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Journal of Chromatography A, 1035 (2004) 63–73
JOURNAL OF CHROMATOGRAPHY A
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Simultaneous separation and quantitation of amino acids and polyamines of forest tree tissues and cell cultures within a single high-performance liquid chromatography run using dansyl derivatization

Rakesh Minocha*, Stephanie Long

US Department of Agriculture, Forest Service, Northeastern Research Station, P.O. Box 640, 271 Mast Road, Durham, NH 03824, USA

Received 26 May 2003; received in revised form 5 February 2004; accepted 6 February 2004

Abstract

The objective of the present study was to develop a rapid HPLC method for simultaneous separation and quantitation of dansylated amino acids and common polyamines in the same matrix for analyzing forest tree tissues and cell cultures. The major modifications incorporated into this method as compared to previously published HPLC methods for separation of only dansyl amino acids include: use of a 10 cm column to reduce the total run time by approximately 15 min; modification of the dansyl derivatization process and gradient profile to elute amino acids and common polyamines within the same run; addition of steps for column cleaning within each run; shorter re-equilibration time; and finally, column cleaning and physically reversing the column at the end of a loop of samples. These changes improved peak resolution and increased column longevity by several-fold. Over 1000 foliar samples from mature forest trees could be analyzed with the same column as compared to only 200–250 samples before the incorporation of these changes. This method eluted 22 amino acids within 40 min plus all three common polyamines between 44 and 47 min. The total run time is 53.6 min for amino acids only and 55.6 min for both amino acids and polyamines.

Keywords: Derivatization, LC; Amino acids, Dns derivatives; Polyamines; Amines; Putrescine; Spermidine; Spermine

1. Introduction

Various reagents like dansyl chloride (Dns-Cl), dabsyl chloride (Dbs-Cl), fluorescamine, fluorenyl-methyl chloroformate (FMOC), and different SH-group containing *o*-phthalaldehydes (OPA) have been used for pre- or post-column derivatization of free amino acids and amines for HPLC separations based on fluorescence [1–4]. In the nmol concentration range, quantitation of amino acids using either the postcolumn derivitization with OPA or precolumn Dns or Dbs derivatization is equally suitable. However, in the lower pmol range, Dns or Dbs derivatization may produce significantly better results [5,6]. In addition, dansyl and dabsyl derivatives are very stable as compared to products formed by OPA [6–8]. Both Dns and Dbs chloride react with primary and secondary amines whereas OPA does not react with secondary amino acids such as proline and hydroxy-proline [7], thus making their detection impractical. In general, *o*-phthalaldehyde derivatization has been a popular method for the obvious reasons of simplicity, timesaving and labor reduction.

More recently, new reagents have been reported for fluorescence-based amino acid quantitation. *N*-Hydroxysuccinimidyl-α-naphthylacetate (SINA) was used to detect amino acids with a detection limit in the fmol range [9]. Underivatized amino acids have also been detected by an HPLC method based on a displacement reaction between the eluted amino acids and copper(II)–L-tryptophan complex [10].

Precolumn derivatization of free amino acids with Dns-Cl is economical and can be successfully used for quantitation of amino acids and polyamines over a wide range of relative concentrations of dansyl chloride [1,11]. The reaction requires an alkaline pH in the range of pH 9.5, an excess of dansyl chloride and a reaction time of about one hour. However, at a pH of above 10.0, the desired reaction of primary amine groups with dansyl chloride is in direct

* Corresponding author. Tel.: +1-603-868-7622; fax: +1-603-868-7604.
E-mail addresses: rminocha@hopper.unh.edu, rminocha@fs.fed.us (R. Minocha).

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doi:10.1016/j.chroma.2004.02.026

competition with hydrolysis of dansyl chloride by water or hydroxyl groups [12]. Therefore, a pH of 9.5 is optimal for the dansylation of unprotonated amine groups.

Dansyl amino acids and dansyl polyamines are routinely separated and quantified using different HPLC methods [1,2,14]. An HPLC method that can simultaneously separate and quantify both dansyl amino acids and dansyl polyamines within a single run would not only save time but also costs of solvents and supplies, and waste disposal. Here we describe an HPLC method that has been developed for the simultaneous separation and quantitation of dansyl amino acids and dansyl polyamines in the same matrix for analyzing forest tree tissue and cell culture samples.

2. Experimental

2.1. Chemicals

The following chemicals were used without additional purifications: amino acid standards kit (Fluka, Milwaukee, WI, USA[1]); ornithine, γ-aminobutyric acid, polyamine standards, α-methyl-DL-phenylalanine and dansyl chloride (Sigma, St. Louis, MO, USA); sodium acetate, sodium carbonate (Aldrich, Milwaukee, WI, USA); acetic acid, perchloric acid (J.T. Baker, Phillipsburg, NJ, USA). The following HPLC-grade solvents were used after filtration with a 0.45 μm nylon filter (Pall-Gelman Labs., Ann Arbor, MI, USA): acetonitrile (Burdick and Jackson, Muskegon, MI, USA), 1-propanol, and methanol (EM Science, Gibbstown, NJ, USA), Photrex toluene and acetone (J.T. Baker). All chemicals were greater than 99% in purity except for putrescine (98%), heptanediamine (98%), spermidine (98%), dansyl chloride (95%) and spermine (95%).

2.2. Sample preparation and derivatization

Approximately 200 mg fresh mass of cells, leaf discs or chopped needle samples were placed in 1 ml of 5% (v/v) ice-cold perchloric acid (PCA). The samples were stored at −20 °C until the time of analyses. Prior to analyses, the frozen samples were thawed and frozen (−20 °C) three times [13], centrifuged at 13 500 × *g* for 10 min and the supernatant was used for dansylation of both amino acids and polyamines according to the procedure described in [1] for polyamine analyses with the following modifications. Briefly, 20 μl aliquots of a mix of two internal standards (0.1 mM heptanediamine for polyamines and 1 mM α-methyl-DL-phenylalanine for amino acids in 5% PCA) and 100 μl aliquots of sample extracts or 100 μl of a mix of 23 amino acids and three common polyamines standards in 5% perchloric acid were placed in microfuge tubes and vortexed. One hundred microliters of saturated sodium carbonate solution and 100 μl of dansyl chloride (20 mg ml^{-1} in acetone) were then added to each tube. The tubes were capped, vortexed and incubated in a water bath at 60 °C. After 60 min, 50 μl of L-alanine (100 mg ml^{-1} in water) was added to the reaction mix to react with excess dansyl chloride. Following an additional 30 min incubation, acetone was evaporated from the tubes in a SpeedVac Evaporator (Savant, Farmingdale, NY, USA) for 3–4 min. A 400 μl volume of toluene was then added to each tube, vortexed for 1 min and centrifuged at 13 500 × *g* for 1 min. After the aqueous and organic phases separated, 200 μl of the toluene phase containing only polyamines was transferred to another microfuge tube and the toluene was completely evaporated under vacuum for 15 min. Into each tube containing dry dansyl polyamines, 730 μl of methanol was added and the tubes were vortexed for 2 min to thoroughly dissolve the polyamines. At this point one half of the toluene-extracted aqueous fraction (135 μl) was added along with 135 μl of 2.9 M acetic acid to bring the total volume to 1 ml. This order of addition is very important for complete dissolution of dansyl polyamines. Acetic acid was added to remove excess carbonate. The tubes were kept open for 4 min to let any CO_2 escape before mixing the solution and filtering with a 0.45 μm nylon syringe filter to remove particulate matter. Dansylated polyamines were extracted separately into an organic phase because in the aqueous phase they stick to the walls of the original microfuge tube in which the reaction was run and do not proportionately get transferred to the new tube when half of the aqueous fraction is transferred for further processing (Table 1). The blank HPLC runs were conducted using dansylated 5% PCA.

