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Note

Reversed-phase ion-pair high-performance liquid chromatography of the plant hormones indolyl-3-acetic and abscisic acid

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The analysis of endogenous plant hormones usually requires their purification as trace components of plant tissue extracts. Modern techniques of high-performance liquid chromatography (HPLC) are being increasingly utilized in both the preparation and analysis of plant extracts. For the naturally occurring auxin indolyl-3-acetic acid (IAA) and the growth inhibitor 2-*cis*-4-*trans*-abscisic acid (*c,t*-ABA) HPLC methods using ion-exchange[1,2] and reversed-phase[2–4] columns have been described. Since both IAA and *c,t*-ABA are weak carboxylic acids reversed-phase chromatography can be performed either with a mobile phase of low pH using the ion-suppression principle[5] (see refs. 2–4) or with a mobile phase with a pH greater than 5 (when the acids are in their ionic dissociated form) if the ion-pairing[5,6] technique is used. This paper describes the application of reversed-phase ion-pair chromatography to the analysis of IAA and *c,t*-ABA in partially purified plant tissue extracts.

EXPERIMENTAL

Chromatographic techniques

Chromatography was performed using equipment manufactured by Pye Unicam (Cambridge, Great Britain): LC-XPD dual reciprocating pump, Model 7125 sample injection valve fitted with a 20-μl injection loop, and LC–UV variable-wavelength absorption detector. The column was 250 × 4.6 mm I.D. stainless steel packed with Partisil-10 ODS bonded reversed-phase material (particle size 10 μm, Whatman, Maidstone, Great Britain). The mobile phases were: (for ion-supression reversed-phase chromatography) 5% acetic acid (pH 3.0)–methanol (70:30 and 60:40); for reversed-phase ion-pair chromatography, 0.01 *M* tetramethylammonium phosphate or tetrabutylammonium hydrogen sulphate (in 0.001 *M* K_2HPO_4/KH_2PO_4, pH 6.6)–methanol (90:10–60:40). IAA, *c,t*-ABA and a mixture of *c,t*-ABA with the non-naturally occurring isomer 2-*trans*-4-*trans*-ABA (*t,t*-ABA) were purchased from Sigma (London) (Poole, Great Britain). Injections were made in 10 μl methanol and the column effluent monitored at 254 nm (ABA isomers) or 278 nm (IAA). A flow-rate of 1 ml/min was used throughout. The time equivalent of the void volume was determined by injecting 10 μl methanol and measuring the time from injection to the first deviation from the baseline.

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Plant tissue extraction and purification

Extracts were prepared from stem bark of dormant apple (*Malus domestica* Borkh., rootstock MM104) trees and leaf petiole of tobacco (*Nicotiana tabacum* cv Xanthi-nc). After the periderm had been removed the inner bark of apple stems was excised, frozen by immersion in liquid nitrogen and ground in a pestle and mortar. 1 g of the frozen powder was extracted overnight at −15°C with 5 ml redistilled methanol, the extract centrifuged and the supernatant added to 15 ml 0.5 *M* K_2HPO_4 (pH 8.5). After partitioning against 2 × 15 ml and 1 × 5 ml redistilled diethyl ether the aqueous phase was adjusted to pH 3 with 3 *M* H_3PO_4 and partitioned against 2 × 15 ml ether. The pH 3 ether-soluble fraction was reduced to dryness under a stream of nitrogen at room temperature, redissolved in 1 ml redistilled methanol and applied to a column of insoluble polyvinyl-pyrrolidone (50 × 10 mm I.D.) equilibrated with methanol[7]. The column was eluted with 12 ml redistilled methanol and the eluate reduced to *ca.* 1 ml volume by rotary film evaporation at room temperature before transfer to a 1.5-ml microvial and reduction to dryness under a stream of nitrogen. The residue was dissolved in 50 µl redistilled methanol and 10 µl injections were used for analysis. The same procedure was used for 100-mg amounts of lyophilized tobacco petiole.

RESULTS AND DISCUSSION

Chromatography on the Partisil-10 ODS column in aqueous methanol–acetic acid resolved IAA and the isomers of ABA (Table I). In 0.001 *M* phosphate (pH 6.6)–methanol (80:20) IAA was unretained whereas the isomers of ABA eluted later and

TABLE I

REVERSED-PHASE HPLC SEPARATION OF INDOLYL-3-ACETIC ACID AND ABSCISIC ACID ISOMERS ON C_{18} PARTISIL-10 COLUMN

TMA = Tetramethylammonium phosphate; TBA = tetrabutylammonium hydrogen sulphate. Flow-rate: 1 ml/min. Time equivalent of void volume: 3.2 min.

