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QUANTITATION OF POLYAMINES USING THIN-LAYER CHROMATOGRAPHY AND IMAGE ANALYSIS

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SUMMARY

Efficient separation of dansylated polyamines can be achieved by thin-layer chromatography (TLC). Quantitation, however, can be laborious because it requires removal of the silica gel and the fluorescing derivative from the glass plates, elution in a suitable solvent, and estimation with a fluorescence spectrophotometer. We report here a relatively simple and rapid method for the quantitation of dansylated polyamines that employs an image analyzer without removal from the glass TLC plates.

INTRODUCTION

Several methods have been used to quantitate polyamines (PAs) including dansylation of the amine groups and separation by thin-layer chromatography (TLC)[1], or high-performance liquid chromatography (HPLC)[1,2] and benzoylation of the amine groups followed by their separation by HPLC[1]. Chromatography of benzoyl derivatives by HPLC affords good resolution of six of the major PAs found in plants: putrescine (PUT), cadaverine (CAD), 1,3-diaminopropane (DAP), spermidine (SDP), spermine (SPM) and agmatine (AGM). Dansylation and separation by TLC or HPLC gives good resolution of five major PAs[1,2]. Each method is reliable and sensitive. One disadvantage in the separation of dansylated PAs by TLC or HPLC, however, is that agmatine is missed because of its poor reactivity with dansyl chloride. Although benzoylation and separation of PAs by HPLC permits its detection, resolution requires more than 20 min. A relatively large number of samples of dansylated PAs can be separated on TLC plates, but the usual method of measurement requires removal of the UV-fluorescent dansyl PAs, elution in a suitable solvent and quantitation by fluorescence spectroscopy. We report here a method for the detection and quantitation of PAs that eliminates the tedium and health hazard associated with their removal from the plates. Using this method we have been able to chromatograph and quantify over 100 samples for PA analysis in a single day.

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MATERIALS AND METHODS

Polyamine extraction

Plant samples were ground in 10% perchloric acid at a ratio of 1:10 (w/v) with a variable-speed Brinkman HM-46 polytron. The homogenate was centrifuged at 27 000 *g* for 10 min and the supernatant liquid containing the soluble polyamines was removed from the pellet and a portion was dansylated[1] or benzoylated[4] as previously described.

Chromatographic procedures

TLC separation of dansylated standards and samples was performed on Whatman LK6D 20 × 20 cm glass TLC plates using triethylamine–chloroform (4:25, v/v) or ethyl acetate–cyclohexane (5:4, v/v). The PAs were chromatographed for 1 h in 30 × 30 × 10 cm glass tanks containing 100 ml of solvent.

HPLC separation of benzoylated standards and samples was performed using a programable Varian Model 5500 liquid chromatograph. The benzoyl PAs were passed through a 250 × 4.6 mm I.D., 5 μm, C_{18} reversed-phase column (ODS ultrasphere, Beckman) and eluted with a water–methanol gradient program that cleaned and regenerated the column after each run (25 min): 60% methanol for 7 min, 60–100% methanol in 4 min, 100% methanol for 4 min, 100–60% methanol in 4 min and a 6-min equilibration time. A Hewlett-Packard 3390A integrator was used for quantitation.

Quantitation of polyamines

Quantitative measurement of the dansyl PAs from the TLC plates was performed using the DARWIN image analyzing program, Density Scan[3], run by an Apple computer. Each chromatographed TLC plate (Fig. 1, A) was placed at one end of a darkened box (106.5 × 32.5 × 30 cm) (B). The plate was back-illuminated by a Sylvania 9″ UV lamp (C) with the UV light passing through a Corning UV transmitting filter (365 nm, No. CS-7-60) (D). Fluorescence from the dansyl PAs was viewed through an RCA TC 1005 video camera with a Fujinon TV lens (F 1.8/17.5,

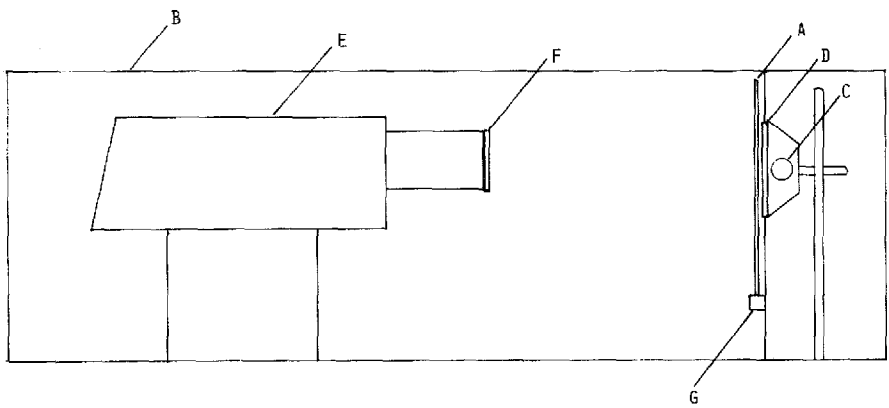

Fig. 1. Schematic diagram of UV fluorescence video scanner.

