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Screening for amines by dansylation and automated high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic procedure for the determination of a broad range of amines after derivatization with dansyl chloride is presented. Forty-five amines were tested over the concentration range 2–16 μM. Linear calibration graphs passing through the origin were obtained with 25 amines. The method was applied to the identification of biogenic amines from *Agrobacterium*-transformed plant tissue. Reliability and ease of operation make the procedure particularly well suited to automation, permitting the rapid analysis of a large number of samples.

INTRODUCTION

Transformation of plants by *Agrobacterium* sp. has a profound effect on their development and nitrogen metabolism [1,2]. An investigation of the role of amines in the transformation required an automated procedure to analyse a large number of plant tissue extracts for the broadest possible range of primary and secondary amines, with the option of recovering fractions for spectroscopic characterization.

The simultaneous determination of all biogenic amines is difficult because of their structural diversity [3], and in the absence of any other common moiety their determination has tended to rely on derivatization of the amino group [4,5]. Fluorescamine [6], *o*-phthalaldehyde (OPA) [7] and naphthalene-2,3-dicarboxaldehyde (NDA) [8] have been used for this purpose, but only react with primary amino groups. 4-Chloronitrobenzoxadiazole (NBD) [9] also reacts with secondary amines but, like OPA, the derivatives are often unstable, and not suitable for spectroscopic analysis. Dabsyl [10] and benzoyl [11,12] derivatives cannot be detected at as low a concentration or as selectively as the corresponding fluorescent derivatives [13,14].

Dansyl chloride is a particularly well established reagent [15] and gives highly fluorescent sulphonamide derivatives with both primary and secondary amines that are relatively stable, have improved chromatographic properties and are readily isolated from the hydrolysis product, dansyl sulphonate (dansyl-OH), by solvent extraction. Sensitive and

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selective detection is possible with a fluorescence detector, and dansylated derivatives have also proved useful for spectroscopic identification [16,17].

However, although well established, the separation of dansylated amines by high-performance liquid chromatography (HPLC) is not particularly suited to automation. Aqueous buffers are often used to improve separation, but continuous use can corrode pumps or, worse, give gradual precipitation [18]. The low solubility of some dansylated amines in aqueous solvent mixtures can also lead to gradual over-pressure problems. We have therefore developed a new method for the determination of a broad range of amines which is compatible with the use of automated HPLC. Over 40 amines were tested, of which 25 gave linear calibration graphs over the concentration range 2–16 μM. Most earlier methods have tended to focus selectively on a small group of amines, particularly catecholamines [19] or the polyamines [20], and few have been applicable to automation [18].

We also observed that the reaction of agmatine (4-guanidinobutyl-1-amine) with dansyl chloride led to a cleavage of the quanido group and, therefore, the major dansylation product of agmatine was always didansylputrescine.