2.3. Instrumentation

For the simultaneous separation of dansyl amino acids and dansyl polyamines the liquid chromatographic system consisted of: a series 410 pump (Perkin-Elmer, Norwalk, CT, USA); an AS-4000 auto-sampler (Hitachi, Tokyo, Japan) fitted with a 20 μl loop (10 μl injection volume); a column heater (Bio-Rad Labs., Hercules, CA, USA) set at 40 °C; a Luna C_{18}, 3 μm, 100 mm × 4.6 mm i.d. column (No. 00D-4251-E0, Phenomenex, Torrance, CA, USA) or a Synergi Hydro-RP, 4 μm, 100 mm × 4.6 mm i.d. column (Phenomenex, No. 00D-4375-E0); a C_{18} Securityguard, 5 μm, 4 mm × 3 mm i.d. cartridge guard column (Phenomenex, No. AJO-4287); a C_{18} Scavenger, 10 μm, 33 mm × 4.6 mm i.d. cartridge column (Perkin-Elmer, No. 0258-0202); and an RF-10A XL fluorescence detector (Shimadzu, Columbia, MD, USA). The excitation and emission wavelengths were set at 340 and 515 nm, respectively. A Gilson 712 HPLC System Controller (Gilson, Middleton, WI, USA) was used to integrate the data.

The separation of polyamines was also carried out using a Perkin-Elmer series 200 pump and autosampler fitted

[1] The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the US Department of Agriculture or the Forest Service of any product or service to the exclusion of others that may be suitable.

Table 1
Data on recovery of dansyl polyamines from an aqueous environment

Treatment	Recovery of polyamines	10 pmol per injection			
		Putrescine	Heptanediamine	Spermidine	Spermine
A	Treatment A	100 ± 4	100 ± 1	100 ± 2	100 ± 3
B	Percentage of Treatment A	61 ± 5	30 ± 4	20 ± 11	21 ± 9
C	Percentage of Treatment A	153 ± 4	167 ± 2	189 ± 3	192 ± 3
B + C	Percentage of Treatment A	107	99	104	106

Treatment A: routine procedure followed in our laboratory for preparation of samples for separation and quantitation of dansylated polyamines. After termination of the dansylation reaction, all of the aqueous phase (270 μl) was extracted with 400 μl toluene. A 200 μl volume of toluene was transferred to a separate microfuge tube evaporated to dryness and redissolved in 1 ml MeOH. Treatment B: after termination of dansylation reaction, 135 μl from the aqueous phase was transferred to a new microfuge tube and extracted with 400 μl toluene. A 300 μl volume of toluene was transferred to a separate microfuge tube, evaporated to dryness and redissolved in 750 μl MeOH to yield same final concentration as in Treatment A. Treatment C: after transfer of the first (135 μl) aqueous fraction for Treatment B the remaining aqueous portion (135 μl) was also extracted with 400 μl toluene and processed the same way as for Treatment B. This experiment was repeated three times. Data presented here are mean ± S.E. of $n = 5$ for only one representative experiment.

with 20 μl loop (10 μl injection volume); a Perkin-Elmer Pecosphere: 3 × 3 CR C_{18}, 33 mm × 4.6 mm i.d. cartridge column (3 μm particle size) and a fluorescence detector (LS-1, Perkin-Elmer). The excitation and emission wavelengths were set at 340 and 510 nm, respectively. A TotalChrom HPLC software package (Perkin-Elmer, version 6.2.1) was used to interpret the data.

2.4. HPLC conditions

Dansylated free amino acids and polyamines were separated simultaneously within a single run using a modification of the HPLC method described in [14]. The original method was developed primarily using a mix of commercially available pre-dansylated amino acid standards (Sigma). No data were provided on the use of this method for any type of free amino acid standards or tissue samples that were dansylated in the laboratory. Therefore, in order to run tissue samples using this HPLC procedure for simultaneous analyses of amino acids and polyamines, both the dansylation procedure and the gradient profile described in [14] had to be optimized. The details of the gradient profile used in the present study are described in Table 2. Briefly, a 0–40% gradient of mobile phase A [100% acetonitrile (ACN)] and B [25 mM sodium acetate buffer (pH 5.94) containing 3% 1-propanol, and 10% ACN] was run for 40 min at 1 ml min^{-1} to elute all amino acids. In order to elute the polyamines within the same run as amino acids, at step 3 in the pump method, the gradient was raised from 40 to 100% ACN in 4.5 min at 2.5 ml min^{-1}. If no toluene fraction was collected for simultaneous polyamine separations within the same run as amino acids then step 3 was reduced to 2 min (Table 2). After this step the column was washed with 100% ACN for 4 min at 2.5 ml min^{-1}. The column was then washed for 2 min with distilled and deionized water containing 10% ACN and equilibrated with mobile phase B for 4 min at 2.5 ml min^{-1} and for 1 min at 1 ml min^{-1}.

At the end of each loop of sample runs (50–80 injections), a cleaning method was used before shutting down the system. This column cleaning method consisted of three steps: distilled and deionized water containing 10% ACN; 100% ACN; and ACN–water (150:50); each for 20 min at a flow rate of 1 ml min^{-1} in the order given here. Before beginning a new loop of sample injections, the column was physically reversed and flushed using a two-step wash method: 100% ACN followed by 10% ACN in distilled and deionized water each for 20 min at a flow rate of 2 ml min^{-1}. The next loop of samples was then run using the column still in the reverse direction. Therefore, on a routine basis, the column was used in both directions for sample analyses. The life of the column was prolonged several-fold by combining these steps with a water wash step within the separation method.

Table 2
Solvent gradient profile for simultaneous separation of dansyl amino acids and dansyl polyamines in a single HPLC run

Step	Time (min)	Flow (ml min^{-1})	ACN (%)	Buffer (%)	Water[a] (%)	Curve[b]	Cumulative time (min)
1	0.1	1.0	0	100	0	0	0.1
2	40.0	1.0	40	60	0	1	40.1
3	**4.5**[c]	**2.5**	**100**	**0**	**0**	**1**	**44.6**
4	4.0	2.5	100	0	0	0	48.6
5	2.0	2.5	0	0	100	0	50.6
6	4.0	2.5	0	100	0	0	54.6
7	1.0	1.0	0	100	0	0	55.6

[a] Denotes that distilled and deionized water contained 10% acetonitrile.
[b] 1 = linear and 0 = step.
[c] Time for step 3 was reduced to 2.5 min if polyamine analyses were not required. This reduced the total run time to 53.6 min.

