples of 0.2 g were extracted for 4 h by ultrasonication in 3 ml of aqueous 2% ascorbic acid and 5% trichloroacetic acid. The extracts were centrifuged at 17,000 *g* for 15 min and the pellets re-extracted (3 ml) once since only trace quantities of β-phenethylamines were detected after further extractions. The two supernatants from each sample were combined and a 4-ml aliquot removed for

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analysis. Mescaline (0.2 mg in aqueous solution) was then added to the aliquot as an internal standard. The pH was adjusted to 10 (1 ml of 29.4% NH_4OH) and the final basic solution extracted with ethyl acetate (5 ml). The ethyl acetate layer was removed and dried under anhydrous nitrogen gas and the residue dissolved in 0.5 ml of water–methanol–acetic acid (90:10:2). Each sample was filtered through a Millipore filter (0.5 μm) and applied directly to HPLC (20-μl injection).

Reagents and solvents

All chromatographic solvents were "Baker Analyzed" HPLC grade. PIC B-7 (heptanesulfonic acid), purchased from Waters Associates (Milford, MA, U.S.A.), was used as an ion-pairing agent for chromatography. The reference compound N-methyltyramine (NMT) was provided in pure form (as revealed by HPLC analysis) by Jerry L. McLaughlin. Tyramine (TYR), 3,4-dimethoxy-β-phenethylamine (DMP) and mescaline (MES) were purchased from Sigma (St. Louis, MO. U.S.A.). Hordenine (HOR) was purchased from Pfaltz & Bauer (Stamford, CT, U.S.A.).

Instrumentation and chromatographic conditions

The equipment used was a Waters Associates Model 244 liquid chromatograph equipped with a programmable gradient elution system, and a UV absorbance detector set at 280 nm. A Hewlett-Packard Model 3390A reporting integrator was used for measuring retention times and peak areas. Analytical scale injections (20 μl) were separated on a μBondapak C_{18} column (300 × 3.9 mm I.D.) with a linear gradient from A–B (95:5) to A–B (70:30) [A = methanol–water–PIC B-7 (10:90:5); B = methanol–PIC B-7 (100:5)] over 40 min at 1.5 ml/min.

Compound identification

Plant extracts of *M. microcarpa* and *M. tetrancistra* were screened on thin layers (Merck, silica gel 60, 0.2 mm) in various solvent systems and the compounds of interest were visualized upon spraying with fluorescamine, dansyl chloride and iodoplatinate[13]. Tentative compound identification was accomplished by cochromatography of reference standards and crude extracts on TLC and HPLC. Further confirmation was supported by HPLC peak enhancement studies.

Quantitative determination

Quantification of alkaloid concentrations within tissue extracts was achieved by the internal standard technique. This technique, however, only accounted for relative losses due to sample processing and HPLC. To estimate the true alkaloid content of tissue extracts, it was necessary to correct for losses due to ethyl acetate partitioning. This was accomplished by implementing eqns. 1 and 2:

$$C_i = C_{is} \cdot \frac{A_i}{A_{is}} \cdot \frac{RF_i}{RF_{is}} \tag{1}$$

$$CC_i = C_i \cdot \frac{P_{is}}{P_i} \tag{2}$$

where C_i = concentration of constituent i; C_{is} = concentration of internal standard (MES); A_i = peak area of constituent i; A_{is} = peak area of internal standard (MES); RF_i = response factor of constituent i as determined by the integrator; RF_{is} = response factor of internal standard (MES) as determined by the integrator; CC_i = constituent i corrected for losses due to ethyl acetate partitioning; P_{is} = per cent of internal standard (MES) recovered from ethyl acetate partitioning and P_i = per cent of constituent i recovered from ethyl acetate partitioning.