Mobile phase	*Retention time (min)*		
	IAA	*t,t-ABA*	*c,t-ABA*
Ion suppression			
5% Acetic acid (pH 3.0)–methanol (70:30)	12.8	19.0	27.0
5% Acetic acid (pH 3.0)–methanol (60:40)	8.0	9.5	12.0
Ion pairing			
0.001 *M* Phosphate (pH 6.6)–methanol (80:30)	2.9	4.0	5.7
0.01 *M* TMA in 0.001 *M* phosphate (pH 6.6)–methanol (80:20)	6.9	11.8	18.2
0.01 *M* TMA in 0.001 *M* phosphate (pH 6.6)–methanol (90:10)	9.6	24.7	45.2
0.01 *M* TBA in 0.001 *M* phosphate (pH 6.6)–methanol (60:40)	7.0	7.3	9.1
0.01 *M* TBA in 0.001 *M* phosphate (pH 6.6)–methanol (80:20)	26.7	35.1	54.3

were resolved, suggesting that adsorption or other effects also influenced the elution pattern. Including 0.01 *M* tetramethyl- or tetrabutylammonium counterion greatly increased the retention times of IAA and the isomers of ABA while maintaining the order of elution observed in ion-suppression reversed-phase chromatography (Table I). The more lipophilic counterion, tetrabutylammonium, increased the retention time of IAA relatively more than those of the ABA isomers (Table I).

Reversed-phase ion-pair chromatographic procedures gave excellent separations of IAA and *c,t*-ABA from other UV-absorbing peaks when applied to partially purified apple (Figs. 1 and 2) and tobacco (Fig. 3) extracts. Apple bark extracts contained very little *c,t*-ABA which moreover was not fully separated from adjacent UV-absorbing peaks in reversed-phase chromatography in aqueous methanol–acetic acid mixtures; an estimated tissue level of $\leq$ 8 ng/g fresh weight could with reversed-phase ion-pair chromatography be reduced to $\leq$ 5 ng/g. The purity of the peaks co-chromatographing with IAA or *c,t*-ABA was not established. The wavelength ratios of suspected IAA or *c,t*-ABA peaks were, however, similar to those observed for

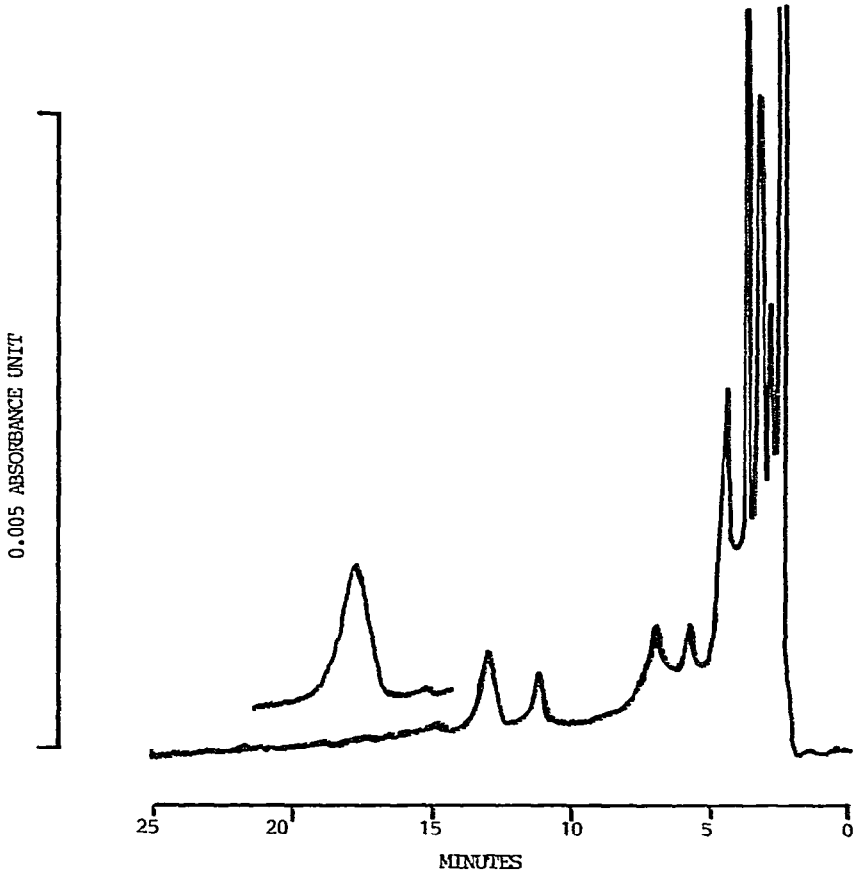

Fig. 1. Chromatogram of extract of apple (*Malus domestica* Borkh.) stem bark. Column: Partisil-10; mobile phase: 0.01 *M* tetramethylammonium phosphate in 0.001 *M* phosphate (pH 6.6)–methanol (80:20). 10-μl extract injected. UV absorption monitored at 254 nm. Upper trace: 8 μl extract and 2 μl (25 ng) *c,t*-ABA.

