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SEPARATION OF PLANT HORMONES BY COUNTER-CURRENT CHROMATOGRAPHY*

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SUMMARY

Counter-current chromatography (CCC) using the toroidal coil planet centrifuge was applied to the separation of plant hormones. Indole auxins were separated in either hexane–ethyl acetate–methanol–water (0.6:1.4:1.0:1.0) or chloroform–acetic acid–water (2:2:1). The latter solvent system was especially useful for the separation of abscisic acid from indole-3-acetic acid. Gibberellins (GA_3, GA_4 and GA_7) were separated from each other in ether–methanol–phosphate buffer (pH 7) (3:1:2). The CCC method was suitable for the separation of four cytokinins in ethyl acetate–methanol–phosphate buffer (pH 7) (3:1:3). Abscisic acid from Zoysia grass seed was successfully analysed by CCC. The potential practical application of CCC to plant hormone analysis is discussed.

INTRODUCTION

Plant hormones and other growth substances that are present in plant tissues at ppm and ppb** concentrations regulate the growth and development in all higher plants. They are required to study several metabolic changes, and other biochemical and physiological implications during the plant growth and developmental processes. Because of their presence in trace amounts, it is often very difficult to analyze and monitor their levels. Several analytical approaches including chromatographic spectroscopic methods have been suggested for their detection and analysis[1]. Recently, high-performance liquid chromatography (HPLC), gas chromatography with an

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** In this article, the American billion (10^9) is meant.

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electron-capture detector (GC–ECD) and selective ion monitoring (SIM) have been used to analyze various groups of plant hormones, especially to detect abscisic acid (ABA)* and indole-3-acetic acid (IAA), with reasonable success[2–4]. However, there are some inherent difficulties in these methods. Although HPLC has been found to be a superior method over other chromatographic systems, the choice of columns and solvent systems is limited, especially for separations of polar compounds and, moreover, other purification methods are needed prior to HPLC use for the determination or analysis of plant hormones from crude plant extracts. Both GC–ECD[5,6] and SIM methods[4–7] are very sensitive and can detect the trace substances at subnanogram levels. However, to utilize these methods, prior to introduction into the GC system, the compounds have to be converted into suitable derivatives in order to make them readily volatile and thus enable them to pass through the GC columns easily. Additionally, for SIM analysis, a thorough knowledge of mass spectrometry, suitable internal standards and above all a large investment of money in mass spectrometric (MS) instrumentation are required.

As an on-going project, we have been investigating analytical methods with a view to either refining the existing ones or developing new methods that would be better suited for plant hormone analysis. In this connection, we have examined a recently developed method, termed counter-current chromatography (CCC)[8], which is based on the principles of counter-current distribution (CCD) and liquid chromatography (LC). This paper deals with the application of CCC to the separation of standard mixtures of plant hormones and other related compounds.

MATERIALS AND METHODS

Instrument design

A simple tabletop model of the toroidal coil planet centrifuge[9,10] was used (Fig. 1). It has a rotary frame consisting of a pair of circular aluminum plates rigidly bridged with multiple aluminum links. The rotary frame is driven by a motor around the stationary pipe mounted on the central axis of the centrifuge and holds a pair of symmetrically spaced cylindrical holders (10 cm from the central axis), one of which (15 cm O.D.) has a coiled column while the other holder (10 cm O.D.) carries a counterweight to balance the centrifuge system. Each holder is equipped with a plastic gear (Winfred N. Berg, Inc.) which is coupled to an identical sun gear mounted around the central stationary pipe. This gear arrangement produces the desired planetary motion of the holders, *i.e.*, revolution around the central axis of the apparatus and rotation about its own axis at the same angular velocity in the same direction. For mechanical stability of the centrifuge, the free end (right side) of the rotary frame is coaxially connected to a short coupling pipe, which is supported by a stationary wall member of the centrifuge through a ball bearing.

The separation column is prepared by winding PTFE tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) of 0.55 mm I.D. on to a flexible core which is again

* Abbreviations: ABA = abscisic acid; GA_3 = gibberellic acid; GA_4 and GA_7 = gibberellins A_4 and A_7; IAA = indole-3-acetic acid; IA = indole-3-acetamide; IAN = indole-3-acetonitrile; IAcA = indole-3-acrylic acid; IBA = indole-3-butyric acid; ICA = indole-3-carboxylic acid; IPA_3 = indole-3-propionic acid; Z = zeatin; ZR = zeatin riboside; IP = 6-isopentenyladenine; IPA = 6-isopentenyladenosine.

