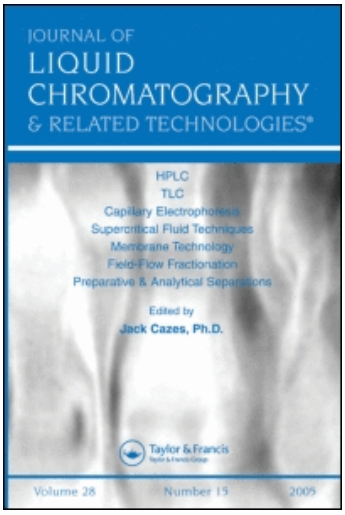


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# The Analysis of Putrescine in Plant Samples by Automated Hplc

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# THE ANALYSIS OF PUTRESCINE IN PLANT SAMPLES BY AUTOMATED HPLC

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ABSTRACT

The quantitative analysis of putrescine from plant tissue can be achieved using isocratic reversed-phase high performance liquid chromatography (HPLC). After preliminary extraction and clean up involving an ion exchange purification step, the isolated diamines are derivatised with benzoyl chloride for determination by HPLC. Automation of the HPLC step has led to a considerable saving in time for the total analysis. Potential problems associated with the analytical procedure are described.



## INTRODUCTION

The diamine, putrescine, has been found to accumulate in a wide range of plants when grown in potassium deficient conditions (1). Although other nutritional deficiencies such as magnesium, calcium, phosphorus and sulphur have been found to enhance the formation of putrescine to a small extent (2), potassium deficiency appears to be the major factor. Therefore, it has been suggested (3, 4) that the concentration of putrescine in the plant could provide a sensitive biochemical indicator of potassium status. It was decided to examine this possibility with pasture plants.

Putrescine has been determined in a variety of biological samples by several methods (5), while determinations from plant samples have used paper chromatography (4), thin-layer chromatography (6) and gas chromatography (7). Published procedures for the analysis of putrescine by high performance liquid chromatography (HPLC) have relied on the separation of the tosyl (8, 9), dansyl (10-13) or

benzoyl (14) derivative or estimation of the o-phthalaldehyde derivative after post-column derivatisation (15, 16). These procedures have all been applied to body fluids, and no HPLC method for the analysis of putrescine from plant samples has been reported. In addition, while most described methods were satisfactory for standards, several authors (11, 13) reported difficulties when real samples were analysed. Methods shown to be satisfactory with body tissue samples (10-12) employed gradient elution and fluorescence detection, and in one case (11) the need for preliminary derivative purification prior to HPLC was reported. Since fluorescence detection was unavailable in our laboratory, we investigated methods employing UV detection. The tosyl derivatisation described for urine (9), although employing an elaborate preliminary purification step similar to one commonly used for the analysis of polyamines from plant samples (7), gave highly variable results with plant samples. We have adapted the benzoylation derivatisation, previously described only for standards (14), and combined this with an adapted batch process for preliminary purification of the plant extract (7) prior to derivatisation.



This paper describes the total extraction, purification, derivatisation and analysis of plant samples using isocratic reverse phase HPLC with UV detection. Application of automated HPLC to decrease the overall analysis time is described. Recovery and reproducibility data for analysis of standards, both without and with plant material, taken through the complete procedure are also given.

## METHODS

### Apparatus

The liquid chromatograph was a modular system consisting of two Spectra Physics Model 740B pumps, a Spectra Physics Model 744 solvent programmer and a Model 714 pressure monitor. Sample injection was by a Valco 7000 p.s.i. injection valve fitted with a 10 µl

sample loop or by a Micromeritics Model 725 autosampler fitted with a 10 µl sample loop. Detection was with a Tracor 970A variable wavelength detector. Integration of peaks was done with a Spectra Physics Minigrator; the external events of this instrument could be used, if desired, to operate the solvent programmer in auto-analysis. Incorporation of the External Data Interface option allowed sample identification to be included in the integrator printout for automation and for the integrator start to be synchronised with the autosampler inject signal. The detector output passed via the minigrator to a Houston Omniscribe two pen recorder.