Dansyl polyamines were also separated using a gradient of acetonitrile and heptanesulfonate buffer (10 mM containing 10% ACN, 3.4 pH) or sodium acetate buffer (25 mM containing 3% 1-propanol, and 10% ACN, pH 5.94) according to the gradient profile published earlier [1]. The standards mix contained the equivalent of 10 pmol for polyamines and the internal standard, heptanediamine per 10 μl injection.

3. Results and discussion

3.1. Optimization of the procedure for complete recovery of dansyl polyamines

The dansylation procedure that has routinely been used in our laboratory for polyamine analyses [1] had to be optimized in order to achieve simultaneous separation of both polyamines and amino acids in one HPLC run. As described in Section 2.2, sample preparation and derivatization, the dansylated polyamines had to be extracted in the toluene phase prior to further processing of samples for dansylated amino acids. The reason for this being that when, after the termination of the dansylation reaction, half of the aqueous phase (135 μl) was further processed without the toluene extraction step, a disproportionately high amount of polyamines always remained behind in the second half of the aqueous phase in the original tube. Data presented in Table 1 show that about 40% of the expected amount of putrescine and 70–80% of heptanediamine, spermidine and spermine stayed behind in the original tube under these conditions (Treatment B). When toluene was added to the second half remaining in the original tube (Treatment C), about 150% of the expected amount of putrescine and 170–180% of heptanediamine, spermidine and spermine was recovered, the average of fractions B and C yielding full recovery which compared well with our routinely used method (Treatment A). Therefore, dansylated polyamines in aqueous solutions do stick to the walls of polypropylene microfuge tubes; the larger size polyamines like spermine bind more strongly due to a higher charge than putrescine.

However, when the entire aqueous reaction mixture in the original tube (270 μl) was processed further without

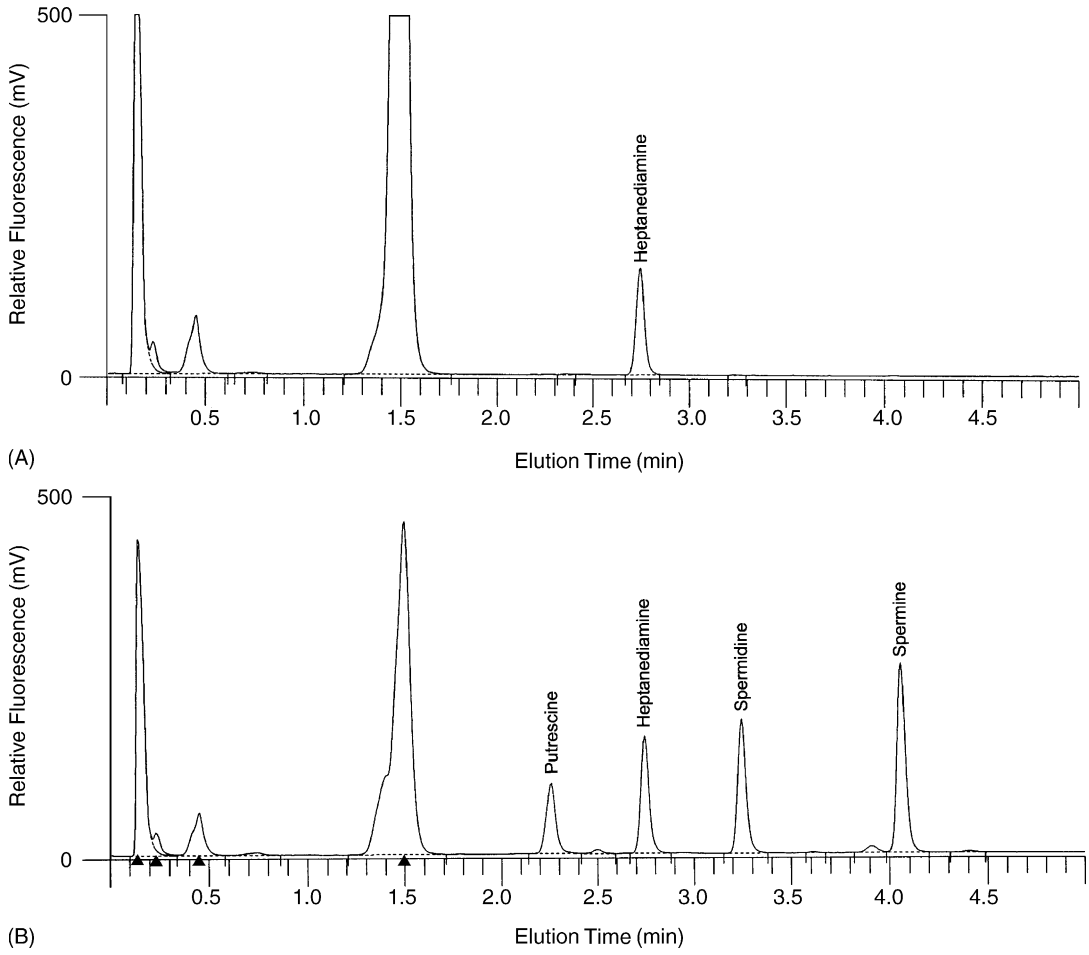

Fig. 1. (A) 5% PCA blank. (B) Separation profile of three common dansyl polyamines along with an internal standard using the gradient profile described in Minocha et al. [1]. The standards mix contained the equivalent of 10 pmol for polyamines and the internal standard, heptanediamine, per 10 μl injection. Run conditions are as described in the text. The (▲) indicates the presence of blank peak.

the toluene extraction step by adding an equal volume of acetic acid (270 μl) and 460 μl of MeOH (to bring the total volume to 1 ml), two major problems were encountered: (1) there was increased sample loss due to increased effervescence and sample splattering due to doubling of sample and acetic acid volume within 1.7 or 2 ml size microfuge tubes being used for these analyses; (2) the recovery of polyamines was still incomplete because the final concentration of the organic phase (in this case, 46% methanol) was still not sufficient to solubilize dansylated polyamines. Therefore, dansyl polyamines had to be extracted separately in toluene and then added back into the dansylated amino acids before running HPLC analyses.