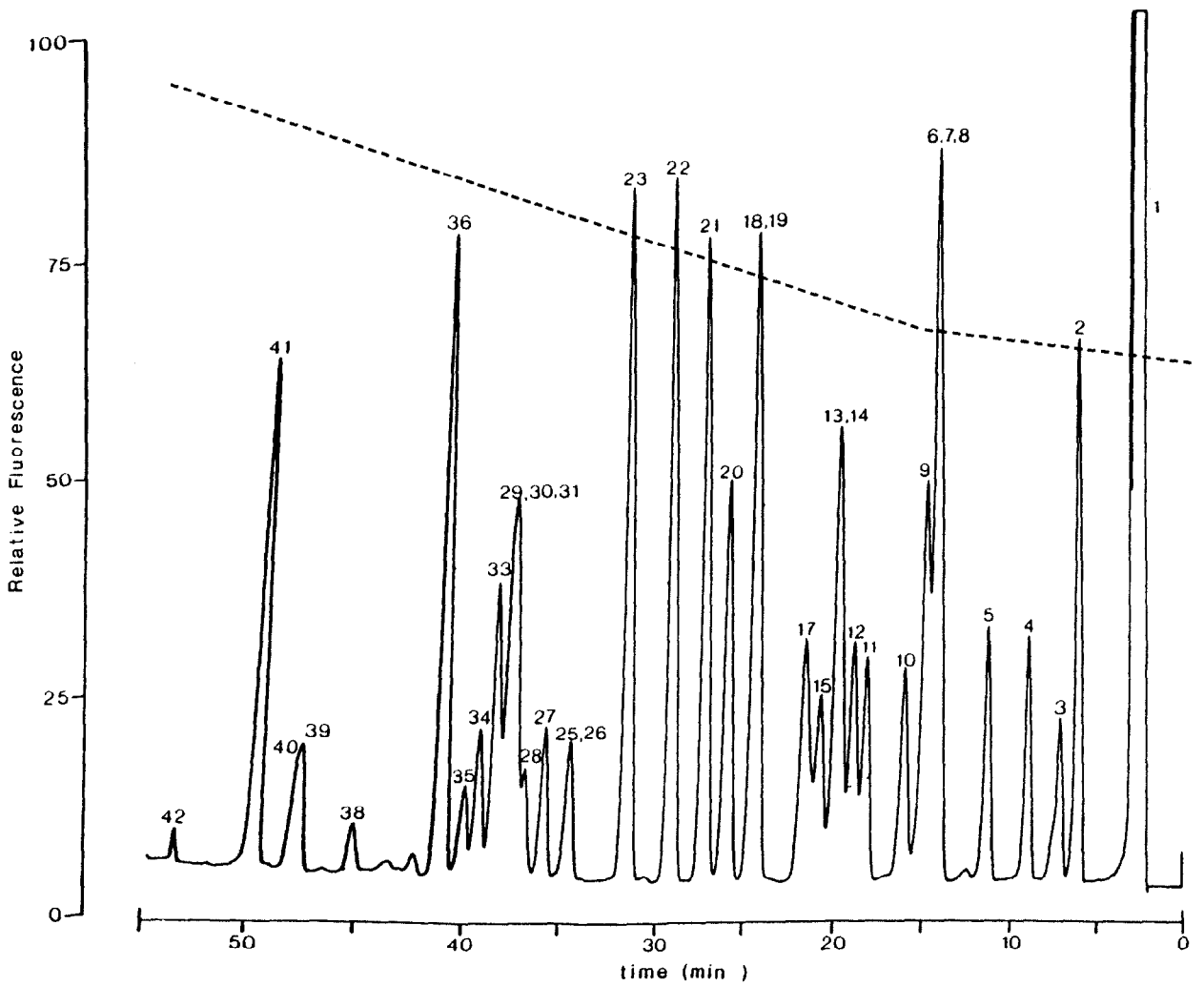

Fig. 1. HPLC of dansylated amine standards separated on a Spheri-5 RP-18 reversed-phase column. The methanol–water gradient shown as a broken line is as described under Experimental. Each peak represents 0.9 nmol of amine. Peak numbers refer to Table I.

EXPERIMENTAL

Agmatine was obtained from Sigma (Poole, UK) (95% pure grade) and from Aldrich (Poole, UK) (99% pure grade). All other chemicals were obtained from Sigma.

The HPLC system consisted of a Model 401 diluter and Model 231 autoinjector (Gilson, Villiers-le-Bel, France), a Rheodyne (Cotati, CA, USA) Model 7010 injector valve fitted with a 50-μl loop, and two LDC IIIG Constametric pumps (LDC-Milton Roy, Riviera Beach, FL, USA). A 25 cm × 4.6 mm I.D. Brownlee Spheri-5 RP-18 reversed-phase C_{18} (5 μm particle size) column was used, protected by a similarly packed 3 cm × 4.6 mm I.D. guard column (Brownlee, Santa Clara, CA, USA). Detection was accomplished with a Perkin-Elmer (Beaconsfield, UK) LS4 spectrofluorimeter (3-μl cell) set at 340 nm excitation and 540 nm emission (10-nm bandwidth).