## Solvents and Chemicals

Methanol was analytical grade and water was from a Millipore Milli-Q water purification system. Putrescine and 1,6-diaminohexane were better than 98% purity (Aldrich Chemical Co., Milwaukee, WI). All other chemicals were laboratory reagent grade. Benzoyl chloride was redistilled. Standards of dibenzoyl putrescine and dibenzoyl-1,6-diaminohexane were prepared by scaling up the derivatisation reaction and isolating and recrystallising the products which were characterised by melting point and mass spectra.



## Chromatographic Conditions

The columns used were Zorbax C-8 (25 cm x 4.6 mm ID) (Dupont, Wilmington, DE), RP-8 (25 cm x 4.6 mm ID) (Brownlee Labs, Santa Clara, CA), and µ Bondapak C18 (30 cm x 3.9 mm ID) (Waters Assoc., Milford, MA). The analyses were performed at room temperature between 19-22$^{o}$C using isocratic solvent mixtures of water-methanol. Similar solvent compositions were employed with all three columns, namely 40:60 water-methanol for preliminary tests without plant, and 44:56 water-methanol for final analysis of plant samples. The flow rate was generally 1 ml/min. The detector was set at 230 nm which is close to the absorption maximum for both derivatised diamines. At least two injections were made from each sample.

Extraction and Preliminary Purification of Putrescine

Plant material (3 g) was macerated with 5% trichloroacetic acid (24 ml) in a 60 ml centrifuge tube, and the mixture held at 2°C for 18 h. After centrifuging, an aliquot (15 ml) of the supernatant layer was removed onto Dowex - 50 x 8 ion exchange resin (1 g, 20-50 mesh, $H^+$ form) in a 50 ml test tube, and 1,6-diaminohexane (150 µg in 50 µl of 5% trichloroacetic acid) added as internal standard. After shaking for 1 h, the liquid was removed by suction with a medium porosity sintered gas distribution tube. The resin was then washed with water (5 ml), and this solution similarly removed. Concentrated hydrochloric acid (10 ml) was added to the resin and the mixture shaken for 2 h. An aliquot (5 ml) of the resulting solution was transferred to a 50 ml test tube and evaporated to dryness under vacuum at 70°C.



Derivatisation of the Amine Fraction

The dried extract was dissolved in water (1 ml), and 2N sodium hydroxide (5 ml) and benzoyl chloride (50 µl) added. The mixture was shaken and allowed to stand at room temperature for 0.5 h. Saturated sodium chloride (10 ml) was added and the mixture extracted with ethyl acetate (10 ml). The organic layer (8 ml) was removed and evaporated to near dryness under vacuum at about 40°C, then at 70°C for a further 5 min. The residue was redissolved in methanol (2 ml) for HPLC analysis.

## RESULTS

Recovery in Absence of Plant

Standard solutions containing a range of concentrations of both putrescine (3-1200 µg) and diaminohexane (20-200 µg) were taken through the complete clean-up, derivatisation and analysis procedure. The absolute theoretical recovery of both compounds relative to a 50 µg/ml external standard mixture of dibenzoyl

putrescine and dibenzoyl diaminohexane was calculated and found to be similar for both compounds (Table 1) in the ranges tested. The percentage recovery of putrescine calculated using the internal standard method was 101% (CV = 8%) when the response ratio of the external standard mixture of derivatives was used, and 99% (CV = 6%) when the response ratio of a processed standard containing initially 120 µg of both putrescine and diaminohexane was used. With the detector set at 230 nm, $10^{-3}$ µg of both benzoylated diamines gave approximately 1% full scale deflection at 0.02 a.u.f.s., and the linear range for both compounds extended to 5 µg per injection.



## Recovery from Plant Samples

To test the reproducibility of the analysis method for plant samples as well as the recovery of both putrescine and 1,6-diaminohexane internal standard from real samples, a series of lucerne (*Medicago sativa* L.) and red clover (*Trifolium Pratense* L.) samples, both spiked and unspiked, were analysed. Either four or five replicate subsamples from each plant sample were analysed, and the results are summarised in Table 2.

TABLE 1

Absolute Recoveries* of Putrescine and 1,6-Diaminohexane Standard Mixtures.

| | Mean Recovery (%) | CV (%) | Range (%) |
|---|---|---|---|
| Putrescine | 79 | 9 | 68-97 |
| Diaminohexane | 76 | 9 | 69-93 |

* Calculated from 30 analyses.