The CC_i term best described the true alkaloid content of tissue extracts. P_{is} and P_i were experimentally determined by measuring the amount of alkaloid standard recovered in ethyl acetate after partitioning once with a known concentration of alkaloid standard in basic aqueous solution (pH 10). P_{is} (MES) is equal to 0.65 ± 0.03 ($\bar{x}$ ± s.e., n = 3); P_i is equal to 0.62 ± 0.02 (x ± s.e., n = 3), 0.39 ± 0.03, 0.76 ± 0.02 and 0.71 ± 0.03 for TYR, NMT, HOR and DMP, respectively.

Statistical analysis

All analyses were done on an IBM 3081 computer using the Statistical Analysis System (SAS) package[14]. Normality testing was performed with the program UNIVARIATE. The two-way analysis of variance and Tukey's multiple range test were based upon the general linear models program (GLM).

RESULTS AND DISCUSSION

Chromatographs

Ion-paired alkaloids of tissue extracts were resolved within 25 min with the described elution system. The five constituents of the standard mixture gave symmetrical and well resolved peaks (Fig. 1). Retention times varied from 7.5 to 20.4 min (Table I). High reproducibility in the separation of each compound is shown by the low coefficients of variation for the retention times.

Calibration graphs

Straight-line calibration graphs (Fig. 2) were obtained from alkaloid standards over a range of 0.2–1.0 μg (free base) by using UV detection at 280 nm. The graphs constructed represent a plot of peak area counts from integrator response factors (mV · sec). A significant regression ($P < 0.0001$ for each F-statistic) of UV response on alkaloid concentration was observed for each standard with the r^2 in each case approaching unity (0.984–0.998). Detection limits (signal-to-noise ratio > 2) were in the nanogram range.

Tissue analysis

In the quantitative analysis of 24 tissue samples, it was found that NMT and HOR, previously reported in *M. microcarpa*[15], were the predominant β-phenethylamines in the two cacti. TYR and DMP were also detected in *M. microcarpa*.

Frequency distributions for each of the alkaloids were significantly non-normal ($\alpha = 0.05$), skewed to the right. A logarithmic transformation normalized these data and made the alkaloid variances homoscedastic. Statistical analysis of the transformed data (two-way analysis of variance [ANOVA] for NMT and HOR) did not reveal significant differences between species for any given tissue type (Fig. 3). How-

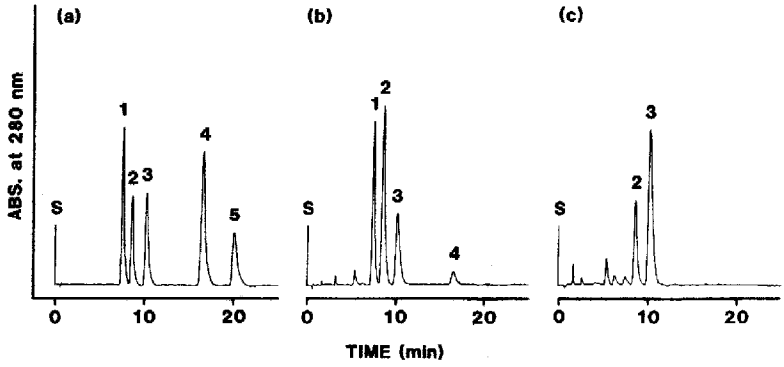

Fig. 1. HPLC separation of cactus alkaloids at 280 nm (0.02 a.u.f.s.): a, mixture of standards; b, extracts of *M. microcarpa*; c, extracts of *M. tetrancistra*. Peaks: S = inject; 1 = tyramine (1 μg); 2 = N-methyltyramine (1 μg); 3 = hordenine (1 μg); 4 = 3,4-dimethoxy-β-phenethylamine (1 μg); 5 = mescaline (5 g).

ever, significant differences ($\alpha = 0.05$) among tissues were observed for both compounds. The results of a Tukey's multiple range test ($\alpha = 0.05$) showed that levels of NMT were significantly greater in CORT than in ROOT or TUBE. HOR levels were found to be significantly greater in ROOT than in TUBE. Alkaloid levels were generally lower in the chlorophyllous tubercule tissue (TUBE).

