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Note

Analysis of plant hormones using high-performance liquid chromatography*

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Since the introduction of high-performance liquid chromatography (HPLC), there has been interest in the use of this instrument for the analysis of plant growth hormones[1–5]. Analysis by HPLC has several advantages over other chromatographic methods including speed of separation, high sensitivity, ease of collecting separated compounds, and often detection without derivatization. It has been noted that certain plant hormones are detected in procedures for the extraction of abscisic acid (ABA) from plant tissue[1,3,5]. Indole-3-acetic acid and kinetins such as the zeatins are present in the final phase of extraction along with the ABA. Separation of these hormones from a single sample would facilitate studies of the relationships of the hormone levels. These procedures could also be used for screening cultivars, investigating plant development, and determining effects of plant growth regulants. The purpose of this investigation was to develop a technique using HPLC for isolating some of the major plant hormones with one chromatographic separation.

MATERIALS AND METHODS

Standards of indole-3-acetic acid (IAA), zeatin (*cis,trans*-Z), zeatin riboside (*cis, trans*-ZR), kinetin (K), and abscisic acid (*cis,cis*- and *cis,trans*-ABA) were obtained from Sigma (St. Louis, MO, U.S.A.) and suspended in methanol (10^{-3} *M*). An HPLC unit (Waters Assoc.) equipped with two pumps sequenced by a solvent programmer was used in this study. The hormones were detected by absorbance at 254 nm with a UV absorbance detector. Both a standard μBondapak C_{18} column (Waters Assoc.) and a Radial Compression Module (RCM-100, Waters Assoc.) equipped with a Radial-Pak A cartridge were used to separate standards, and the results were compared.

The solvent system developed for the μBondapak C_{18} column consisted of a 15-min linear gradient from 25% methanol (in 0.67% acetic acid) to 32% methanol. At 16 min 40 sec after injection, the methanol concentration was increased to 55% methanol to elute the ABA rapidly. Plant hormone separation using the RCM-100 was accomplished by 0.5% acetic acid ($6 \cdot 10^{-3}$ *M* ammonium acetate)–acetonitrile (80:20) and methanol–acetonitrile (58:42). A 20-min concave gradient was initiated to a final solvent of 68 parts 0.5% acetic acid and 32 parts acetonitrile–methanol. The

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concave gradient was gradient 4 on the Waters solvent programmer. Total run time for both separations was approximately 20 min at flow-rates of 2 ml/min. Methanol and acetonitrile were spectrophotometric grade solvents (distilled in glass).

The plant sample was prepared by a modification of the procedure reported by Wightman[5]. One gram of soybean tissue was ground with 10 ml of acetone, 5 ml of water were added, the extract filtered, and the acetone evaporated. The sample was then filtered through Celite. A 0.22-μm Millipore filter was used to retain large particles. The pH of the solution was adjusted to 2.5 and extracted with diethyl ether. The ether was evaporated under a stream of nitrogen gas and the hormones were resuspended in 80% methanol.

The following chromatographic data were calculated on the various components

$$k' = \frac{t_r - t_0}{t_0} \qquad \alpha = \frac{k'_2}{k'_1} \qquad R_s = \frac{\frac{1}{4}\cdot(\alpha - 1)}{\alpha}\cdot\sqrt{N}\cdot\frac{k'_2}{1 + k'_2}$$

where k' = capacity factor, t_0 = retention time of non-sorbed solvent, t_r = retention time of component, α = relative retention, R_s = resolution of two peaks and N = theoretical plate count (3000 for μBondapak C_{18}, 5000 for Radial-Pak A).

RESULTS AND DISCUSSION

Chromatograms using both columns are presented in Fig. 1. It is visually apparent that the separation with the RCM-100 was superior to that using the μBondapak C_{18} column. Baseline separation of all components was achieved using the

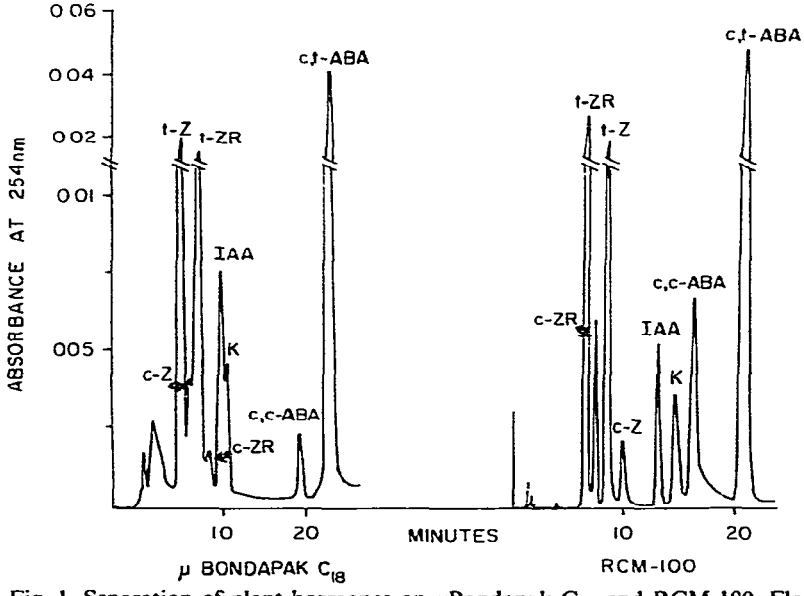

Fig. 1. Separation of plant hormones on μBondapak C_{18} and RCM-100. Flow-rate 2 ml/min. t-ZR = *trans*-Zeatin riboside; c-ZR = *cis*-zeatin riboside; t-Z = *trans*-zeatin; c-Z = *cis*-zeatin; IAA = indole-3-acetic acid; K = kinetin; c,c-ABA = *cis*,*cis*-abscisic acid; c,t-ABA = *cis*,*trans*-abscisic acid.