TABLE 2

Recoveries of Putrescine and 1,6-Diaminohexane[1] from Plant Samples.

| Sample[2] | Mean IS Recovery[3] | | Mean putrescine conc.[4] (µg/g plant) | | | |
|---|---|---|---|---|---|---|
| | % | CV (%) | Total | CV (%) | Spike | CV (%) |
| A | 75 | 7 | 10 | 14 | - | - |
| A + spike[5] | 76 | 4 | 53 | 2 | 43 | 3 |
| B | 88 | 6 | 88 | 11 | - | - |
| B + spike[5] | 87 | 9 | 118 | 9 | 30 | 35 |
| C | 90 | 3 | 64 | 11 | - | - |
| D | 60 | 3 | 174 | 13 | - | - |
| E | 55 | 3 | 35 | 13 | - | - |
| E stems[6] | 59 | 12 | 57 | 24 | - | - |
| E leaves[6] | 55 | 13 | 10 | 4 | - | - |
| F | 75 | 13 | 3 | 38 | - | - |
| F + spike[5] | 76 | 7 | 42 | 7 | 39 | 6 |

1. Added as internal standard (IS) before preliminary purification.
2. Sample A was red clover; samples B, C, D, E, F, were lucerne.
3. Absolute recovery relative to a 50 µg/ml external standard of dibenzoyl derivative.
4. Total putrescine concentration calculated by the internal standard method and using the response ratio of a standard mixture of putrescine and diaminohexane taken through the complete procedure. Concentration of the spike was determined using the individual putrescine responses from the spiked samples less the mean of the responses from unspiked samples and corrected for internal standard response.
5. Spike of 50 µg putrescine/g fresh plant, added before maceration.
6. Mean ratio of weights of stems : leaves was 1:1.65.

The absolute recovery of the internal standard was in the range 55-90%. The gross variations in recovery occurred between batches processed on different days, and within run variability was usually below 10%. Estimates of the concentration of putrescine usually varied by less than 15% for subsamples for the same plant sample. Recovery of the spike was in the range of 60-86%, and was higher and less variable when the originally unspiked plant material contained less putrescine. When the method was employed for routine analysis of plant samples, the putrescine concentrations were estimated using the internal standard method and using the response ratio obtained from low putrescine plant samples (less than 5 µg/g) spiked with 50 µg of putrescine/g of fresh plant, and corrected for natural putrescine content. For the analysis of plant samples the mobile phase used was 44:56 water-methanol. As Figure 1 shows,



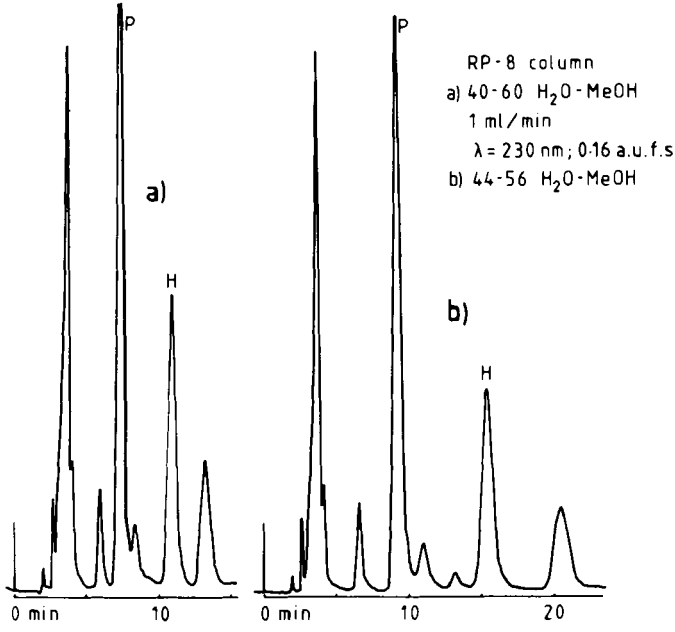


FIGURE 1. Separation of the dibenzoyl derivatives of putrescine (P) and 1,6-diaminohexane (H) from lucerne extract.

this gave greater resolution of the peaks of interest than the 40:60 water-methanol mobile phase used for the analysis of standards. The total sample analysis time was 23 min.

