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Note

Use of reversed-phase ion-pair high-performance liquid chromatography for the removal of compounds inhibitory to the formation of the 2-methyl indolo-α-pyrone derivative of indole-3-acetic acid

DAVID BLAKESLEY, ADRIAN JOHN ALEXANDER ALLSOPP, JOHN FREDERICK HALL, GEOFFREY DONALD WESTON and MALCOLM CHARLES ELLIOTT*
School of Life Sciences, Leicester Polytechnic, P.O. Box 143, Leicester LE1 9BH (U.K.)
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The levels of indole-3-acetic acid (IAA) in plant tissue are frequently assayed by measuring the fluorescence of 2-methyl indolo-α-pyrone (2-MIP), the derivative formed from the reaction of IAA with acetic anhydride[1,2]. However, there are two problems associated with this method. Firstly, in the derivatised extracts of some plant tissues, such as *Chamaecyparis lawsoniana* there is evidence for the presence of contaminating fluorescent compounds. These can be removed by high-performance liquid chromatography (HPLC) of the derivatised extract[3]. Secondly, in extracts of tissues such as etiolated *Zea mays* and *Triticum vulgare*, severe inhibition of the derivatisation reaction has been reported[4]. Extra purification steps, such as thin-layer chromatography have been used to free extracts of inhibiting compounds[5,6].

Reversed-phase ion-pair chromatography (IPC)[7,8] has been shown to be a powerful tool for the separation of carboxylic acids[9], and has been used as a purification[10] or an analytical step[11,12] in the analysis of IAA in plant tissue. This paper reports the use of IPC for the removal of compounds which inhibit the formation of 2-MIP.

EXPERIMENTAL

Vegetative tissue of *Cotinus coggygria* cv Royal Purple, roots of etiolated three day old seedlings of *Zea mays* cv LG 11 and coleoptiles of five day old etiolated seedlings of *Triticum vulgare* cv Sicco were harvested, frozen immediately in liquid nitrogen, freeze dried and stored at −20°C. The extraction and purification procedures for IAA have been previously reported[3]. Essentially, following a methanol extraction, and addition of an internal standard of [2-^{14}C]IAA (Radio-chemical Centre, Amersham, U.K.) samples were washed with diethyl ether and light petroleum (b.p. 60–80°C) at pH 8.0, extracted into diethyl ether at pH 3.0, concentrated under reduced pressure and passed through a column of poly-N-vinyl pyrrolidone (Polyclar AT) and finally extracted into diethyl ether at pH 3.0. Water was removed from the samples by freezing, the diethyl ether evaporated to dryness under reduced pressure, and the residue taken up in 50 mm^3 of methanol and stored at −20°C.

Tetraethylammonium chloride (TEA) was found to be the most suitable quat-

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ernary ammonium salt which could be added to the HPLC eluent as a counter ion. The mobile phases for IPC were 0.01 *M* TEA in 0.001 *M* K_2HPO_4–KH_2PO_4 at pH 6.6, running as a gradient with 15–35% methanol at a flow-rate of 2 cm^3/min rising from 15% at 1%/min for 20 min, held at 35% for 5 min and returned to 15% at 2%/min. Samples were injected in 50 mm^3 of methanol via a Rheodyne valve and 200 mm^3 injection loop onto a preparative 5 μm Hypersil ODS column (150 × 22.5 mm I.D.), fitted with a 55 × 5 mm I.D. pre-column packed with LiChroprep RP-18 (Merck). The HPLC instrument (Applied Chromatography Systems) was connected to a detector system consisting of a UV absorbance monitor (Perkin-Elmer LC 75) set at 254 nm (ABA) or 280 nm (4-Cl-IAA) and a spectrophotofluorimeter (Perkin-Elmer MPF 43A) adjusted to an excitation wavelength of 280 ± 10 nm and an emission wavelength of 350 ± 10 nm. Fractions collected following IPC were diluted with distilled water, adjusted to pH 3.0 and extracted into diethyl ether. Preparation of the 2-MIP derivative has previously been reported[3]. For separation of the 2-MIP derivative, the mobile phase was a gradient of methanol and water pH 3.5, and the column a semi-preparative 5 μm Hypersil ODS column (250 × 10 mm I.D.). The spectrophotofluorimeter excitation wavelength was adjusted to 445 ± 5 nm, and the emission wavelength to 480 ± 10 nm.