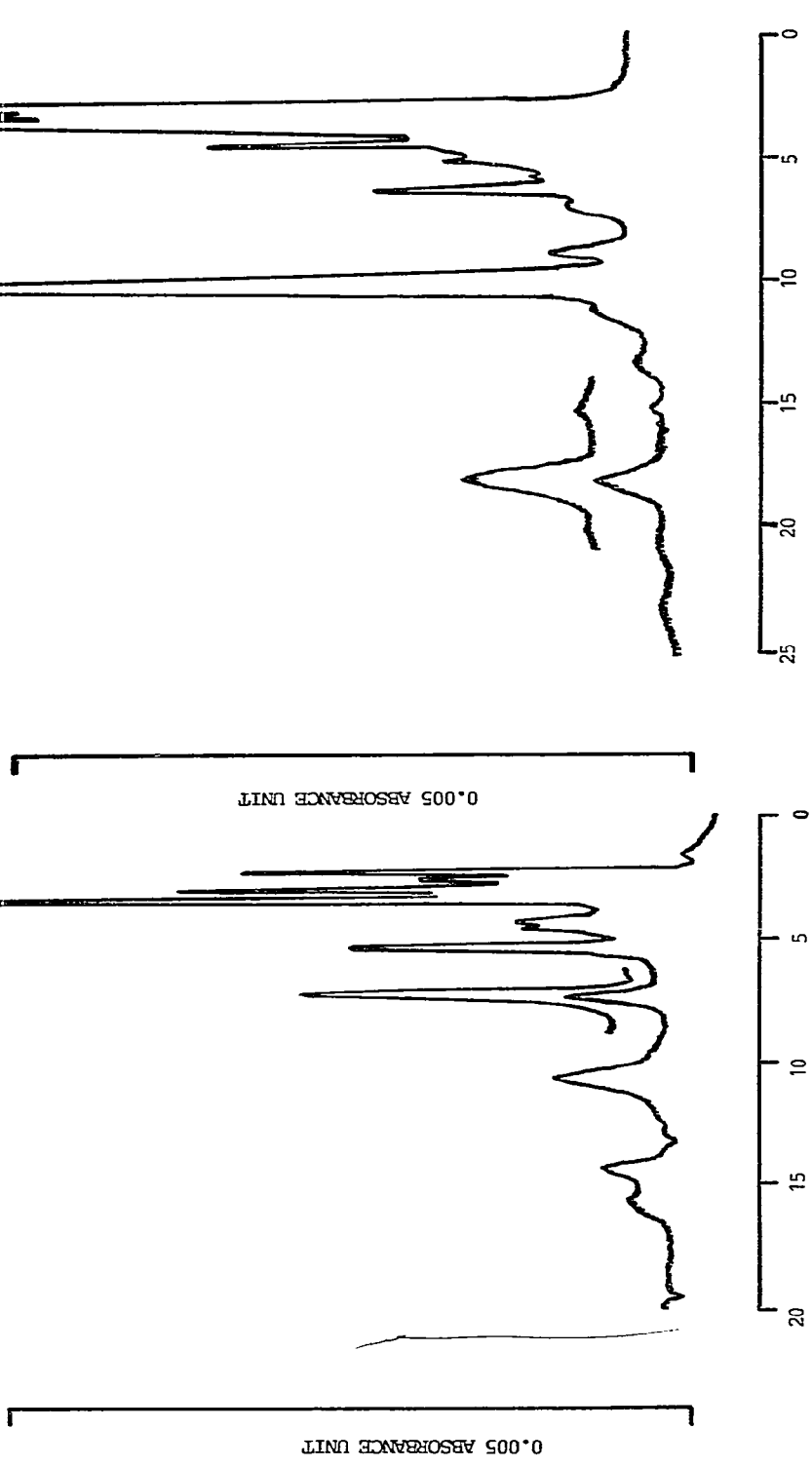

Fig. 2. Chromatogram of extract of apple stem bark. Mobile phase: 0.01 *M* tetramethylammonium phosphate in 0.001 *M* phosphate (pH 6.6)–methanol (85:15). 10-μl extract injected. UV absorption monitored at 278 nm. Upper trace: 8 μl extract and 2 μl (25 ng) IAA.

Fig. 3. Chromatogram of extract of tobacco (*Nicotiana tabacum*) petiole. Mobile phase: 0.01 *M* tetramethylammonium phosphate in 0.001 *M* phosphate (pH 6.6)–methanol (80:20). 10-μl injected. UV absorption monitored at 254 nm. Upper trace: 8 μl extract and 2 μl (12.5 ng) *o,p'*-ABA.

authentic samples: for *c,t*-ABA peak height at 254 nm/peak height at 278 nm was 2.1 (observed 1.9); for IAA peak height 254 nm/peak height 278 was 0.4 (observed 0.33). Further manipulation of mobile phase composition or the nature or concentration of the counter ion may result in superior chromatographic separations and may be required to meet the demands posed by extracts of other plant tissues. The results presented here demonstrate, however, the potential value of reversed-phase ion-pair chromatography as a supplement to reversed-phase chromatography in the ion-suppression mode of analysis of weak acid solutes.

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