## Potential Sources of Error

The number of glassware changes have been reduced to a minimum to avoid losses and cross-contamination due to transference. The main potential source of cross-contamination is from bumping during vacuum evaporation of the concentrated hydrochloric acid amine fraction. Of more importance is the need to follow the final derivative evaporation with a period of higher temperature (70°C) evaporation to remove high boiling interferences. The value of this step is illustrated in Figure 2.

Comparison of injections 1 and 2 shows failure to use the higher temperature for evaporation leaves three prominent impurities in the sample at retention times of approximately 7, 13 and 28 min. This results in interference with the quantitation of putrescine as well as an unnecessarily long analysis time. Injections 3, 4 and 5 illustrate a more serious effect that would result in automated analysis if occasional samples were not processed correctly; the impurity at 28 min. from injection 3 would make accurate quantitation of injection 4 impossible.



## Automation

The extraction, preliminary purification and derivatisation procedures described require care, and are therefore time consuming. For example, 30 samples would commonly require 3 man-days to process. The additional time required for HPLC analysis meant that 7-8 man-days were required to fully analyse 30 samples - clearly an unacceptable period. Incorporation of an automatic sampling device into the HPLC system, and the coupling of this to a computing integrator, meant that the total analysis and quantitation effectively could be completed in little more than the time required for the extraction, purification and derivatisation process. In addition,

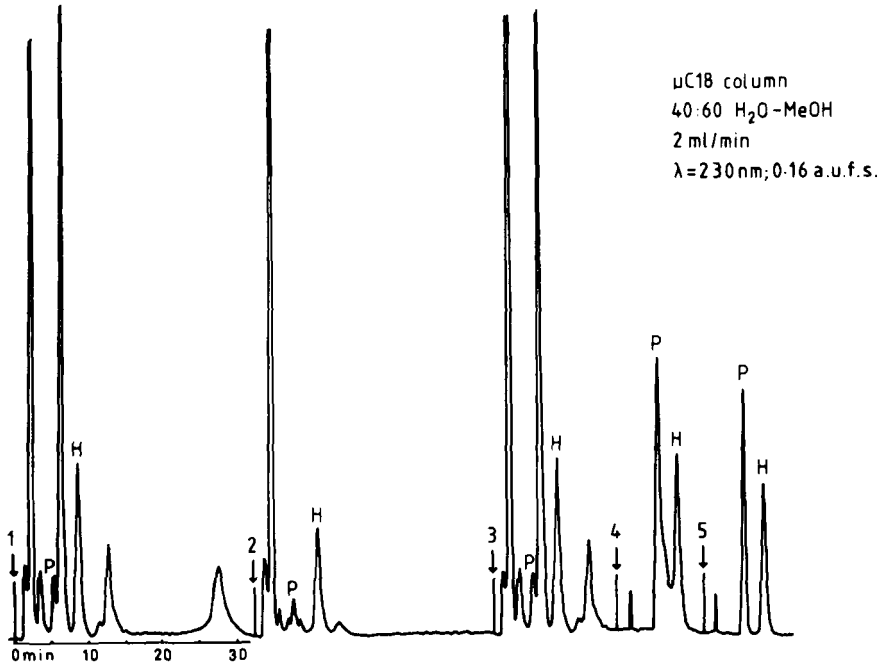


FIGURE 2. Illustration of the effect of failure to follow the 70°C evaporation of derivative solutions. Retention time scale identical for all samples. Injections marked by arrows, 1 : low putrescine content plant sample without high temperature evaporation of derivative solution; 2 : same sample with high temperature evaporation; 3 : as for 1; 4, 5 : 50 μg/ml standard mixture of derivatives. P = dibenzoyl putrescine; H = dibenzoyl 1,6-diaminohexane.

the greater inherent stability of an HPLC system in continuous operation compared to one in stop-go operation meant that fewer test standards were required in the analysis run. With two injections per sample, within sample variation was less than 1%, and reproducibility throughout the run was high. In three runs, each of 40 h duration, the variation in eight test standards spaced throughout was less than 3.3% in each case. For automated analysis a sample run time of 26 min. was set to allow complete sample analysis and peak area printout before the next injection occurred.

