Communication

High Speed HPLC Analysis of Polyamines in Plant Tissues

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ABSTRACT

A high speed high performance liquid chromatography (HPLC) method for the quantification of putrescine, spermidine, and spermine in biological samples is described. The dansylation is followed by a sample cleanup. The isotropic HPLC-analysis with acetonitrile-H2O (72:28 volume/volume) on 10 cm tlc plates allows only 4.5 min. By our method about 100 analyses can be done in 1 day.

Polyamines are known to be ubiquitous in plant and animal cells. Although there has been an increasing interest in polyamines during recent years as illustrated by the large number of survey articles on this subject, their exact function is still unknown (2, 8–10). Our interest in the polyamine content during the adventitious root formation on the hypocotyl of intact mung bean seedlings growing in corn silo solution (4) confronted us with a huge number of analyses. Therefore, we needed a fast, inexpensive, and reliable method for polyamine quantification.

Fluorometric quantification of TLC eluates of dansylated polyamines (6) did not give valuable results due to impurities as revealed by fluorescence scanning of the eluates (similar peak shapes were not always obtained). This throws doubt upon some results obtained by measuring the fluorescence at a fixed wavelength only, so often done by plant physiologists. Moreover, this method is rather laborious. If two samples of one extract are dansylated, the quantification of putrescine, spermidine, and spermine necessitates 2 x 3 scans of the unspiked TLC eluates to check their purity, and at least 24 readings on the fluorometer (one for each unspiked TLC eluate and three times for each eluate spiked with three known amounts of standards). The use of an internal standard is excluded.

Polyamine analysis was improved by using labeled dansylchloride and measuring the radioactivity of the bands after chromatography on RP-TLC (C18) in acetone-water (70:30). However, no internal standard can be used and the method is rather expensive.

The analysis and quantification of dansylated polyamines can be much improved by using a Dual-Wavelength TLC Scanner with an activating wavelength of 360 nm and a fluorescent wavelength of 500 nm as used by Katoh et al. (3). This method also allows the use of an internal standard.

HPLC on conventional RP-columns was too slow for our purpose (15–20 min analysis time) and required gradients causing loss of time while re-equilibrating the column between runs. Gradients require expensive HPLC solvents and the solvent consumption per analysis is high.

As we had no access to a fluorimetric HPLC-detector, we choose to work with dansyl derivatives that are measured at 337 nm, allowing the use of low cost solvents (benzoates as used by Redmond and Tseng [5] and Flores and Galston [1] would require very pure solvents to measure in the low UV). Gradients were avoided because they require more expensive and extensive equipment, enhancing the risks of instrumental breakdown, and they make re-equilibration necessary.

MATERIALS AND METHODS

Extraction and Derivatization. Tissue (200–250 mg) frozen in liquid N2 is homogenized in 2 ml 4% HClO4 containing 1,7-diaminoheptane -2HCl (5 mg/l) as internal standard. After 1 h at 4°C the homogenate is filtered by sucking over glass wool plugged into 5 ml pipet tips. To 0.2 ml homogenate 1 ml of carbonate buffer pH 9 and 1 ml of dansyl chloride solution (10 mg/ml acetone) is added. After vigorous shaking (Vortex) the mixture is heated for 1 h at 60°C in the dark, cooled and stored in a refrigerator. The dansylated polyamines are extracted with 3 ml toluene.

Post Derivatization Cleanup. The cleanup step is based on the method described by Seller et al. (7), which was adapted for allowing very quick routine analyses. The toluol extract is purified over 0.5 g silica gel columns made in 5 ml pipet tips plugged with glass wool at the constricted end (silica gel: 0.063–0.2 mm diameter). Ten columns at a time are rinsed, under reduced pressure, with 5 ml toluol and 5 ml toluol-triethylamine (10:1 v/v). These eluates are discarded. The rack with pipet tips is then placed on a device to elute the dansylated polyamines under reduced pressure into 20 ml vials with 2 times 3 ml ethylacetate. The volume ethylacetate is reduced under a stream of N2 at 50°C, transferred into 1 ml sample vials and completely dried. The sample cleanup takes approximately 1 d for 300 samples.