A methanol–water gradient (flow-rate 1 ml/min) was used, consisting of three linear sections: 0 min, 60% methanol; 15 min, 67% methanol; 54 min, 95% methanol; 60 min, 60% methanol. A period of 10 min was allowed to re-equilibrate the system after each run.

Derivatization procedure

Amines in aqueous solution (100 μl; 15 nmol) were derivatized by adding dansyl chloride in acetone (400 μl; 5 mg/ml). After saturation with solid sodium hydrogencarbonate, the mixture was reacted in the dark for 15 h at room temperature. Excess of acetone was then removed by warming at 60°C for 10 min and the mixture was diluted to 1 ml with water. Dansylated amines were extracted by vortex mixing for 20 s with 3 × 2 ml of toluene. The phases were separated by centrifugation (2700 *g*; 10 min) and the upper layer was removed and evaporated to dryness under a stream of air. Plant tissue extracts were derivatized by the same procedure. At all stages exposure to light was kept to a minimum.

The dansylated samples were redissolved in 500 μl of methanol and centrifuged (9000 *g*; 4 min). The autoinjector made 30-μl (0.9-nmol) injections.

RESULTS AND DISCUSSION

A typical chromatogram of dansylated amines is shown in Fig. 1. Forty-five amines were tested, of which 30 were sufficiently separated to allow recognition from retention data (Table I). Gramine, indole and creatinine did not dansylate under the reaction conditions. Every other amine, with the exception of agmatine, gave a single peak after correcting for the blank, indicating that amines able to undergo dansylation at more than one position were in fact completely derivatized.

In addition to the hydrolysis product, the dansylation reaction always gives rise to dansyldimethylamine and dansylmethylamine from partial cleavage of the dimethylamino moiety [15]. All chromatograms therefore had peaks at 2.2 min (dansyl-OH), 8.6 min (dansylmethylamine) and 13.7 min (dansyldimethylamine). Excess of dansyl chloride co-eluted with the dansyldimethylamine by-product. Ammonia is a common contaminant of samples and reagents, so a peak was usually observed at 5.9 min for dansylated ammonia. Phenolics and thiols are also derivatized, but are easily distinguished from dansylated amines because they fluoresce at higher wavelenths [15].

An application of the method is shown in Fig. 2. Biogenic amines from a crown gall culture of *Cinchona ledgeriana* transformed with *A. tumefaciens* strain A6 were readily separated. Fifteen peaks were identified by reference to the standard retention times in Table I.

Agmatine samples always gave a major didansylputrescine peak (identified by spiking), and sometimes a minor peak presumed to be monodansylagmatine (Fig. 3). The agmatine samples were checked by OPA derivatization [7] and did not contain significant amounts of putrescine, suggesting that the didansylputrescine arose during the dansylation reaction, probably from partial cleavage of the agmatine guanido group. Similar decomposition to a putrescine derivative occurs when agmatine is benzoylated [11,12].

It is common HPLC practice to dissolve samples prior to injection in the solvent mixture to be used at the start of the HPLC gradient. Consequently, aqueous methanol (60%, v/v) was initially used for this purpose. However, it was noted that the detector response was non-linear under these conditions, and that the preinjection solutions were often cloudy. This suggested that some or all of the dansylated amines were at best only partially soluble in 60% (v/v) aqueous methanol. The solubilities of selected

TABLE I

HPLC RETENTION TIMES (t_R) OF DANSYLATED AMINES

All retention times quoted (except for dansylated agmatine) are the means of eight separate readings. Standard deviations (S.D.) were calculated for the same data. The derivative numbers refer to the numbered HPLC peaks shown in Fig. 1.