Alternative Clean-up Procedures

Several methods were tried in an attempt to simplify the preliminary clean-up procedure. A paired ion extraction of the diamines with hexane sulphonic acid was unsuccessful, as was a chloroform extraction after prewashing the initial plant extract with chloroform and basifying with 2N sodium hydroxide. Acceptable recoveries of both putrescine and 1,6-diaminohexane were obtained by simply derivatising an aliquot of the trichloroacetic acid extract either with or without a prior ether wash. However, in the case of direct derivatisation of the extract, several minor co-extractives interfered with the quantitation of putrescine in particular in the HPLC analysis. Preliminary washing with ether before derivatisation often gave less interferences in the vicinity of the dibenzoyl putrescine and dibenzoyl diaminohexane peaks, although not reproducibly so. In addition, both these sets of samples contained significant co-extractive peaks at higher retention times which would considerably lengthen the total analysis time. In automated runs, these co-extractives interfered with the quantitation of samples up to three injections later. Samples prepared by these shorter methods also produced a considerable quantity of methanol insoluble material after derivatisation and therefore each sample required filtration before submission for HPLC analysis.



REFERENCES

1. Murty, R.S., Smith, T.A. and Bould, C. The Relation Between the Putrescine Content and Potassium Status of Black Current Leaves, Ann. Bot. (London), 35, 687, 1971.

2. Smith, T.A. The Occurrence, Metabolism and Functions of Amines in Plants, Biol. Rev. Cambridge Philos. Soc., 46, 201, 1971.

3. Basso, L.C. and Smith, T.A. Effect of Mineral Deficiency on Amine Formation in Higher Plants, Phytochemistry, 13, 875, 1974.

4. Hoffman, M. and Samish, R.M. Free Amine Content in Fruit Tree Organs as an Indicator of the Nutritional Status with Respect to Potassium. Recent Advances in Plant Nutrition, Vol. 1, Samish, R.M., Ed., Gordon and Breach Science, New York, 1971, p. 189.

5. Extensive references to all these methods are given in (8), (11) and (13).

6. Smith, T.A., Amine Levels in Mineral-deficient Hardeum valgare leaves, Phytochemistry, 12, 2093, 1973.

7. Smith, T.A., Putrescine, Spermidine and Spermine in Higher Plants, Phytochemistry, 9, 1479, 1970.

8. Sugiura, T., Hayashi, T., Kawai, S. and Ohno, T., High-Speed Liquid Chromatographic Determination of Putrescine, Spermidine and Spermine, J. Chromatogr., 110, 385, 1975.

9. Hayashi, T., Sugiura, T., Kawai, S. and Ohno, T., High-Speed Liquid Chromatographic Determination of Putrescine, Spermidine and Spermine in Human Urine, J. Chromatogr., 145, 147, 1978.

10. Newton, N.E., Ohno, T. and Abdel-Monem, M.M., Determination of Diamines and Polyamines in Tissues by High-Pressure Liquid Chromatography, J. Chromatogr., 124, 277, 1976.

11. Seiler, N., Knodgen, B. and Eisenbeiss, F., Determination of Di- and Polyamines by High-Performance Liquid Chromatographic Separation of Their 5-Dimethylaminonaphthalene-1-sulphonyl Derivatives, J. Chromatogr., 145, 29, 1978.

12. Saeki, Y., Uehara, N. and Shirakawa, S., Sensitive Fluorimetric Method for the Determination of Putrescine, Spermidine and Spermine by High-Performance Liquid Chromatography and its Application to Human Blood, J. Chromatogr., 145, 221, 1978.

13. Vandemark, F.L., Schmidt, G.J. and Slavin, W., Determination of Polyamines by Liquid Chromatography and Precolumn Labelling for Fluorescence Detection, J. Chromatogr., Sci. 16, 465, 1978.

14. Redmond, J.W. and Tseng, A., High-Pressure Liquid Chromatographic Determination of Putrescine, Cadaverine, Spermidine and Spermine, J. Chromatogr., 170, 479, 1979.

15. Stripe, J.R. Jr. and Savory, J., High Performance Liquid Chromatographic Separation and Fluorescence Detection of Polyamines in Plasma and Erythrocytes, Ann. Clin. Lab. Sci., 10, 128, 1980.

16. Shaw, G.G., Al-Deen, J.H.S. and Elworthy, P.M., The Construction and Performance of a Low Cost Automated HPLC System for Polyamine Assay, J. Chromatogr. Sci., 18, 166, 1980.