Thus, while the two species differed in alkaloid composition, their magnitude of quantitative variation was similar. Such a quantitative parallelism indicates that some genetic or environmental factor is contributing to β-phenethylamine regulation in a manner common to both species. HPLC studies to investigate the effects of environment on chemical production in the genus are currently in progress.

CONCLUSION

Gradient elution combined with ion-pairing via HPLC was effective in the rapid separation and quantification of cactus alkaloids from small samples (0.2 g dry wt.) of plant material. The technique should prove useful as a method for studying β-phenethylamine variation in the *Cactaceae*.

TABLE I

RETENTION TIMES AND CORRESPONDING VALUES OF COEFFICIENTS OF VARIATION (C.V.) FOR THE ALKALOID STANDARDS

*Compound**	*Peak No.*	t_R *(min)***	*C.V.*
TYR	1	7.5	2.2
NMT	2	8.8	1.6
HOR	3	10.5	2.0
DMP	4	17.2	1.8
MES	5	20.4	1.6

* See text for abbreviations.

** Average retention times based on 15 chromatographic runs using the mixture of standards as in Fig. 1c.

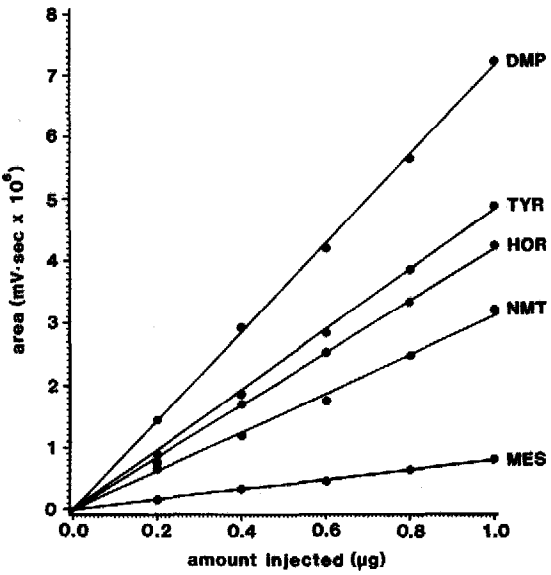

Fig. 2. Calibration graph of the reference alkaloids (each data point a mean of three determinations). The regression r^2 of each compound is as follows: TYR = tyramine, 0.998; NMT = N-methyltyramine, 0.984; HOR = hordenine, 0.993; DMP = 3,4-dimethoxy-β-phenethylamine, 0.995; MES = mescaline, 0.994.

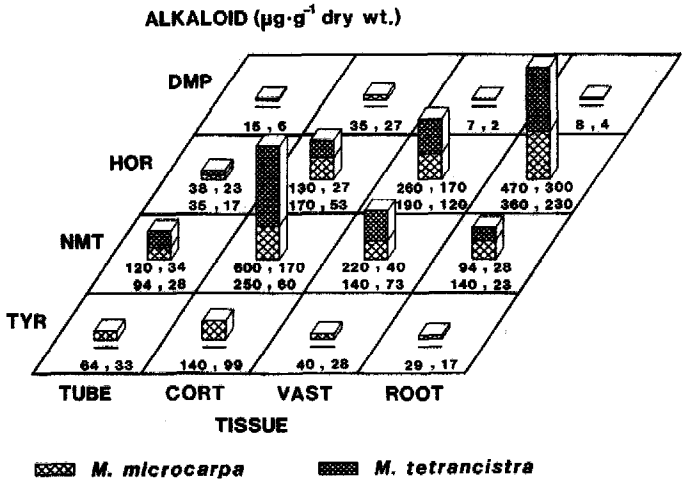

Fig. 3. Alkaloid content contrasted between both species for all tissue types. Values within cells, cited above represent means and standard errors ($\bar{x}$, s.e., n = 3) for *M. tetrancistra* (TYR and DMP were not detected); values below for *M. microcarpa*. See text for abbreviations.

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