No.	Derivative	Mean t_R (min)	S.D. (min)	No.	Derivative	Mean t_R (min)	S.D. (min)
1	Dansyl-OH	2.2	0.10	23	1,6-Diaminohexane	31.1	0.19
2	Ammonia	5.9	0.04	24	N-Methylputrescine	34.0	0.12
3	Ethanolamine	6.9	0.08	25	D,L-Octopamine	34.0	0.16
4	Methylamine	8.6	0.05	26	Histamine	34.4	0.12
5	Ethylamine	11.0	0.04	27	3-Methoxy-4-hydroxy-benzylamine	35.3	0.17
6	Dimethylamine	13.7	0.08				
7	Iso-propylamine	13.7	0.08	28	Serotonin	36.3	0.13
8	Dansyl-Cl	13.7	0.08	29	Metanephrine	36.9	0.11
9	*n*-Propylamine	14.5	0.11	30	3-Hydroxy-4-methoxy-phenylethylamine	36.9	0.11
10	Phenylethanolamine	15.6	0.09				
11	Norephedrine	17.8	0.11	31	3-Methoxy-*p*-tyramine	37.3	0.13
12	Isobutylamine	18.9	0.18	32	1,7-Diaminoheptane	37.4	0.12
13	*n*-Butylmamine	19.4	0.11	33	D,L-Synephrine	37.7	0.11
14	Benzylamine	19.4	0.11	34	*p*-Tyramine	38.5	0.09
15	Tryptamine	20.4	0.08	35	*o*-Tyramine	39.2	0.11
16	Agmatine	20.8	–	36	Spermidine	40.4	0.13
17	L-Ephedrine	21.3	0.08	37	Homospermidine	41.5	–
18	Isoamylamine	23.8	0.10	38	Norepinephrine	44.4	0.13
19	2-Phenylethylamine	23.8	0.10	39	D,L-Epinephrine	46.6	0.15
20	1,3-Diaminopropane	25.5	0.11	40	Dopamine	47.0	0.12
21	Putrescine	26.7	0.11	41	Spermine	48.6	0.13
22	Cadaverine	28.6	0.17	42	5-Hydroxydopamine	53.6	0.04

