CHROM. 16,342

Note

Analysis of indole-3-acetic acid by reversed-phase preparative ion suppression and analytical ion-pair high-performance liquid chromatography

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(First received August 25th, 1983; revised manuscript received October 4th, 1983)

The analysis of plant growth regulators, such as indole-3-acetic acid (IAA), is difficult due to the small amounts found in plant tissue (20–250 ng g^{-1} fresh weight). Recently, high-performance liquid chromatography (HPLC) has been shown to be an important tool in the analysis of plant growth regulators[1,2]. A number of separation mechanisms such as ion-exchange[3-6], normal-phase[7,8] and reversed-phase systems[9-16] have been reported for HPLC analysis of plant growth regulators. The most popular mode has been reversed-phase HPLC employing C$_{18}$ packing materials due to their stability, separation efficiency and reproducibility[1,2].

Although analytical HPLC has been widely used in the analysis of IAA in plant extracts, purification of plant extracts to the degree required for HPLC analysis has been accomplished by an extensive protocol including liquid–liquid partitioning, conventional column chromatography and thin-layer chromatography[15]. The use of both column chromatography and thin-layer chromatography is time consuming and results in substantial and variable losses[17]. The time required in the multistep process necessary to obtain sufficiently purified extracts suitable for analytical HPLC has been a major limitation in plant growth regulator research[7]. Because of these problems the suggestion has been made that preparative HPLC be used to partially or totally replace the more conventional methods of purifying plant extracts[1,2].

Reversed-phase preparative HPLC has been shown to be of some utility in the purification of acidic plant growth regulators from crude plant extracts[1,10,18]. Preparative HPLC can considerably reduce the time required for the analysis of plant growth regulators. Furthermore, present day HPLC columns, with efficiencies in excess of 30,000 theoretical plates per meter, provide greater resolving power than either thin-layer or conventional column chromatography[1].

When preparative HPLC is used in the purification of plant extracts, it has

been proposed that the analytical step employ a different separatory mode than that used in the preparative step to facilitate resolution of coeluting substances[2]. Recently, ion-pair chromatography (IPC) has been reported to be superior to ion-suppression chromatography (ISC) in analytical reversed-phase HPLC analysis of IAA[15,16]. Ion-pair reversed-phase HPLC using tetrabutyl ammonium (TBA) as the counter ion has successfully separated IAA from other indole derivatives found in plants, such as the 4-chloro and 5-hydroxy derivatives of IAA. It was also reported that if gel filtration column chromatography is followed by thin-layer chromatography in the purification of plant extracts, ion-pair reversed-phase analytical HPLC may be used to quantify levels of IAA in small amounts of plant tissue (400 mg fresh weight)[15].

This paper reports on the use of reversed-phase preparative ISC in conjunction with reversed-phase analytical IPC to isolate and quantify levels of IAA in plant tissue. This method allows the use of reversed-phase HPLC, while exploiting the differential separation mechanisms of ISC and IPC. Employing conifer roots, which are rich in many potentially interfering phenolic and terpenoid compounds, analysis of the acidic terpenoid phytohormone IAA, via this procedure, is rapid, precise and sensitive.

MATERIALS AND METHODS

Plant material

Shortleaf pine (*Pinus echinata* Mill.) seedlings were grown in a glasshouse for 18–20 weeks under supplemental light (18 h, 750 μE m^{-2} sec^{-1} photon flux density). Immediately after harvest, samples were immersed in liquid nitrogen and stored at $-20°C$ in the dark. Tissue was freeze dried prior to extraction.

Extraction procedure

Plant material was homogenized in 50 ml of 80% methanol and 5.0 kBq (300,000 dpm) [2-^{14}C]IAA (Amersham, Arlington Heights, IL, U.S.A.) were added as an internal standard. The amount of plant material used ranged from 50 to 300 mg dry weight. The homogenate was agitated for 24 h on a wrist action shaker in the dark at 4°C. Samples were then centrifuged at 10,000 g for 0.5 h. The supernatant was reduced *in vacuo* to an aqueous solution, diluted to 50 ml with 0.05 N dipotassium hydrogen phosphate, and the pH was adjusted to 8.5. The solution was partitioned (2 ×) against equal volumes of diethyl ether. The aqueous fraction was slurried with polyvinyl polypyrrolidone, filtered, and adjusted to pH 2.8 with orthophosphoric acid. The filtrate was then partitioned with ethyl acetate (3 ×). The ethyl acetate fractions were pooled and partitioned against 50 ml of HPLC grade water to remove any buffers from the ethyl acetate phase. The ethyl acetate was reduced in volume with a rotary film evaporator, transferred to a conical vial, and evaporated to dryness under nitrogen; the residue was diluted with 500 μl of 100% methanol.