3.2. Peak detection, integration and data analyses

Polyamine samples are routinely dansylated and analyzed in our laboratory using the dansylation and HPLC procedures described by Minocha et al. [1]. Peak quantifications are done by an internal standard method using a five-point calibration curve. Fig. 1 shows the separation of dansyl polyamines using this procedure where all three common polyamines and heptanediamine eluted within 4 min with a total run time of 8.7 min. For simultaneous separation of amino acids and polyamines, the peak quantifications were done by an external and/or internal standard method using four- to five-point calibration curves. The standards mix

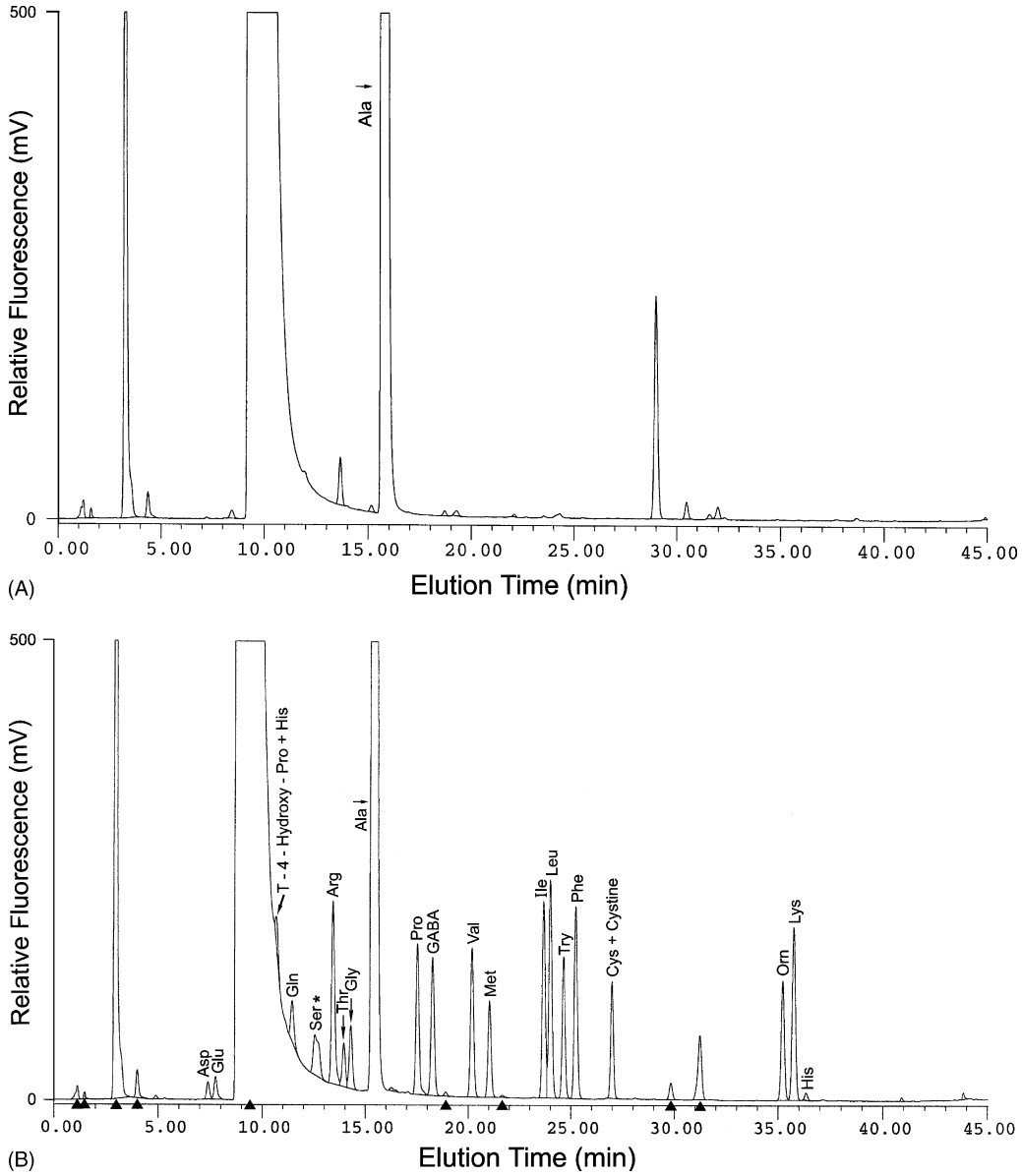

Fig. 2. (A) 5% PCA blank. (B) Separation profile of 22 dansyl amino acids along with an internal standard using the gradient profile described in Table 2. A Phenomenex Synergi Hydro-RP column was used. The standards mix contained the equivalent of 50 pmol for each amino acid except arginine and the internal standard, α-methyl-DL-phenylalanine, which were 100 pmol per 10 μl injection. Run conditions are as described in the text. The (▲) indicates the presence of blank peak. (*) Denotes that Ser could be identified but could not always be quantified. Sometimes Ser coeluted with another peak that is generally a byproduct of Met dansylation reaction.