HPLC Analysis of Dansylated Polyamines. Small columns of 10 cm length and 3 mm internal diameter filled with 3 μm reverse phase material of Chrompack-Nederland (C.P. Microsphere) were used. This material was selected because of its well suited retention characteristics to perform the analysis in an isocratic run within 4.5 min. The column was heated at 50 ± 0.1°C in a water jacket, so reducing the column back-pressure and the solvent viscosity and accelerating the separation while the resolution remained sufficient for our analytical problem. The solvent flow was 2 ml/min (acetonitrile-H2O 72:28 v/v).
The samples were dissolved in 50 μl methanol. The injection volume was 10 μl.

RESULTS AND DISCUSSION

In Figure 1 a typical chromatogram of dansylated reference compounds is shown (Dns-putrescine, Dns-diaminoheptane, Dns-spermidine, Dns-spermine). The most retained compound of our mixture (Dns-spermine) is completely eluted within 4.5 min, and this by an isocratic solvent run at 50°C. This is much faster than any published method. At room temperature (20°C) the analysis time was more than 10 min. The reduction in retention time by placing the column in a water jacket at 50°C and by insulating the tubing did not affect the peak resolution very much, but had a positive effect on the lower detection limit as the peak heights increased. The higher temperature also lowered the column back-pressure from 400 kg/cm² to 200 kg/cm² due to the lower viscosity of the solvent, and thus allowing a higher solvent flow (up to 2 ml/min).

The detector response was linear for polyamine concentrations between 0 and 100 μM, thus covering the endogenous concentration range in plants. Figure 2 shows a chromatogram of a dansylated extract of leafs from 3 d old etiolated mung bean seedlings (Phaseolus aureus Roxb.), grown at 25°C. The first large peak mainly consisting of dansylammonia and dansyldimethylamine (7) is sufficiently separated from putrescine to allow a correct integration. In our mung bean seedlings putrescine, spermidine and spermine were detected, but no cadaverine. As we used a spectrophotometer equipped with 8 μl flow-through cells as UV-detector, the practical sensitivity limit was 0.1 AUFS. This restricted the lower detection limit to about 1 nmol/g fresh weight for putrescine and spermidine, and to about 10 nmol/g fresh weight for spermine. By using a modern UV-detector or fluorescence detector the lower detection limit will be improved at least 50 times. With Dns-heptanediamine as internal standard straight calibration curves were obtained up to 100 μM (putrescine).

Table 1. Retention Times and Amounts of Putrescine, Spermidine, and Spermine in 3 Day Old Etiolated Mung Bean Seedlings

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Retention Time</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>Leaf Cotyledons</td>
</tr>
<tr>
<td>Dns-putrescine</td>
<td>0.65 ± 0.01</td>
<td>519 ± 31 80 ± 6</td>
</tr>
<tr>
<td>Dns-heptanedi</td>
<td>0.94 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Dns-spermidine</td>
<td>1.54 ± 0.01</td>
<td>1229 ± 37 389 ± 22</td>
</tr>
<tr>
<td>Dns-spermin</td>
<td>4.19 ± 0.02</td>
<td>552 ± 26 81 ± 7</td>
</tr>
</tbody>
</table>
As we encountered some problems with the reproducibility of the dansylation of some plant extracts, we found the yield of these dansylations to be strongly influenced by the pH. Therefore, we used a carbonate buffer at pH 9 as also used by Rosier and Van Peteghem (personal communication). In our further work this carbonate buffer at pH 9 is always used thus avoiding all our previous problems with reproducibility. We have also experienced difficulties with the derivatization itself. Often the dansylation is not carried out under strictly defined conditions (e.g. overnight at room temperature). We have found varying results due to the duration and temperature of derivatization, 1 h at 60°C being the best condition for quantitative and reproducible results. By working under controlled conditions we obtained very reproducible results. The retention times calculated for 10 different plant extracts (3 d old etiolated mung bean cotyledons and leaves) are given in Table I. In this table the reproducible quantitative results are also demonstrated, the SE varying between 3 and 8.6%. The use of this high speed isocratic HPLC analysis with a peak detection at 337 nm enabled us to work with low cost solvents that could even be redistilled and reused. About 100 samples can be analyzed in 1 d. Then, however, an automatic injector becomes very desirable.

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LITERATURE CITED