# A Simple Analysis of Purine and Pyrimidine Nucleotides in Plant Cells by High-Performance Liquid Chromatography*

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Purine and pyrimidine nucleotides, extracted from cultured plant cells with 6% perchloric acid, were separated directly with HPLC using anion-exchange Shimpack WAX-1 column. More than fifteen nucleoside mono-, di-, and triphosphates and nucleotide sugars were clearly separated and quantified without any interference from plant phenolic compounds.

## Introduction

Evidence is accumulating that free nucleotides play a central role in the regulation and integration of cell metabolism. Nevertheless, only a few reports on nucleotide levels in plant cells have been published. Compared with animals and microorganisms, the nucleotide level is low in plant cells and large amounts of UV-absorbing substances, *i.e.,* plant phenolics, interfere with the analysis of plant nucleotides. As a substitute for the traditional and rather laborious ion-exchange chromatography [1], new high-performance liquid chromatography (HPLC) systems have recently been introduced for plant nucleotide analysis [2, 3]. Although these systems are of tremendous importance, pretreatment for the isolation of nucleotides from crude plant extracts, and more than two different kinds of column, are required to separate a series of nucleotides. Here, we report a simple, rapid and reproducible assay method for the determination of nucleotides in plant cells.

## Materials and Methods

For the HPLC equipment, we used the Shimadzu HPLC system, type LC-4A, with a variable-wavelength UV-detector, type SPD-2AS, and a computing integrator, Chromatopack, type C-R 1B, attached. The anion exchange column (5 cm × 4 mm ∅) was a Shimpack WAX-1 (3 μm, 100-Å, poresize) obtained from Shimadzu Corporation, Kyoto, Japan.

Nucleotide assay was performed with gradients of phosphate buffer. Solution "A", 20 mM $KH_2PO_4$–$Na_2HPO_4$ buffer (pH 7.00) and solution "B", 480 mM $KH_2PO_4$–$Na_2HPO_4$ buffer (pH 6.85) were used as elutants. They were filtered through a cellulose nitrate membrane filter, pore size 0.2 μm (Toyo Roshi Kaisha Ltd., Tokyo) before use. The elution programme was as follows: 0 min, "A" only; 0–10 min, linear increase in "B" from 0 to 50%; 10–15 min, linear increase in "B" from 50 to 60%; 15–20 min, linear increase in "B" from 60 to 100%; 20–26 min, "B" only; 26–60 min, "A" only. The flow rate was 1.0 ml · $min^{-1}$, and column temperature was 45 °C. The absorbance at 260 nm was monitored.

Tissue culture of *Datura inoxia* (strain DX-1) was grown in 0.7% agar medium containing Murashige-Skoog nutrients as described in our previous paper [4], and subcultured every 14 days. Suspension culture of *Catharanthus roseus* (strain UN-1) cells was maintained as previously [5]. The plant samples (400–750 mg fresh weight) were homogenized with 4 volumes of 6% perchloric acid in a glass homogenizer. ITP (25 μl, 87.1 μmol) was added to the homogenate as an internal standard for recovery determination. After the homogenate had been centrifuged at 30000 g for 15 min, the supernatant was neutralized and concentrated as described in our previous paper [5]. The resulting residue was dissolved in 1.0 ml of solution "A", centrifuged, and the supernatant obtained was transferred to small culture tubes. The tubes were frozen at −20 °C for 20 min to remove the rest of the $KClO_4$ solubilized in the plant

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extracts. After thawing, the upper part of the extracts was collected and filtered through a Millipore Columngard, SJHU 004NS, pore size 0.45 μm (Millipore Japan Ltd., Tokyo) and aliquots (5–25 μl) of the filtrate were injected into the HPLC system.

The level of nucleotides was calculated from the chromatographic area obtained from known amounts of standard nucleotides (Sigma Chem. Company, St. Louis, U.S.A) using a computing integrator, and corrected according to the recovery of the internal standard (ITP). The concentration of each of the standard nucleotides was calibrated using the reported extinction coefficients at 260 nm.

## Results and Discussion

Fig. 1 shows the separation of standard purine and pyrimidine nucleotides by the Shimpack WAX-1 column, using phosphate buffer gradients. Nucleotide sugars (UDP-glucose and ADP-glucose), nucleoside monophosphates, diphosphates and triphosphates were eluted in that order, except that UTP appeared before GDP. NAD (retention time 0.79 min) and NADP (6.89 min) were clearly separated from the nucleotides mentioned above; NAD appeared before UDP-glucose, and NADP emerged between AMP and GMP. ITP could not be detected in any of the plant materials examined, and it was therefore used as an internal standard for recovery. ITP appeared between CTP and ATP.