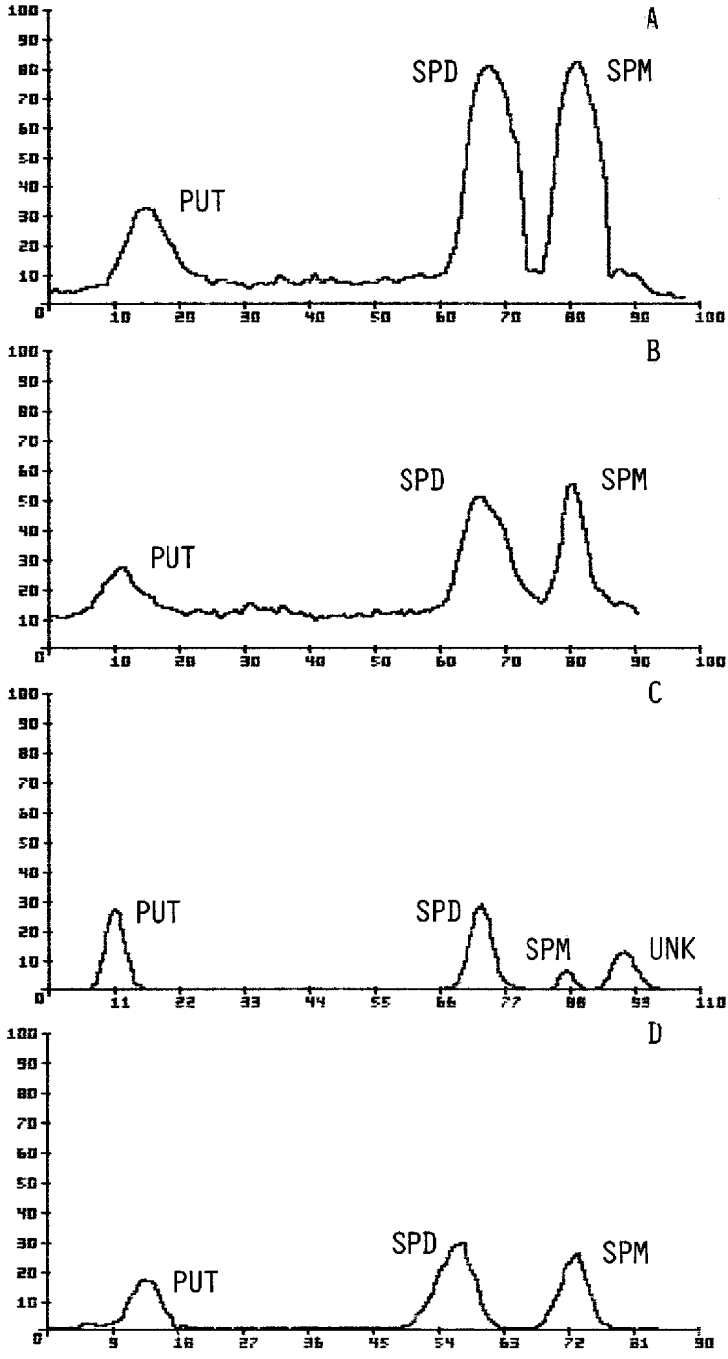

Fig. 2. Graphic output of polyamine fluorescence intensity peaks. *Y*-axis, intensity; *X*-axis, calibrated scale in mm. (A) Standards, (B) wheat, (C) bean, (D) oat. PUT = putrescine, SPD = spermidine, SPM = spermine, UNK = unknown.

105 mm zoom) and a Hoya 58 mm +2 close-up lens (E). A yellow transmitting filter (465 nm, Turner No. 110-813) was attached to the camera lens to reduce extraneous excitation light (F). Output from the video camera was displayed on an Electrohome high-resolution monochromatic monitor as well as being digitized and stored in the Apple computer.