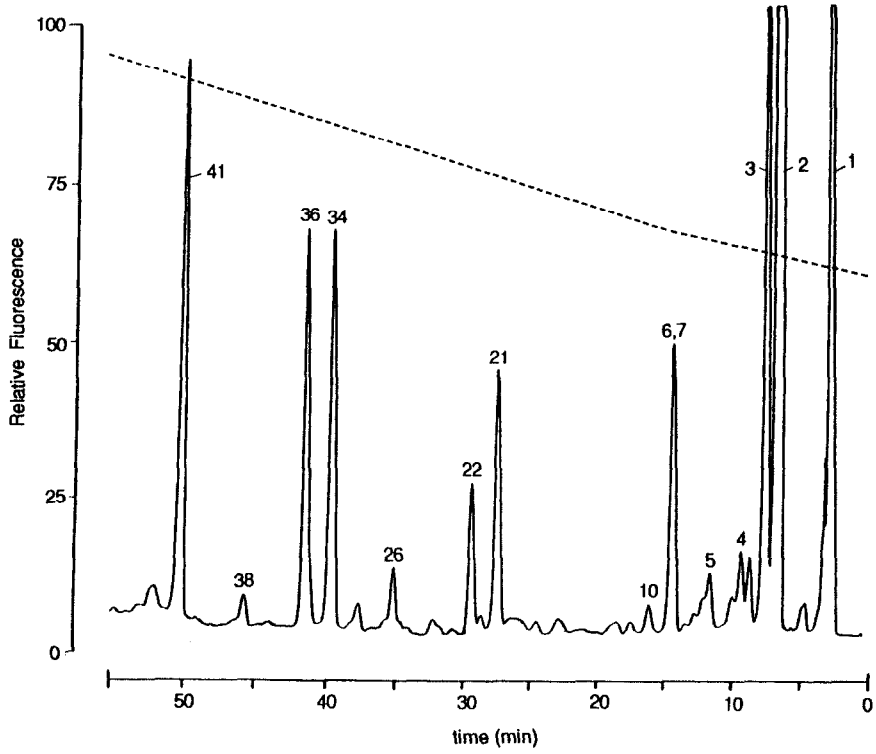

Fig. 2. HPLC separation of dansylated amines from *Cinchona ledgeriana–A. tumefaciens* strain A6 crown gall culture (40 mg fresh weight). Conditions as described in the text. Peak numbers refer to Table I.

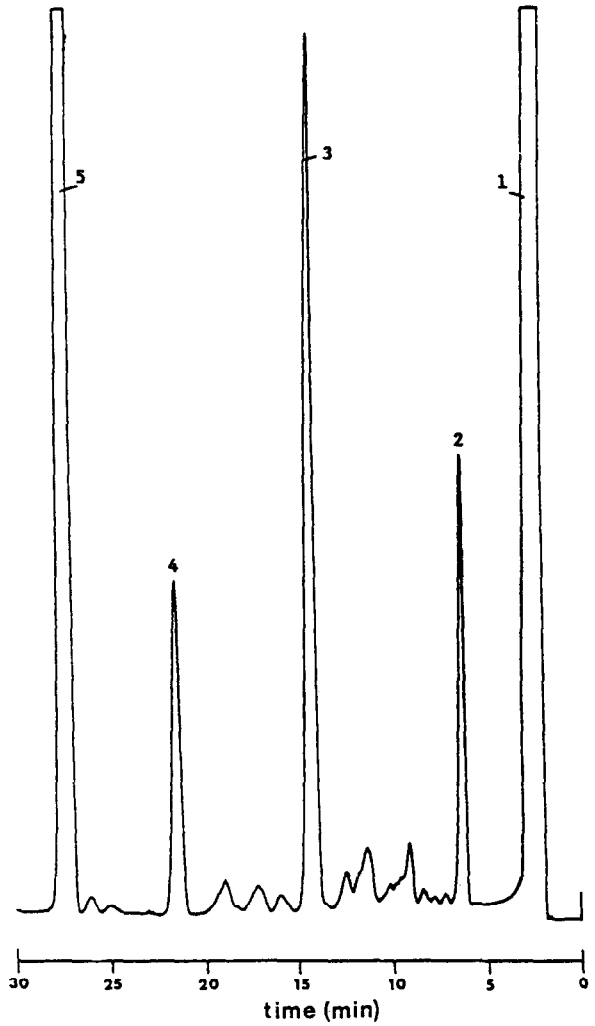

Fig. 3. HPLC of dansylated agmatine. Partial decomposition of the guanido group gives rise to didansylputrescine. Details are described in the text. Peaks: 1 = dansyl-OH; 2 = dansylated ammonia; 3 = excess of dansyl chloride/dansyldimethylamine; 4 = monodansylagmatine; 5 = didansylputrescine.

TABLE II

SOLUBILITIES OF DANSYLATED AMINES AT 25°C

Excess of solid dansylated amine was shaken overnight at 25°C with 0.2 ml of the stated solvent. Duplicate aliquots were taken and diluted to 1.5 ml. The weight of dansylated amine present was determined by measuring the absorbance at the λ_{max} and applying the previously determined molar absorptivity.