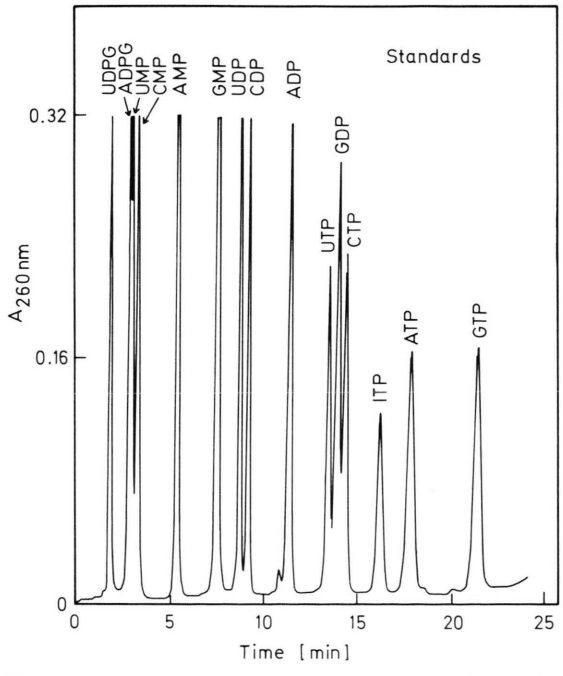


Fig. 1. Separation of standard mixture of purine and pyrimidine nucleotides by HPLC using a Shimpack WAX-1 column. ADPG, ADP-glucose; UDPG, UDP-glucose.

Profiles of perchloric acid soluble nucleotides from 10 day-old tissue culture of *Datura inoxia* and 10 day-old suspension cultured cells of *Catharanthus roseus* are shown in Fig. 2-A and 2-B, respectively. These two samples were chosen because *Datura inoxia* was a typical sample of logarithmic growth phase cells and *Catharanthus roseus* was a sample of stationary phase resting cells where phenolic compounds seemed to be accumulating. In both cases, adenine-, guanine-, uracil-, cytosine mono-, di-, and trinucleotides and UDPG were identified. The profile of *Datura inoxia* (Fig. 2-A) resembles that of several plant materials [2, 3, 6, 7]; major components are UDP-glucose and ATP, and the cytidine nucleotide pool was extremely small. In contrast, the ATP level in the resting cells of *Catharanthus roseus* was fairly low (Fig. 2-B).

The results indicate that the HPLC system using the Shimpack WAX-1 is very useful for the analysis of nucleotides in cultured plant tissues and cells. One of the principal advantages of this method is the clear separation of all major nucleotides with a single column. Furthermore, no pre-treatment of plant extracts is required before HPLC analysis. We tried purification of plant extracts using a "Baker"-10SPE phenyl ($C_6H_5$) disposable column (J. T. Baker Chem. Co., Phillipsburg, U.S.A) as described by Meyer and Wagner [3], but the results were similar to those using crude extracts (Fig. 2). The separation of nucleotides deteriorated after more than 50 samples had been injected into the Shimpack WAX-1 column. This seems to be partly due to the absorption of phenolics on to the surface of the column, and purification of plant extracts may partially prevent this absorption. However, it would seem easier and more practical to remove the upper surface of the resin and to repack with a small amount of fresh resin.

We are currently determinating the nucleotide levels in relation to various physiological conditions in cultured plant cells and tissues using this method. The HPLC system described here was also effective for separation of nucleotides extracted from intact plants, including mung bean seedlings. Furthermore, this system can be used for the nucleotide analysis of other biological materials.

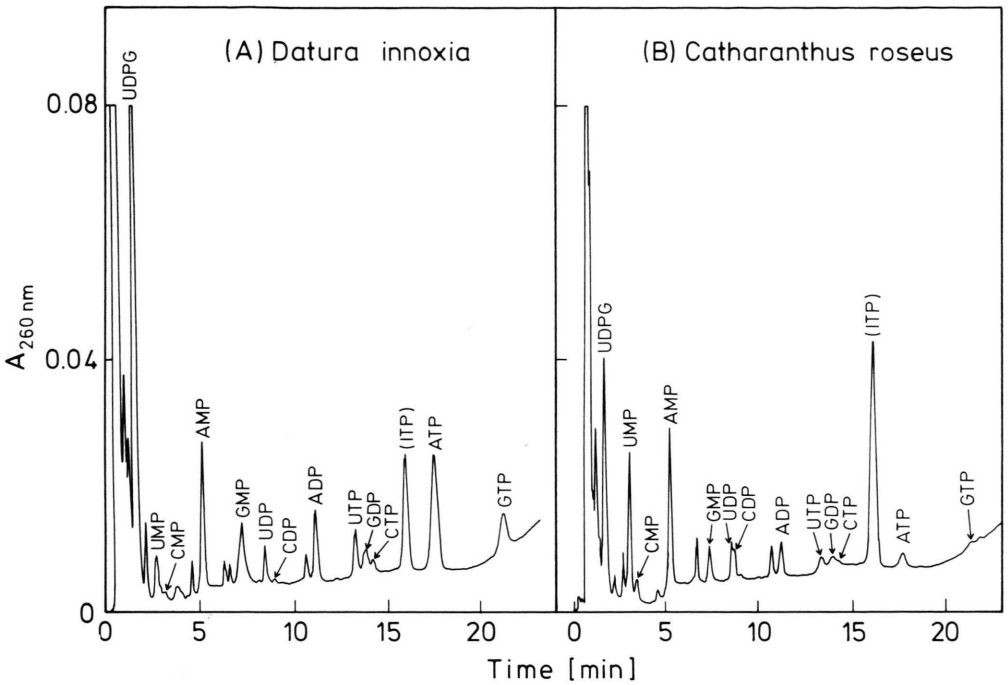


Fig. 2. Separation of nucleotides extracted from 10 day-old tissue cultures of *Datura inoxia* (A) and 10 day-old suspension cultured cells of *Catharanthus roseus* (B) by HPLC using a Shimpack WAX-1 column. ITP is an internal standard.

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