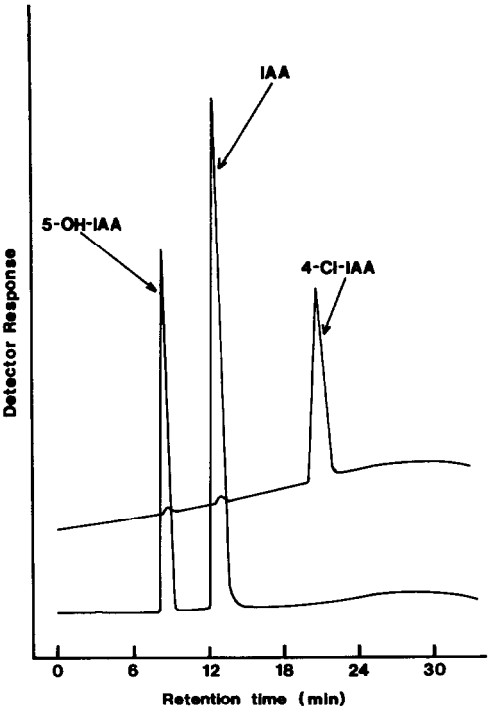

Fig. 1. Chromatogram of IAA, 5-OH-IAA and 4-Cl-IAA standards separated by ion-pair-chromatography. Column 5 μm Hypersil ODS (150 × 22.5 mm I.D.). Flow-rate, 2 cm^3/min. Mobile phase: gradient of 15–35% methanol in an aqueous solution of 0.01 *M* TEA, 0.001 *M* K_2HPO_4–KH_2PO_4, pH 6.6, at a rate of 1%/min for 20 min, 0%/min for 5 min. Spectrophotofluorimetric detection at 350 nm following excitation at 280 nm. 4-Cl-IAA detected by UV absorbance at 280 nm.

RESULTS AND DISCUSSION

We have found that with certain plant materials no peak of fluorescence occurs at the same HPLC retention time as that of authentic 2-MIP, even when the procedure which we described previously[3] is followed. The reaction must have been inhibited in such cases since the added [^{14}C]IAA standard should yield a peak even if no IAA was present in the plant material. We were able to devise procedures[3] which were successful when semi-purified extracts of *Cotinus* (clone SBI) were used. Subsequent work with another clone of *Cotinus* (LPI) was not successful because of the presence of material which inhibited the derivatisation reaction. Similar problems have been encountered with extracts of the cultivars of *Zea mays* and *Triticum vulgare* used in the present investigation and may well be widespread.

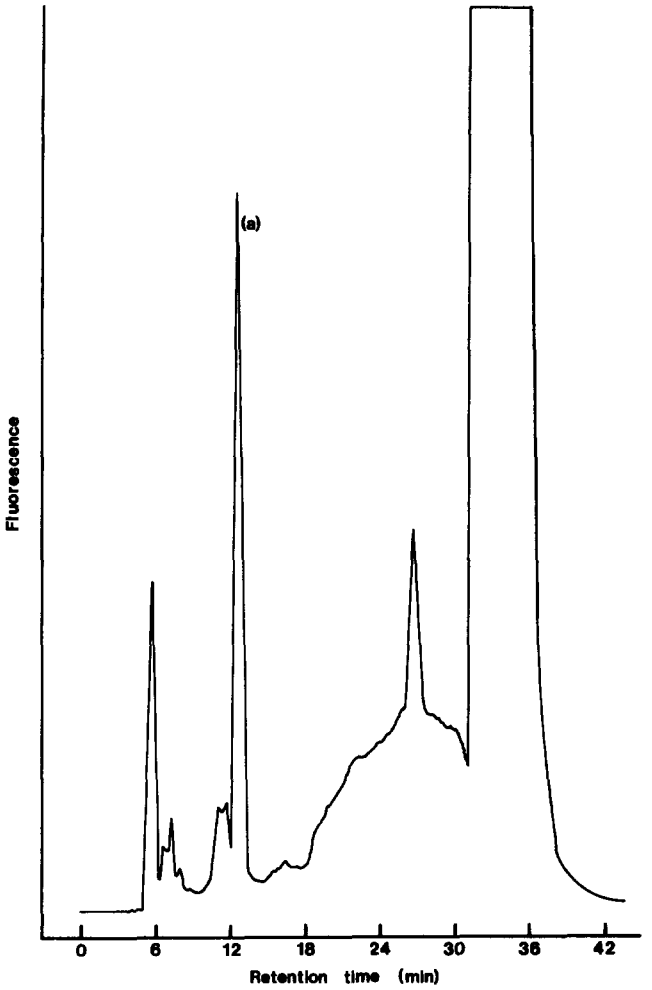

Fig. 2. Chromatogram of a methanolic extract of *Zea mays* cv LG11. Peak a has the same retention time as authentic IAA. HPLC and spectrophotofluorimetric detector conditions as for Fig. 1.

