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Note

Determination of zeatin and zeatin riboside in plant tissue by solid-phase extraction and ion-exchange chromatography

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Kling *et al.*[1] recently reported on a high-performance liquid chromatographic (HPLC) system for analysis of indoleacetic acid (IAA) in plant tissue. One of the benefits of the reported system is that the initial preparative HPLC procedure yields three partially purified fractions; one containing IAA, another containing abscisic acid (ABA) and the third fraction containing the cytokinins zeatin and zeatin riboside. Final quantitative HPLC procedures for analysis of IAA[1] and ABA[2] have been reported. This paper describes a rapid and reproducible system for quantitative analysis of zeatin and zeatin riboside in the above mentioned fraction.

Many systems have been reported for final separation and quantitation of cytokinins. These systems include bioassay[3–8], radioimmunoassay[9,10], ion-exchange chromatography[11], reversed-phase chromatography[12–16], reversed-phase ion-pair chromatography[13] and chromatography on polyvinylpolypyrrolidone (PVPP) columns. Bioassay was not used as a quantitative procedure due to the inherent problems with reproducibility and interfering compounds present in plant extracts[17]. None of the above chromatography systems were effective at separating the cytokinins from interfering compounds present in the partially purified fraction. Insoluble PVPP has been used in open columns for sample purification and final separation in several hormone analysis systems[13,18,19]. PVPP exhibits strong retention of cytokinins at neutral pH, and rapid elution is possible using methanol[18]. The present study reports on the use of PVPP solid phase extraction followed by analytical cation-exchange HPLC to accomplish rapid analysis of zeatin and zeatin riboside, previously purified on our preparative system.

There are many reports of systems for purification and quantitation of the cytokinins however few of these contain information on recovery and variability[3,4,6,10,20,21]. The coefficient of variation (C.V.)[22] indicates the variability of a system and is useful for evaluation of analytical procedures; however, it is seldom included in descriptions of analysis systems. The C.V. can be calculated or estimated from the data presented in some reports. The system reported here has been evaluated for recovery and reproducibility using a radiolabelled internal standard.

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MATERIALS AND METHODS

The entire system for analysis of zeatin and zeatin riboside in plant tissue consists of four steps; extraction, preparative HPLC, solid-phase extraction using PVPP cartridges and ion-exchange chromatography. The extraction and preparative HPLC procedures have been described previously[1]. The following is a description of the PVPP and ion-exchange procedures.

Solid-phase extraction

Dry, methanol-washed PVPP (GAF, New York, NY, U.S.A.) was loaded into 4-ml plastic syringe barrels (PGC Scientifics, Gaithersburg, MD, U.S.A.) (0.25 g PVPP/syringe) with two glass microfibre filters (1.0 cm GF/D, Whatman, Maidstone, U.K.) to hold the packing material in place. The PVPP was packed loosely in the syringe barrel and settled with a 4-ml methanol wash. Before use, the cartridge was washed with an additional 4-ml volume of methanol followed by 4 ml of water. The cytokinin fraction resulting from preparative HPLC[1] was concentrated to approximately 1 ml and was loaded onto the cartridge and drawn through under vacuum. The cartridge was washed with 4 ml of water and the cytokinins were then eluted with a 4-ml methanol wash. The resultant methanol fraction was reduced to dryness *in vacuo* at 35°C and the remaining sample was dissolved in 1.0 ml of water.

Ion-exchange HPLC

A 400-μl aliquot of the above sample was analyzed for zeatin on a Vydac 401TP SCX column (5-μm particle diameter, 150 × 4.6 mm I.D., Alltech, Deerfield, IL, U.S.A.) with an isocratic delivery of 0.05 *M* $NH_4C_2H_3O_2$ (pH 4.1) at a flow-rate of 2.0 ml/min at 40°C. A second 400-μl aliquot was used for zeatin riboside analysis. The same buffer was used at a concentration of 0.01 *M* and a pH of 3.6 with all other parameters the same as for zeatin analysis. The solvent delivery system was composed of a Hewlett-Packard 1082b HPLC system (Avondale, PA, U.S.A.) equipped with a Hewlett-Packard (Model 79870A) 254 nm UV absorbance detector.