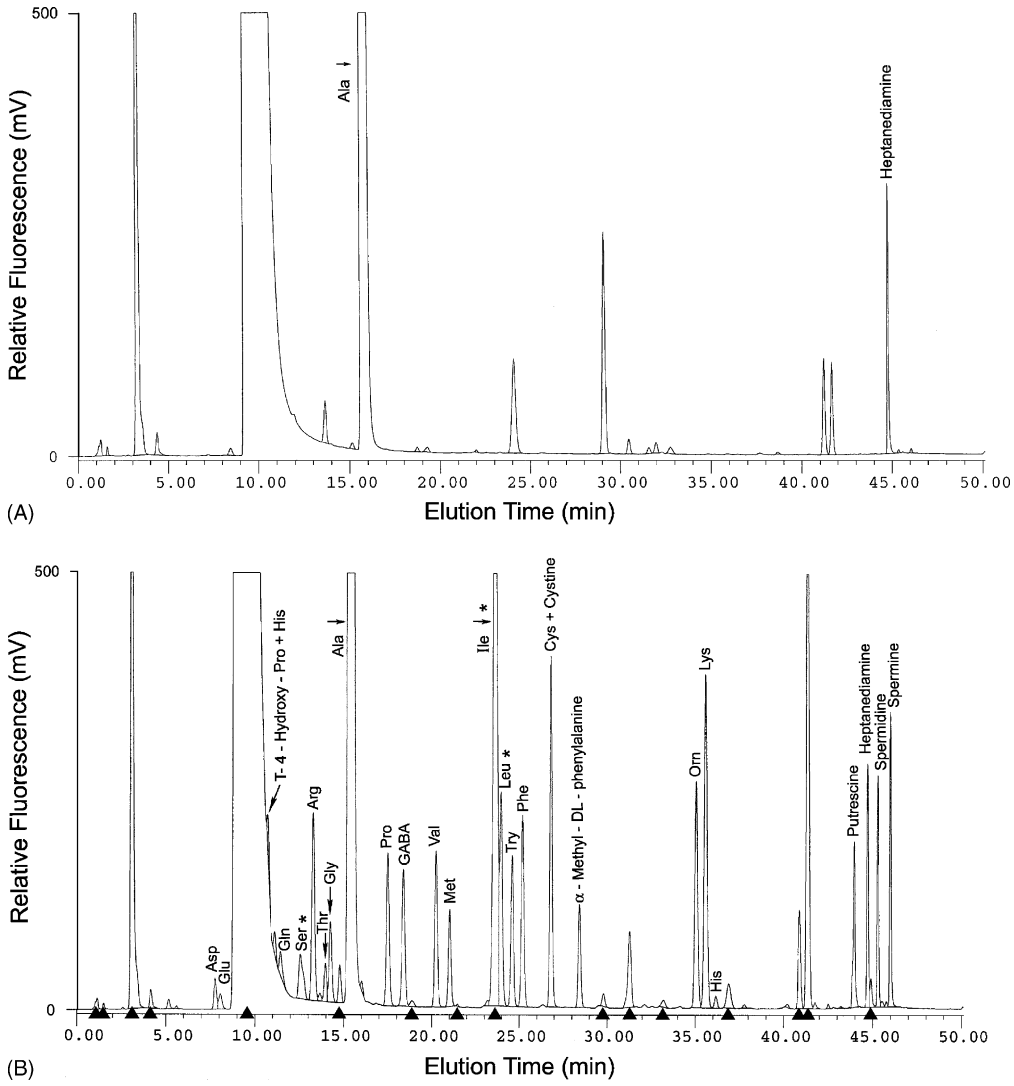

Fig. 3. (A) 5% PCA blank. (B) Separation profile of 22 dansyl amino acids and three common dansyl polyamines along with two internal standards using the gradient profile described in Table 2. A Phenomenex Synergi Hydro-RP column was used. The standards mix contained the equivalent of 50 pmol for each amino acid except arginine and the internal standard, α-methyl-DL-phenylalanine, which were at 100 pmol per 10 μl injection. Polyamines and the internal standard, heptanediamine, were the equivalent of 10 pmol per 10 μl injection. Run conditions are as described in the text. The (▲) indicates the presence of blank peak. (*) Denotes peaks that could be identified (Ser, Ile, and Leu) but could not always be quantified due to poor resolution. Sometimes Ser coeluted with another peak that is generally a byproduct of Met dansylation reaction. Isoleucine coeluted with another larger unidentified peak that only appears when the polyamines containing toluene fraction is added back to the injection mix, affecting the resolution of both Ile and Leu. For retention times and other details refer to Table 3.

contained 23 amino acids and three common polyamines. Alanine was not part of the standard mix since it was used for termination of the dansylation reaction. Twenty-two amino acids including alanine and two uncommon amino acids, ornithine and γ-aminobutyric acid (GABA), could be detected from the elution profile using a Synergi Hydro-RP column (Figs. 2 and 3). However, Ile, Leu, and the uncommon amino acid T4-hydroxyproline could not be reliably quantified due to poor separation and/or resolution especially when toluene fraction was added to the mix (Fig. 3). Alanine could be identified, but since it was added to the reaction mix to remove excess dansyl chloride, it was not quantified. Asparagine and Tyr could not be detected by this method. Two internal standards were used within each run: α-methyl-DL-phenylalanine for the quantitation of amino acids, and heptanediamine for polyamines. The detection limit for most amino acids and polyamines was below 1 pmol (Table 3). The linear range of detection for polyamines was from 2 to 20 pmol. Other than a few exceptions listed in Table 3, most amino acids had a linear range of detection from 10 to 200 pmol. The r^2 value for standard curves was 0.99 or higher for all amino acids. The precision was within 2.5% for repeat samples run on the same day or over a longer period for all amino acids except Met, Cys + cystine, Orn, Lys, and His, where precision was within 5%. For about 30% of the time, the standard calibration curve for these six

Table 3
Detection limits and linear ranges for quantification of dansyl amino acids and dansyl polyamines

Name	Retention time (min)	Mean of replication	Linear curve points	Detection limit (pmol)	Linear range tested (pmol)	r^2		Slope ($\times 10^4$)		Intercept ($\times 10^4$)	
						Mean	S.E.	Mean	S.E.	Mean	S.E.
Asp	7.76	9	5	<2	10–200	0.998	0.001	9.88	0.85	−0.11	1.51
Glu	8.06	9	3–5	<1	10–200	0.991	0.004	4.06	1.12	8.65	2.36
Gln	11.43	10	4	<20	20–200	0.997	0.001	10.23	0.63	9.50	3.69
Ser[a]	12.54	10	5	<1	10–200	0.997	0.001	24.98	0.98	8.32	3.06
Arg[a]	13.28	10	5	<1	10–400	0.998	0.001	27.89	1.19	4.61	4.51
Thr	13.96	10	5	<1	10–200	0.998	0.001	15.19	0.50	−0.61	1.54
Gly	14.25	10	5	<1	10–200	0.999	0.000	28.97	1.14	3.89	2.54
Pro	17.50	10	5	<1	10–200	1.000	0.000	55.69	0.78	1.11	2.54
Gaba	18.40	10	5	<1	10–200	0.999	0.000	54.49	1.56	14.69	6.36
Val	20.24	10	5	<1	10–200	0.999	0.000	56.51	1.18	11.98	4.35
Met[c]	21.02	10	5	<2	10–200	0.997	0.001	40.96	1.76	−26.22	6.02
Ile[b]	23.62	12	5	<1	10–200	0.999	0.000	67.40	1.27	10.47	2.92
Ieu[b]	23.95	12	5	<1	10–200	1.000	0.000	74.08	1.64	8.50	3.17
Try	24.59	10	5	<1	10–200	0.999	0.000	59.32	1.84	−5.07	5.46
Phe	25.18	10	5	<1	10–200	0.999	0.000	69.39	0.97	14.19	3.61
Cys + cystine	26.80	10	3–5	<1	10–50	0.993	0.003	106.24	12.83	43.63	19.89
Orn	35.04	10	3–5	<1	10–50	0.997	0.001	88.05	7.90	16.66	10.67
Lys	35.58	10	3–5	<1	10–50	0.997	0.001	122.32	10.56	39.21	13.79
His[c]	36.15	10	3–5	<10	10–50	0.987	0.004	4.16	0.52	−0.15	1.17
Put	43.95	5	4	<1	2–20	1.000	0.000	208.71	10.40	−1.62	2.90
Spd	45.28	5	4	<1	2–20	0.999	0.000	278.20	8.16	13.32	4.26
Spm	45.98	5	4	<1	2–20	0.999	0.000	340.10	23.23	24.22	5.37

The precision was within 2.5% for repeat samples run the same day or over a longer period for all amino acids except for Lys, His, Orn, Met, Cys + cystine where precision was within 5%.

[a] Reliable peak resolution with the Synergi Hydro-RP column only.

[b] Reliable peak resolution without toluene containing polyamine fraction only.

[c] Dansylation of both Met and His produced two peaks each. A small peak of Met sometimes coeluted with Ser and other times it was separated from it. The major peak of Met was the one used for quantification. The first peak of His eluted with T4-hydroxyproline and could not be quantified as most of it came out as a shoulder to a large peak produced by dansylhydroxy product. The second His peak was used for quantification (see text for more details). Asparagine and Tyr could not be detected by this method.

amino acids was linear only up to 100 pmol. As is evident from Figs. 2–6, a few very small blank peaks did merge with a couple of amino acids (Glu, Arg, and GABA). But since these areas of blank peaks are constant, we addressed this problem by using a nonzero intercept for the linear regressions. However, there were one or two blank peaks whose areas were sample dependent. These peaks never interfered with quantitation of desired peaks.