Using the DARWIN image analyzing program, an initial scan is performed in order to establish a reference to which all scans are compared. The initial scan is performed by applying a single spot of dansyl PA onto a 20 × 5 cm TLC plate and adjusting the brightness scale to achieve approximately 60–70% of maximum brightness compared to a dark baseline. After the reference scan, a TLC plate containing chromatographed PAs is placed in the scanner and the boundaries for the scanning area set. The upper and lower limits of the scanning area correspond to the edges of a single track on the plate, while the right and left boundaries are set to include the PAs for quantitation. Once this area is set it need not be changed. Each track (8 mm) on the TLC plate can then be scanned horizontally by raising or lowering the plate with a precise linear positioning track (Fig. 1, G) (Edmund Scientific) and the fluorescent intensity of each polyamine recorded as peaks of light intensity (Fig. 2). Repeat scans permit increased accuracy and a measure of variability in reading accuracy.

The ultraviolet fluorescence peaks were displayed on an Apple II+ monitor in graphic form with the Y-axis displaying intensity from 0 to 100% and the X-axis displaying a calibrated scale (mm) (Fig. 2). Integration of the peaks can be achieved by determining the two points that form the base of the peak along the horizontal axis. This is accomplished by moving the cursor along the baseline to appropriate positions. These positions are stored in memory and the area between those points and beneath the baseline is integrated. Elevated baselines (Fig. 2A and B) result from an initial reference scan set below the 60–70% total brightness level. In these cases one must rely more on the calibrated scale to ensure precise replication than when the base of the peaks are adjusted to the baseline (Fig. 2C and D). Areas calculated by the computer program for each PA were compared to known standards for quantitation.

RESULTS AND DISCUSSION

The accuracy of this method is reflected in the estimate of parameters for three polyamine standards (Table I) while the precision of the method is found in the

TABLE I

ESTIMATE OF PARAMETERS FOR THE STANDARD CURVES OF PUTRESCINE, SPERMIDINE* AND SPERMINE*

PA standard	*Line equation*	*Correlation coefficient*	*Standard error of estimate*
PUT	$Y = 18.4X - 7.1$	0.99	5.4
SPD	$Y = 35.2X + 167.5$	0.99	21.1
SPM	$Y = 29.1X + 203.7$	0.99	16.9

* Linear portion of curve only.

TABLE II

LEVELS OF POLYAMINES IN VARIOUS PLANT TISSUES QUANTIFIED BY TLC AND HPLC

	nmoles PA/g fresh weight		
	PUT	*SPD*	*SPM*
TLC			
Wheat leaves	34.2 ± 4.0	18.3 ± 6.7	22.6 ± 2.7
Bean leaves	33.4 ± 4.2	149.9 ± 14.8	35.6 ± 4.6
Oat leaves	31.2 ± 2.3	54.1 ± 4.1	11.1 ± 1.6
	nmoles PA/g dry weight		
	PUT	*SPD*	*SPM*
HPLC			
Corn embryos	0.45 ± 0.03	1.24 ± 0.04	0.71 ± 0.07

standard errors of actual samples quantified by the image analysis system (Table II). Standards errors were consistently about 10% of the mean values and, when compared with the HPLC method used in this laboratory, were comparable to those quantified by HPLC. The standard curve for PUT, a diamine, was completely linear while SPD, a triamine, and SPM, a tetramine, were linear only to 0.12 nmoles (Fig. 3). When fluorescence values from unknown samples exceed the linear range of the standard, quantification can be achieved by adjustment of the lens diaphragm before the initial reference scan to bring the fluorescence intensity of the standards into a linear range.

When the separation of polyamines during chromatography is not complete a double peak will be produced which cannot be accurately integrated, resulting in overestimation of peak areas. Prolonged chromatography or chromatography of the same sample by two different mobile phases can, in most cases, resolve this problem.

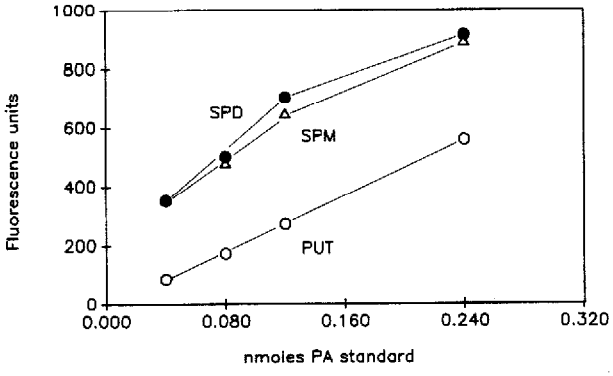

Fig. 3. Calibration curve for polyamine standards. Limit of detection: putrescine, 0.052 nmoles; spermidine, 0.041 nmoles; spermine, 0.047 nmoles.

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