Determination of recovery and reproducibility

An internal standard of [8-^{3}H]adenine (27 Ci/mmol) was used for determination of recovery of cytokinins from each sample. In the preparative HPLC system the cytokinins coelute, approximately 1.5 min following the adenine. Rather than collect a wider fraction to include the cytokinins and the adenine (and additional interfering substances) the radiolabelled adenine was added separately to samples before extraction–preparative HPLC, and before the solid-phase extraction–analytical HPLC, to determine recovery from both procedures.

Recovery of adenine from the extraction and preparative HPLC system was determined in three sets of eight plant samples. Samples (2 g) of *Pseudotsuga menziesii* root tissue were enriched with [^{3}H]adenine (approximately 3000 dpm/g tissue) and processed through the extraction and preparative HPLC procedures as described[1]. Aquasol-2 (15 ml) (NEN Research Products, Boston, MA, U.S.A.) was added to each adenine fraction and ^{3}H determined in a Beckman LS 3800 liquid scintillation counter. Recovery was calculated by comparison with [^{3}H]adenine standards not processed through the extraction and preparative HPLC procedures.

Performance of the entire system for analysis of zeatin and zeatin riboside, extraction through final quantitation, was tested with extracts made from roots and shoot apices of dormant and actively growing *Cornus sericea* plants. Samples (1 g, fresh weight) from roots (2–4 mm diameter) and 2-cm shoot tips of eight dormant *Cornus sericea* plants were harvested and processed according to the method described by Kling *et al.*[1]. Additional plants were placed in hydroponic culture (one half strength Hoagland's solution No. 1, pH 6.5, with aeration) in a growth chamber (Conviron E-15, Pembina, ND, U.S.A.) at 24°C with a 15-h photoperiod. When plants were in an active vegetative state of growth, roots and the terminal 1-g portion of the newly expanding shoots were harvested and processed as above. The experiment was a completely randomized design with eight replications.

Identity of the zeatin and zeatin riboside peaks was verified with coupled gas chromatography–mass spectrometry (GC–MS). The two fractions containing the cytokinins from the ion-exchange HPLC procedure were collected, derivatized with tetramethylsilane (TMS) (Pierce, Rockford, IL, U.S.A.) and separated on a Hewlett-Packard 5985 GC–MS system.

RESULTS

The mean recoveries and C.V. values[22] for the three sets of [^{3}H]adenine-enriched plant extracts processed through the extraction and preparative systems combined were 89.7 (C.V. 1.9%), 88.2 (C.V. 1.8%) and 90.6 (C.V. 2.1%). The C.V. of [^{3}H]adenine recovery for the 24 samples grouped together was 2.1%.

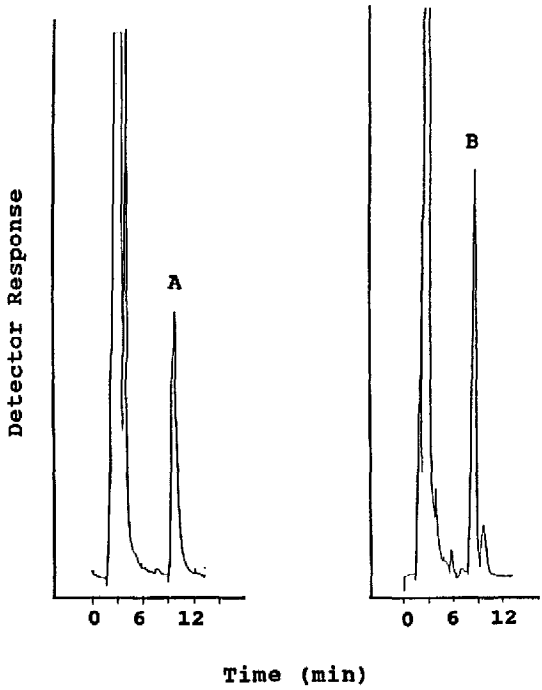

Fig. 1. Chromatograms of zeatin (A) and zeatin riboside (B) separated from *Cornus sericea* plant extracts using cation-exchange HPLC. See text for chromatographic conditions.