RCM-100. The RCM-100 was especially valuable in that the separation of the *cis* and *trans* isomers of Z, ZR, and ABA was achieved. The *cis* isomers of Z and ZR were not sufficiently distinct from the other compounds on the μBondapak C_{18} column. If small amounts of kinetin were chromatographed relative to the amount of IAA present, these two compounds could not be distinguished. Besides using methanol, various other solvents such as acetonitrile and ethanol were used to attempt separation on the μBondapak C_{18} column, but none was satisfactory. Brenner[1] described adequate separation on a column packed with μBondapak C_{18} larger than the one used for this study, and the flow-rate was much faster (5 ml/min) than our flow-rate (2 ml/min).

The use of several solvents for separation was explored with the RCM-100. Different concentrations of acetic acid were used, from 0.67% to 2.0%, with and

TABLE I
RETENTION TIMES AND CHROMATOGRAPHIC PARAMETERS FOR THE SEPARATION OF HORMONES USING RCM-100 AND μBONDAPAK C_{18} (n = 4)
Flow-rate 2 ml/min. t_o = 1 min 30 sec (RCM-100) and 2 min 25 sec (μBondapak C_{18}).

Hormone	*RCM-100*				*μBondapak C_{18}*			
	Retention time (sec)	*k'*	*α*	*R_s*	*Retention time (sec)*	*k'*	*α*	*R_s*
trans-Zeatin riboside	369	3.10	1.19	2.22				
cis-Zeatin riboside	423	3.70	1.22	2.61				
trans-Zeatin	497	4.52	1.24	2.90				
cis-Zeatin	593	5.59	1.39	4.40				
IAA	791	7.79	1.14	1.95				
Kinetin	889	8.88	1.11	1.59				
cis,cis-ABA	979	9.88	1.31	3.88				
cis,trans-ABA	1255	12.94						
trans-Zeatin					407	1.81		
trans-Zeatin riboside					479	2.30	1.27	2.03
IAA					619	3.27	1.42	3.10
Kinetin					656	3.52	1.08	0.79
cis,cis-ABA					1249	7.61	2.16	6.50
cis,trans-ABA					1363	8.40	1.10	1.11

without ammonium acetate. Neither methanol nor acetonitrile alone provided adequate separation; however, a 58:42 mixture was successful. Acetonitrile was added because Z was difficult to elute from the RCM-100 with the methanol alone. The relative positions of Z and ZR are different from the positions of the hormones separated by the μBondapak C_{18} column.

The relative retention times and chromatographic data for the hormones are presented in Table I. The coefficient of variation for the retention times, as determined by either procedure, ranged from 1.7% to 4.8%. The optimal values for k' should range from 1 to 10, inclusive for multi-component separation. The values for *cis,trans*-ABA fell outside this region for the RCM-100. At an R_s value of 1.0, peak overlap was only 2% so that all calculated values, except for IAA and K on the μBondapak C_{18}, represented acceptable separation of the two peaks. Although the resolution data were acceptable for the μBondapak C_{18}, the separation by the RCM-100 was clearly superior. The procedure described using the RCM-100 will detect nanogram quantities of the hormones as other researchers have reported[1-3].

This separation technique has been applied to plant samples extracted for hormones. A chromatogram representing a plant sample is presented in Fig. 2. The hormones tentatively identified were *trans*-ZR, IAA, and *cis,trans*-ABA. One advantage of the RCM-100 procedure is the capability to separate *cis* and *trans* isomers. Isomeric configuration does influence hormonal activity of the compounds. The effect of a chemical treatment or stimulus on a plant could be to alter the production of a

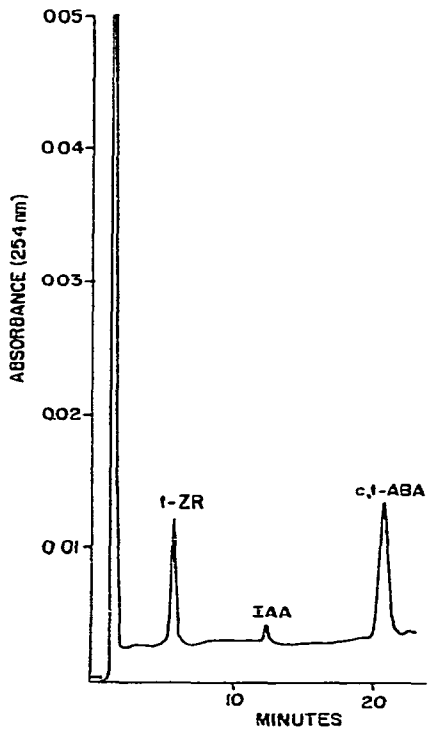

Fig. 2. Separation by RCM-100 of hormones extracted from soybean plant tissue. Abbreviations as in Fig. 1.

hormone toward the less effective isomer. This type of information could not be obtained by using other HPLC procedures[3,4].

This technique would be useful to the physiologist to investigate how different environmental conditions or chemical treatments such as growth regulants, herbicides, insecticides, and fungicides influence the plant and its hormonal levels. Plant breeders could use this procedure in cultivar evaluation programs to develop seasonal hormonal profiles that may be linked with particular characteristics such as high yields, short stature, or resistance to a particular pest. The ability of the present technique to separate component hormones from a given plant sample with speed and accuracy can enhance the studies of the relationships of plant hormones.

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