HPLC equipment and analysis

The mobile phases were delivered by a Perkin-Elmer Series 3B HPLC pump. Columns were maintained at 35°C. A Perkin-Elmer 65-105 fluorescence spectrophotometer (excitation 280 nm, emission 350 nm) was used to monitor the eluate. Peak areas were integrated with a Perkin-Elmer Sigma 10 microprocessor (Perkin-Elmer, Norwalk, CT, U.S.A.).

Partially purified plant extract was injected (250 μl) into a 250 × 22 mm I.D. Partisil Magnum 20 ODS-3 preparative reversed-phase C_{18} HPLC column (Whatman, Clifton, NJ, U.S.A.). The mobile phase was 35% methanol, pH 2.8, with a flow-rate of 10 ml min^{-1}. Fractions eluting with the same retention time as standard IAA were collected, pH adjusted to 7.0, dried under nitrogen, and dissolved in 300 μl of 30% methanol. Twenty microliters of this solution were injected on to an analytical C_{18} column (Unimetrics, Anaheim, CA, U.S.A.). The mobile phase was 30% methanol (pH 6.5, 0.01 N dipotassium hydrogen phosphate, 0.01 N TBA) with a flow rate of 2 ml min^{-1}. A peak corresponding to the retention time of standard IAA was collected for scintillation counting.

Liquid scintillation counting

Corrections for losses of IAA during the analytical procedures were made by counting sample aliquots containing [2-^{14}C]IAA as an internal standard. Sample aliquots were dissolved in InstaGel (Packard, Downers Grove, IL, U.S.A.) and their

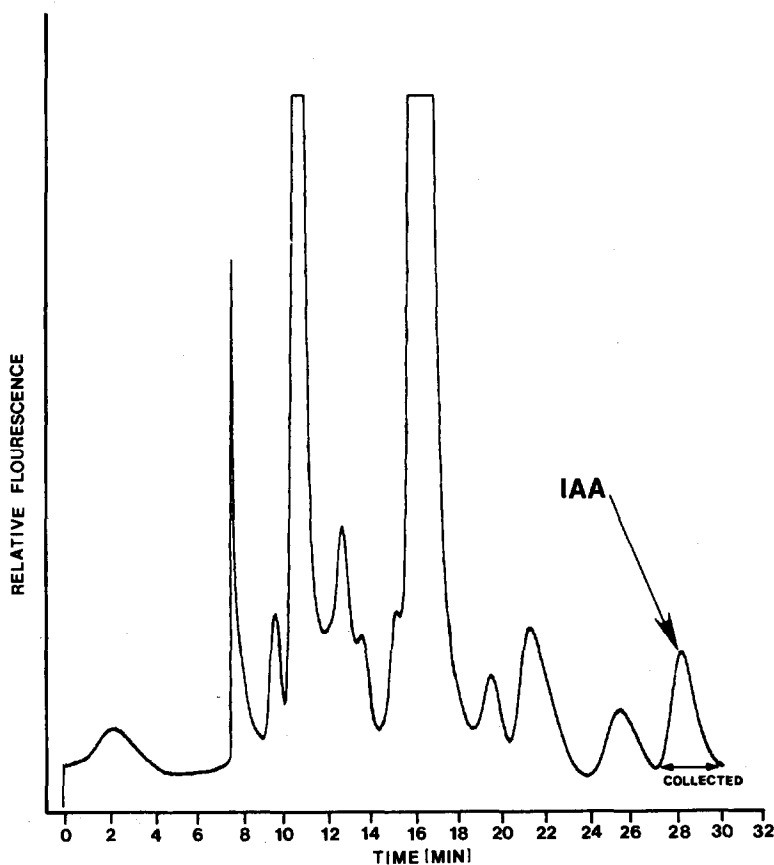
Fig. 1. Preparative HPLC chromatogram of partially purified pine extracts. Column: 250 × 22 mm I.D. Magnum 20 Partisil ODS-3. Eluent: 35% methanol, pH 2.8. Detector: Perkin-Elmer 650-105 fluorescence spectrophotometer (excitation 280 nm, emission 350 nm, attenuation 5, sensitivity 10).

radioactivity was quantitated with a Beckman model LS-100C liquid scintillation counter (Beckman, Fullerton, CA, U.S.A.).