The dansylation of Met and His produced two peaks each. The smaller of the two Met peaks sometimes coeluted with Ser and other times it was separated from it. It was approximately 19% of the combined peak area produced by the two Met peaks. The major peak of Met was the one used for quantification. The first of the two His peaks always co-eluted with T4-hydroxyproline and could not be quantified as most of the time this combined peak eluted as a shoulder to a large peak produced by dansyl hydroxy product [15]. With some columns this shoulder was a bit larger and this combined peak did not even appear. This first His peak constituted only one sixth of the total T4-hydroxylproline plus His peak area and 50% of the total His peak area. The second His peak was used for quantification. The areas of both peaks of Met and the second peak of His increased proportionately with increasing concentration (data not shown).

We were able to use α-methyl-DL-phenylalanine as an internal standard for quantifying amino acids for all tissue samples except sugar maple foliage. A naturally occurring amine was apparently present in sugar maple (*Acer saccharum*) samples that coeluted with this internal standard. The peak area of α-methyl-DL-phenylalanine did not vary much from sample to sample so the quantitations done by an external standard curve were comparable to the ones done by internal standard curve. Heptanediamine was the best choice for an internal standard for polyamine samples since its elution profile is very similar to that of polyamines [1].

This method was applied to analyze amino acids and polyamines from tissue cultures and foliage of several different coniferous and hardwood tree species. The chromatogram in Fig. 4 shows an amino acids profile for poplar (*Populus nigra* × *maximowiczii*) suspension cell cultures. Polyamines are not shown in Fig. 4 because they had already been extracted with toluene and were not added back into the dansylated amino acids fraction. Fig. 5 shows a chromatogram of red spruce (*Picea rubens*) foliage analyzed both for amino acids and polyamines. This method worked equally well with foliage of other coniferous trees such as balsam fir, red pine and hemlock (data not shown). Fig. 6 is a chromatogram of sugar maple leaves that shows

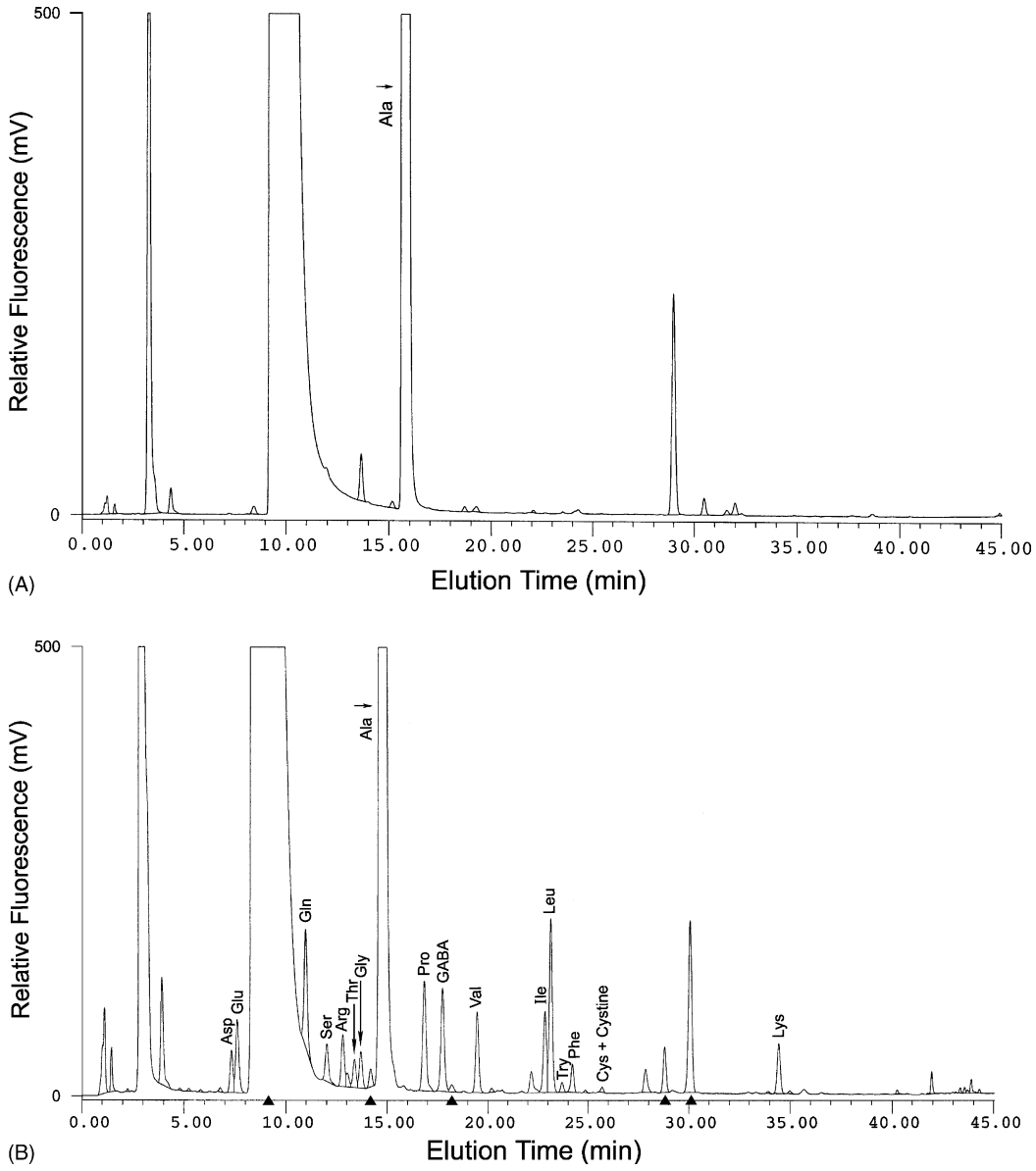

Fig. 4. (A) 5% PCA blank. (B) Separation of dansyl amino acids in hybrid poplar (*Populus nigra* × *maximowiczii*) cell cultures using the gradient profile described in Table 2 (step three was shortened to 2 min since polyamines were not analyzed). A Phenomenex Synergi Hydro-RP column was used for these analyses. Injection volume was 10 μl. Run conditions are as described in the text. The (▲) indicates the presence of blank peak.

the interference of polyphenols and tannins present in the extract with the dansylation process. The binding problems and possible interference with dansylation in the case of sugar maple can be seen clearly by comparing the size of the alanine peak (Ala is added to each sample in excess for termination of dansylation reaction) between sugar maple, red spruce and the standard mix. This peak is same size for red spruce (Fig. 5) and standard mix (Fig. 3) but is very small in comparison for sugar maple (Fig. 6) keeping in mind that the total amines to be dansylated was highest in the standards. This tissue contained a large number of unidentifiable dansylated amines. At the end of dansylation reaction using sugar maple leaf extracts, there is often a small yellow pellet at the bottom of the tube in this species that is not present in a dozen other tree species tested. Sugar maple leaf extract strongly binds polyamines as seen by the size of heptanediamine (internal standard for polyamines) peak in this chromatograph. When exogenous polyamines were added to this extract over 90% of these were not recovered (data not shown). However, this procedure could still be used with sugar maple leaves where effects of plot level treatments on amino acids and polyamines were to be compared. For polyamines, the internal standard, heptanediamine, was very useful in estimating the actual level of polyamines present in the extracts since in many cases the extent of binding was the same for the internal standard and the three polyamines as tested by comparing quantities and trends obtained by external standard and internal