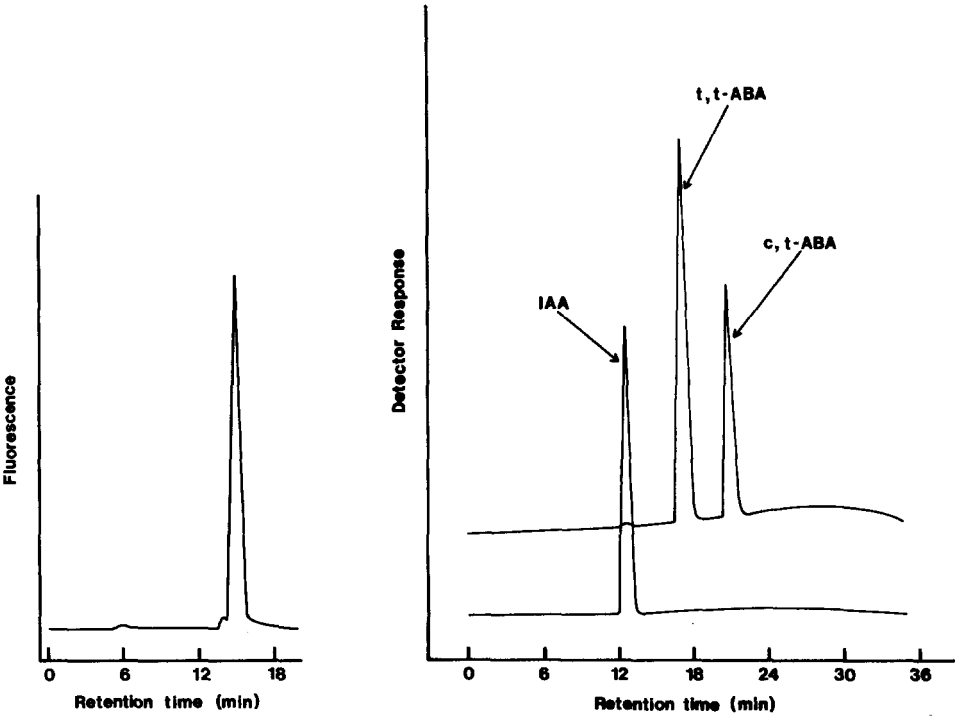

Fig. 3. Chromatogram of the putative IAA fraction collected after ion-pair chromatography, following derivatisation with AA-TFA, Column: 5 μm Hypersil ODS (250 × 10 mm I.D.). Flow-rate: 2 cm^3/min. Mobile phase: gradient of 50–100% methanol in water of pH 3.5 at a rate of 1%/min for 5 min, 2%/min for 10 min and 5%/min for 5 min. Spectrophotofluorimetric detection at 480 nm following excitation at 445 nm.

Fig. 4. Chromatogram of IAA, *cis-trans*-ABA (c,t-ABA) and *trans-trans*-ABA (t,t-ABA) standards separated by ion-pair chromatography. HPLC and spectrophotofluorimetric detector conditions as for Fig. 1. ABA detected by UV absorbance at 254 nm.

IAA, 5-hydroxy-IAA (5-OH-IAA) and 4-chloro-IAA (4-Cl-IAA) were well separated when IPC was used (Fig. 1). Furthermore successful derivatisation occurred when the material yielding the peak with the same retention time as authentic IAA was collected from extracts of *Zea* (Fig. 2), *Cotinus* (LPI) and *Triticum* (Fig. 3). Authentic IAA added to the extracts prior to derivatisation was in fact derivatised confirming that the sample was free from inhibition.

It was reported previously[3] that HPLC could be used to demonstrate the presence, in derivatised plant extracts, of 4-Cl-IAA, 5-OH-IAA or any other compound with a fluorescent derivative. These compounds may be separated from IAA by use of an IPC purification step. The presence of 4-Cl-IAA and 5-OH-IAA may be investigated after collection of peaks with the appropriate retention times for IPC as shown in Fig. 1.

Extraction and purification of abscisic acid (ABA) and IAA are often conducted simultaneously. IPC separates *trans-trans* and *cis-trans* isomers of ABA from each other and from IAA (Fig. 4) and is an excellent purification step prior to ABA analysis by gas chromatography[13].

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