RESULTS AND DISCUSSION

The trend in HPLC analysis is toward an increased use of reversed-phase instead of ion-exchange or straight-phase adsorption. Analysis of acidic plant growth regulators, such as IAA, has been shown to be optimized when used in a reversed-phase ion-pair mode with methanol concentrations between 27.5–40.0%[15]. Clean-up procedures, such as gel-filtration column chromatography and thin-layer chromatography, are required if this chromatographic system is to be successful[15]. Not only are these purification methods time consuming, but substantial losses are often associated with their application[17]. Using the preparative reversed-phase ISC system described here, consistent recoveries of IAA greater than 90% were associated with this single step in contrast to recoveries of as low as 30% from column and thin-layer chromatography noted above[17]. This recovery is comparable to that of abscisic acid in a preparative reverse phase separation reported by Brenner[7]. Another advantage in the use of preparative HPLC is the reduction in analysis time (only 45 min between sample injections is required).

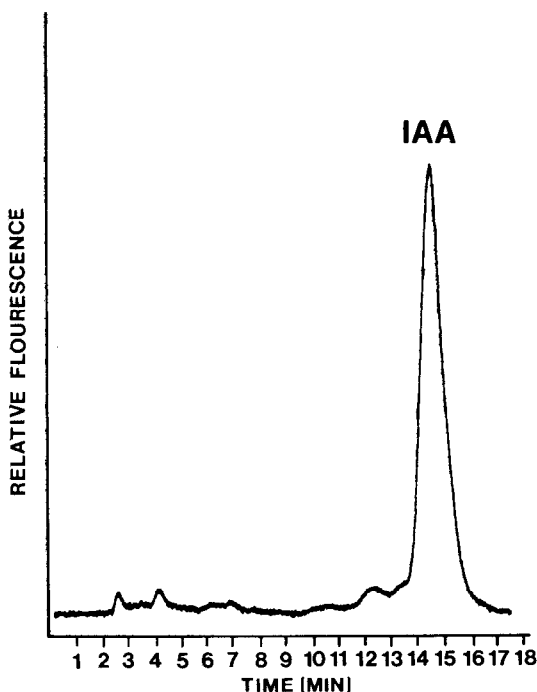The preparative reversed-phase HPLC step eliminated most contaminating

Fig. 2. Analytical HPLC chromatogram of a plant extract. 250 × 2.6 mm I.D. RP-18 Unimetrics analytical C_{18} reversed-phase column. Eluent: 30% methanol, pH 6.5, 0.01 N K_2HPO_4, 0.01 N TBA. Detector: Perkin-Elmer 650-105 fluorescence spectrophotometer (excitation 280 nm, emission 350 nm, attenuation 3, sensitivity 10).

fluorimetrically detectable peaks and yielded a large IAA peak (Figs. 1 and 2). The IAA peak in the analytical step was collected and counted. Overall recovery rates of 50–60% were consistently obtained, with most of the losses occurring during the extraction procedures.

In order to determine purity of the peak tentatively identified as IAA, samples were prepared without an internal standard. These samples were derivatized with N-methyl-N-tert.-butyldimethylsilyltrifluoroacetamide producing the tert.-butyldimethylsilyl derivative of IAA which was then subjected to combined gas–liquid chromatography–mass spectrometry[19]. The mass spectra of samples and standard IAA confirmed the identity of the peak as IAA.

Further experiments determined the standard error of the procedure. Freeze-dried roots (1.2 g dry weight) were homogenized in 100 ml of 80% methanol and 20 kBq [2-^{14}C]IAA were added. This homogenate was extracted for 24 h and centrifuged at 10,000 g for 0.5 h. The supernatant was divided into four 25-ml samples and each sample was analyzed as described. The IAA content of the roots was 90 ± 4.5 ng g^{-1} (5.2% standard error). This error is less than the 14.0% reported by Sandberg et al.[15].

In conclusion, preparative reversed-phase ion-suppression HPLC in conjunction with analytical reversed-phase ion-pair chromatography efficiently separates IAA from (shortleaf pine) roots. When this method of isolation is combined with the sensitivity of fluorimetric detection a precise quantification of IAA can be made.

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