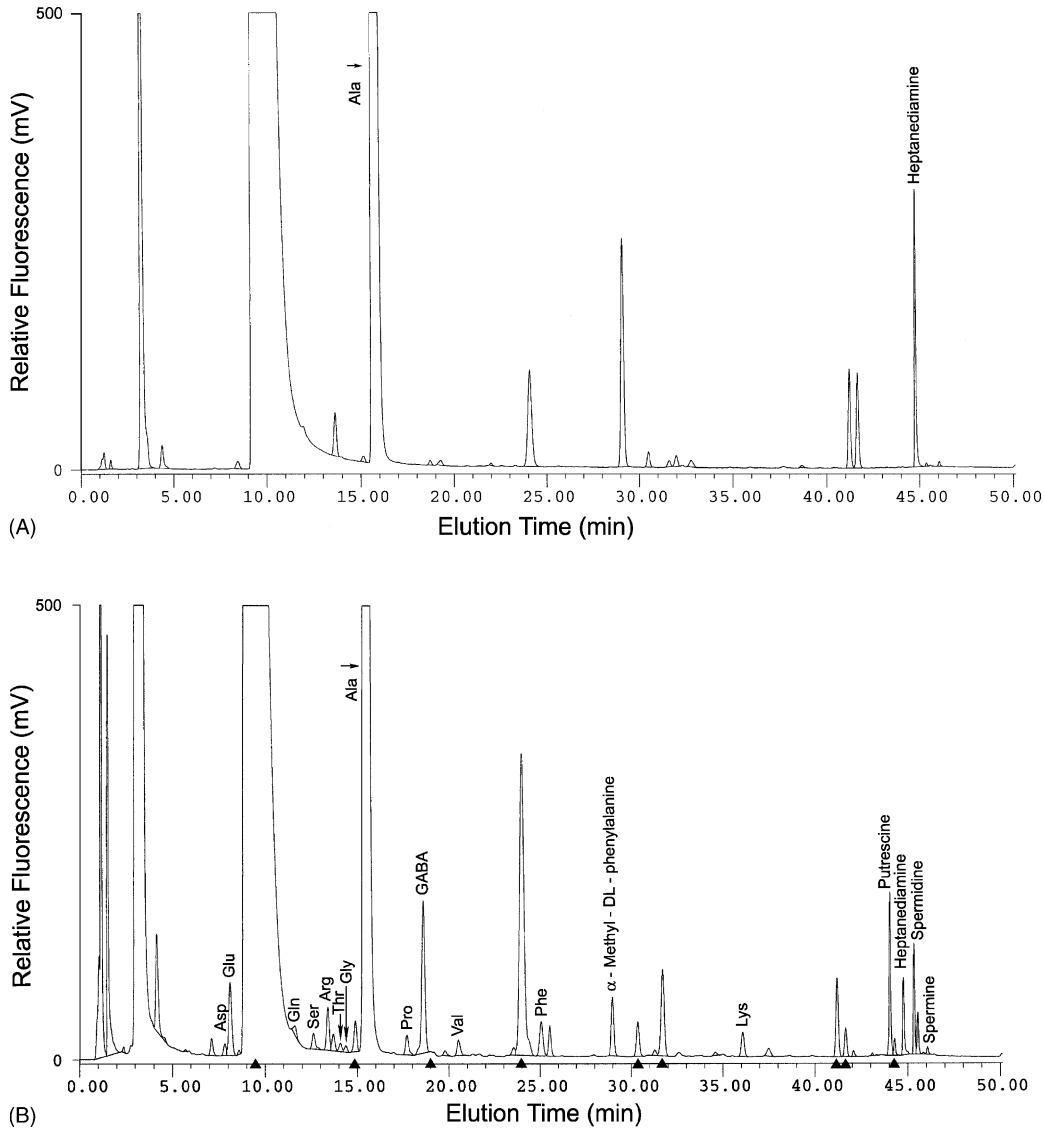

Fig. 5. (A) 5% PCA blank. (B) Separation of dansyl amino acids and dansyl polyamines in foliage of mature red spruce (*Picea rubens*) trees using the gradient profile described in Table 2. A Phenomenex Synergi Hydro-RP column was used for these analyses. Injection volume was 10 μl. Run conditions are as described in the text. The (▲) indicates the presence of blank peak.

standard curves. Wood tissue from sugar maple and foliage of other hardwoods such as oak, cherry, yellow birch, American beech do not seem to have any of these problems for dansylation (data not shown).

Experiments were also conducted to study the recovery of several concentrations of exogenously added standards to various tissue types. These data show that the recovery of amino acids and polyamines was tissue type dependent with sugar maple foliage being at the lowest end of the scale for recovered amines and polyamines (data to be presented elsewhere).

3.3. Column performance

It is important to point out that sometimes the performance of an analytical method relies heavily upon the use of a specific column. We were able to achieve similar separation and performance using two different types of columns by the same manufacturer (Phenomenex). The first column was a Luna C_{18} (RP), 100 mm × 4.6 mm, 3 μm particle size (Phenomenex No. 00D-4251-E0) column. When the manufacturing specifications changed for the silica being used for these columns, the HPLC separation using a new batch of columns resulted in the coelution of serine and arginine under the same elution conditions as described earlier. However, the Synergi Hydro-RP, 100 mm × 4.6 mm, 4 μm particle size (Phenomenex No. 00D-4375-E0) column resulted in satisfactory resolution of these two peaks. The detection limits and linear range for all of the amino acids and polyamines are given in Table 3 for separations using a Synergi Hydro-RP column.