Adenine was separated sufficiently from zeatin and zeatin riboside in the cation-exchange analytical system and did not interfere with quantitation (Fig. 1). The retention times for adenine in the zeatin and zeatin riboside systems were 13.8 and 12.0 min, respectively.

The procedures for final separation and quantitation of the cytokinins proved to be reproducible enough to allow for routine analysis of zeatin and zeatin riboside. This system was able to detect differences in the levels of these hormones in both root and shoot tissue of dormant and actively growing *Cornus sericea* (Table I). Mean recovery of [^{3}H]adenine from the PVPP and ion-exchange procedures was 86.3% with a C.V. of 7.0%. The individual C.V.s for each set of samples are presented in Table I.

TABLE I

LEVELS OF ZEATIN AND ZEATIN RIBOSIDE IN ROOTS AND SHOOT TIPS OF DORMANT AND ACTIVELY GROWING *CORNUS SERICEA* PLANTS

	Dormant		*Growing*[a]	
	Root	*Shoot*	*Root*	*Shoot*
Zeatin (ng/g fresh weight)	45.98 c[b]	36.45 d	127.38 b	163.49 a
C.V. (%)	6.9	7.1	5.8	6.6
Z. riboside (ng/g fresh weight)	40.91 d	65.94 c	153.73 b	171.82 a
C.V. (%)	5.8	7.0	7.4	6.9

[a] See text for growth chamber conditions.

[b] Values followed by different letters are significant at the 5% level (least significant difference).

DISCUSSION

A system described by Dixon *et al.*[12] consisted of solvent partitioning, Dowex 50 cation-exchange chromatography and reversed-phase HPLC. They did not report percent recovery; however, a C.V. of 25% can be estimated from the data presented. Doumas and Zaerr[9] employed DEAE-cellulose chromatography and immunoaffinity chromatography followed by reversed-phase HPLC. They reported 65% recovery and C.V.s ranging from approximately 6% to 60% can be estimated from their data. A system described by Smith and Schwabe[5] consisted of solvent partitioning and thin-layer chromatography, followed by bioassay. The C.V. calculated from their data is approximately 25%. Mousdale and Knee[18] reported 97% recovery from a PVPP column, however they did not publish recovery or variation information for the entire system. Purse *et al.*[7] reported 100% recovery of radiolabelled cytokinins from a column of Sephadex, and recovery from TLC of 65% and 25% for zeatin and zeatin riboside, respectively. Stevens and Berry[16] indicate a C.V. of approximately 9.0% for cytokinins in culture filtrate using reversed-phase HPLC followed by GC–MS. They were able to by-pass much of the usual sample purification because they were working with culture medium filtrate, a relatively clean sample compared with crude plant tissue extracts.

The results from our experiments show lower coefficients of variation than

previous systems with comparable recovery rates. Total recovery of [^{3}H]adenine, extraction through analytical determination, was calculated to be 77.2%. The low variability of the system allowed for significant differences to be found in the levels of cytokinins in several plant tissues in different stages of development (Table I).

The identity of the presumed zeatin and zeatin riboside peaks from plant extracts were confirmed by GC–MS. Comparison of the mass spectra of the TMS-derivative of the plant zeatin, with that of authentic TMS-zeatin, showed both with characteristic ions at 261 (molecular ion, M^+), 230, 216, 188, 162, 135, 133 and a base peak at 73 (TMS). Mass spectra of TMS-derivatized plant sample and authentic zeatin riboside both contained ions of m/z 639 (M^+), 624, 550, 536, 319, 230, 202 and a base peak of 73 (TMS). Similar results were reported by Dixon *et al.*[12].

With the addition of the described cytokinin analysis procedures, we are now able to quantitate four major plant hormones from a single 1-g tissue sample. These methods will greatly facilitate studies of the interactive nature of plant hormones.

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