Dansylated amine	Maximum solubility (mmol/ml)			
	Ethyl acetate	Methanol	60% aq. methanol	30% aq. acetonitrile
Spermidine	>8.63	3.2	0.155	0.12
Histamine	24.2	5.9	1.05	0.34
Diaminopropane	>24.9	>52.0	3.25	4.05
Cadaverine	>9.14	>9.7	1.84	0.22
Putrescine	0.92	0.38	0.10–0.13	0.12
Ammonia	4.51	10.0	2.26	1.78

TABLE III

RELATIVE FLUORESCENCE INTENSITIES OF DANSYLATED AMINES

Dansylated amine	No. of dansyl groups[a]	Relative fluoresence intensity		
		%F[b] per 0.1 nmol	Normalized[c]	r^d
Spermine	4	47.77	100.0	1.000
Spermidine	3	29.17	61.1	0.999
Cadaverine	2	21.23	44.5	0.998
Putrescine	2	20.83	43.6	0.997
1,6-Diaminohexane	2	20.40	42.7	0.999
Ethanolamine	1	18.33	38.4	1.000
n-Propylamine	1	18.13	38.0	0.999
Ethylamine	1	17.90	37.5	1.000
Iso butylamine	1	15.63	32.7	0.999
Synephrine	2	14.57	30.5	1.000
1,3-Diaminopropane	2	12.90	27.0	1.000
Ephedrine	1	12.70	26.6	0.999
3-Methoxy-*p*-tyramine	2	12.30	25.8	1.000
Phenylethanolamine	1	12.06	25.3	0.999
p-Tyramine	2	11.23	23.5	0.998
Norephedrine	1	10.40	21.8	1.000
Tryptamine	1	9.80	20.5	0.998
3-Methoxy-4-hydroxy-benzylamine	2	8.97	18.8	0.997
Epinephrine	3	8.13	17.0	0.999
Dopamine	3	5.83	12.2	0.999
Norepinephrine	3	4.57	9.6	0.999
o-Tyramine	1	4.17	8.7	0.999
5-Hydroxydopamine	4	2.07	4.3	0.997
Histamine	2	1.67	3.5	1.000
Serotonin	2	1.47	3.1	1.000

[a] Number of dansyl moieties on each derivative.
[b] Percentage fluorescence, an arbitary scale based on peak height.
[c] Relative fluorescence intensities normalized on the tetradansylspermine response (nominally assigned as 100).
[d] Correlation coefficients, calculated from four replicates per point.

dansylated amines were measured in 100% methanol, 60% (v/v) aqueous methanol, 100% ethyl acetate and 30% (v/v) aqueous acetonitrile. The results, shown in Table II, suggested that low solubility in 60% (v/v) aqueous methanol might indeed be a factor. After making the preinjection dilutions in 100% methanol, 25 out of 28 amines tested (Table III) gave reproducible ($r > 0.997$) linear calibration graphs over the concentration range 2–16 μM, all with zero intercept. Ammonia, methylamine and dimethylamine derivatives were the exceptions, probably because of the high background levels arising from the dansylation reaction.

The relative fluorescence intensities of the dansylated amines obtained from the gradients of the calibration graphs are given in Table III. With 340-nm excitation and 540-nm emission, settings chosen to optimize the detection of derivatives with a range of different emission wavelengths, tetradansylspermine had the highest relative fluorescence intensity measured, and was 33 times more fluorescent than didansylserotonin. The detection limits varied with the relative intensities, but were generally of the order of 5–10 pmol (signal-to-noise ratio = 3).

Simplicity, ease of operation and reliability are prerequisites of automated HPLC. It is particularly important that there is no gradual accumulation of precipitates. We therefore avoided the use of buffers, and also determined the solubility of some dansylamines in aqueous solvents. We found that

comparable methods used to determine a broad range of amines [13,14] were unsuitable for automation, and eventually led to problems of over-pressure. Other workers [18] have reported that precipitates gradually form in the phosphate buffer used by Hayman *et al.* [14]. In contrast, Seiler [15] used a simple methanol–water gradient, but did not consider the low and variable solubilities of dansylated amines. The method described here overcomes both of these disadvantages. In one instance the HPLC system was run continuously for 72 h, the autoinjector making 66 injections without encountering any problems, and in our experience the method is particularly suited to screening for novel amines from plant tissue.

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