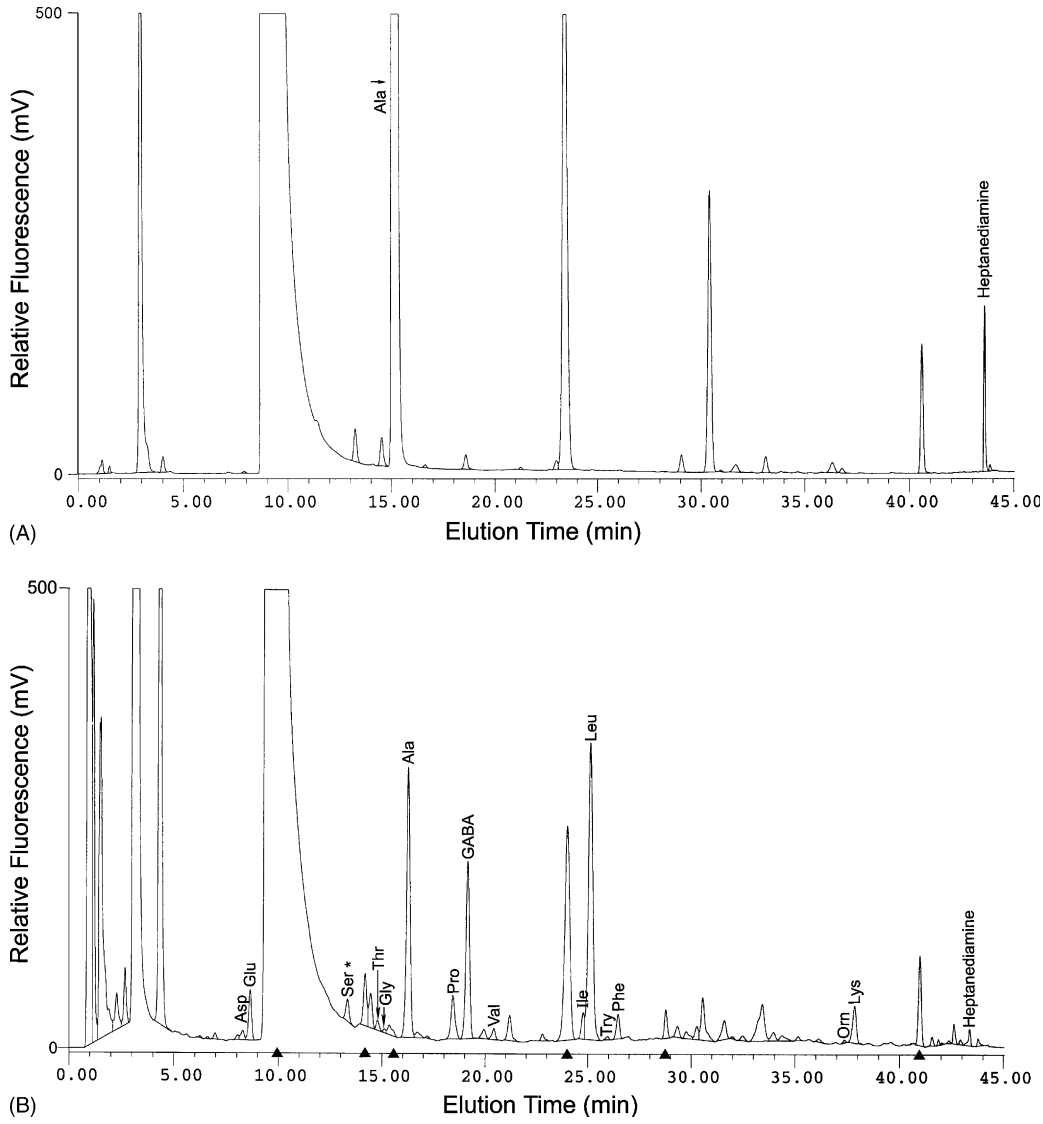

Fig. 6. (A) 5% PCA blank. (B) Separation of dansyl amino acids and dansyl polyamines in foliage of mature sugar maple (*Acer saccharum*) trees using the gradient profile described in Table 2. A Phenomenex Synergi Hydro-RP column was used for these analyses. Injection volume was 10 μl. Run conditions are as described in the text. The (▲) indicates the presence of blank peak.

3.4. Method performance

Analyses of dansylated polyamines and amino acids are routinely performed using two separate HPLC methods [1,2,14,17]. The shortest HPLC method available for dansylated polyamine separations takes only 4 min with a total run time of 8.7 min [1]. Most of the currently available methods for the separation of dansyl amino acids require significantly longer run time than the one presented in this study [2,14]. An efficient and fully automated method has been reported for amino acids separation that takes only 38 min to separate 25 amino acids [18]. However, it requires a sample processor unit for sample dilution, pre-column derivatization of both primary and secondary amine groups with *o*-phthalaldehyde-3-mercaptopropionic acid and 9-fluorenylmethyl chloroformate and on-line dialysis to remove sample impurities.

Methods that use any type of fluorescent tagging agents and have been used for simultaneous separations of amino acids and biogenic amines also have relatively long run times [4] with the exception of one method described by Kultán and Molnár-Perl [19]. This method uses OPA for derivatization and separates 37 compounds in a total run time of 53 min. However, this procedure cannot separate the amino acid, proline, and the common polyamine, spermidine, both of which play an important role in plant metabolism, especially stress physiology, and are critical elements of our research project. The new method presented here simultaneously separates dansylated amino acids and common polyamines, saves time, reduces the costs of solvents, supplies, and waste disposal and is suitable for studying plant stress responses involving changes in polyamines and proline.

The major changes incorporated in the present method as compared to previously published HPLC methods for the

separation of dansyl amino acids are: (I) removal of excess reagent (carbonate) from the reaction mix with acetic acid after derivatization with dansyl chloride. Without the addition of acetic acid to remove excess carbonate, sodium carbonate settled on the column, increasing backpressure and reducing column longevity; (II) removal of particulate matter from dansylated tissue extracts by filtration through a 0.45 μm nylon filter; (III) the pH of the buffer was precisely set at pH 5.94 for better separation of peaks that elute early within the profile, especially Asp and Glu; (IV) use of a 10 cm column to significantly reduce run time; (V) shorter equilibration time by the addition of 3% 1-propanol to the buffer as suggested by Warner and Dorsey [14]; (VI) modification of the gradient profile to elute dansyl polyamines within the same run as dansyl amino acids. To accomplish this, gradient step 3 in the amino acid method was increased by only 2 min (Table 2); (VII) addition of a step for column washing with water containing 10% ACN within each run to remove water soluble impurities that might adhere to the column; (VIII) addition of an extensive washing of column with 100% ACN and distilled and deionized water containing 10% ACN was done and the column stored in ACN–water (150:50) at the end of a batch/loop of samples; (IX) column direction was physically reversed and the column washed with 100% ACN and distilled and deionized water containing 10% ACN before starting the next loop of samples in this reversed direction; and finally (X) as suggested recently by Dolan [16], the column was dedicated to this one method and not used for any other type of analyses. Several other column maintenance points described by Dolan [16] were also implemented. In total, these modifications improved peak resolution and increased column longevity by several-fold. This method eluted 22 dansyl amino acids within 40 min and three common dansyl polyamines between 44 and 47 min. The total run time is 53.6 min for amino acids only and 55.6 min for both. Over 1000 foliar or tissue culture samples from conifer and hardwood trees could be analyzed with the same column.

4. Conclusions

A new method for simultaneous quantitation of amino acids and common polyamines was developed and protocols were optimized for dansyl derivatization mainly to analyze various conifer and hardwood tree tissues and cell cultures. Foliage of mature sugar maples (*Acer saccharum*), contains high concentrations of polyphenols and tannins that interfere either with the dansylation process or bind to and precipitate other compounds present in or added to the cell extracts. The combination of excess reagent removal, sample filtration, and frequent column cleaning steps has enabled us to achieve better separation for dansyl amino acids and dansyl polyamines for these tissues and substantially increase column life. The run time for this method is significantly shorter than most previously published HPLC methods for separation of dansyl or dabsyl amino acids with or without polyamines.

Acknowledgements

The authors would like to thank Dr. John Dolan, Dr. William R. Seitz, Dr. Gary Weisman, and Dr. Sterling Tomellini for excellent guidance during the stages of method development. Thanks are also extended to Benjamin Mayer and Kenneth R. Dudzik for technical assistance, and to Dr. Leland Jahnke, Dr. Sterling Tomellini, Dr. Kevin Smith, and Dr. Subhash Minocha for critical review of the manuscript.

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