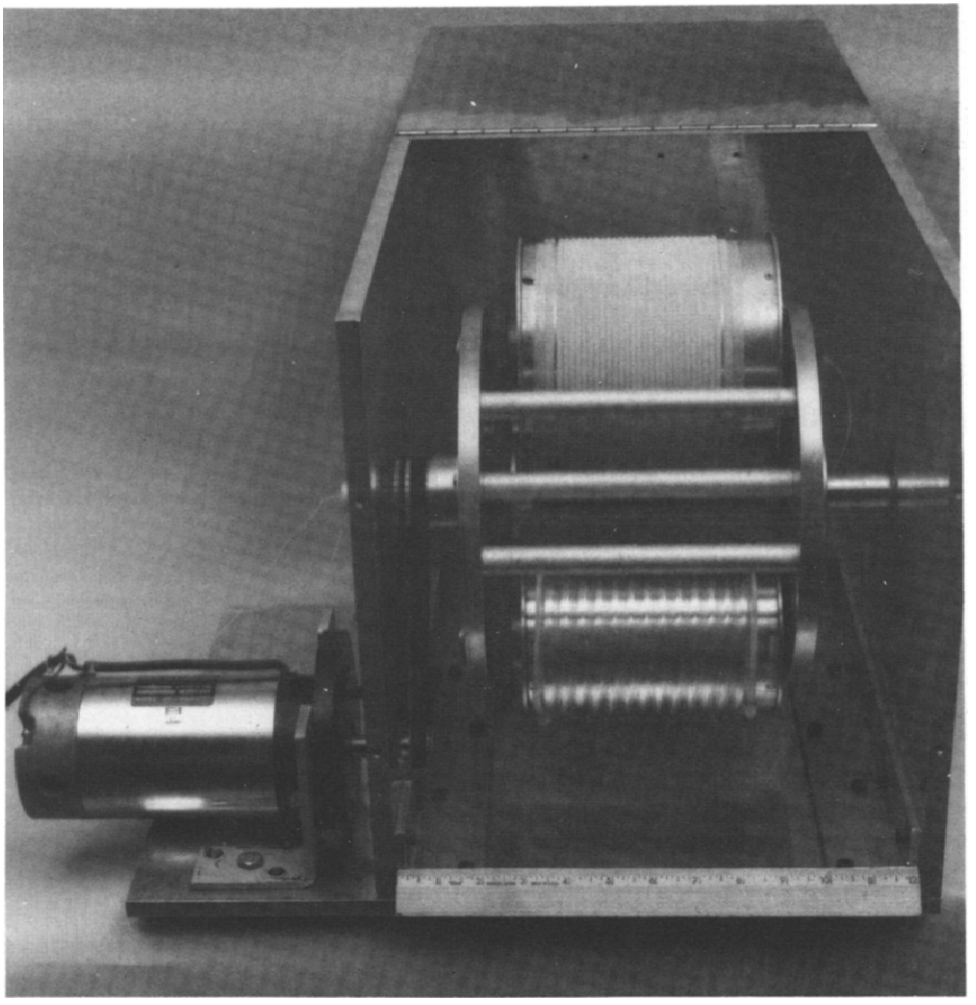

Fig. 1. A table-top model of the toroidal coil planet centrifuge.

coiled around the holder to make a coiled helix or toroidal coil configuration (helical diameter, 1.5 mm; total number of turns, 8500; total capacity, 18 ml; theoretical plates, 2000–6000). A counterweight is applied to the other holder to balance the centrifuge. The flow tubes from the column are first passed through the center of the coil holder shaft and then led into the coupling pipe through a side hole to enter the opening of the stationary pipe. To prevent mechanical damage, the flow tubes are lubricated (silicone grease) and protected with a piece of plastic tubing at each supported position. The planetary motion produced by the gears permits the flow tubes simply to roll around themselves without twisting. The revolutional speed is continuously adjustable up to 1000 rpm (450 *g*) with a Motomatic speed control unit (Electro-Craft).

Operation

The coiled column is first filled with a stationary phase of the pre-equilibrated two-phase solvent with the aid of either a Chromatronix Cheminert metering pump or a Milton-Roy pump. The test sample solution is introduced through a sample injection port and the mobile phase is pumped through the column at 2.4 ml/h while the apparatus is run at a desired revolutional speed (450–500 rpm). The eluate is continuously monitored with either an LKB Uvicord III or an LKB Uvicord S at 206, 260 or 280 nm, depending on the test samples, and then collected with a fraction collector.

Sources of hormone samples

All of the test compounds were obtained from several commercial sources and used without further purification. ABA-containing samples from Zoysia grass seeds were obtained by scarification of the seeds with alkali followed by extraction with diethyl ether–ethyl acetate (1:1). The crude acidic extract was purified by HPLC on a reversed-phase column Bondapak C_{18}, (Waters Assoc.) using methanol–water (80:20) and a fraction corresponding to the retention volume of standard ABA sample was collected and analyzed by CCC. Final proof for ABA was obtained by GC–MS of its methyl ester.

Partition coefficients

Partition coefficients were determined by adding a known amount of the test compounds in the solvent systems used for each group of hormones. After thoroughly mixing the compounds in the desired solvent system, the two phases were separated and the absorbances measured at the desired wavelength (indoles and ABA at 280 nm, cytokinins at 260 nm and gibberellins at 206 nm). The ratio of the absorbance values of the samples from two phases gave their partition coefficient (P.C.) in the respective solvent systems. A typical procedure for determining the P.C. of IAA in chloroform–acetic acid–water (2:2:1) is given here. To a mixture of 2 ml of chloroform, 2 ml of acetic acid and 1 ml of water were added 10 μl of IAA aqueous solution (concentration 20 μg/μl). The compounds and the solvents were mixed vigorously and two phases allowed to separate. A 1-ml volume of each phase was mixed with 3 ml of methanol and the absorbance was measured at 280 nm with a Beckman UV–visible spectrophotometer using a 1-cm light-path quartz cell which gave readings of 0.382 and 0.405 for the lighter phase (Lp) and the heavier phase (Hp), respectively. Hence the P.C. of IAA is 0.94 (Lp/Hp) for the above solvent system.

RESULTS AND DISCUSSION

Indole compounds

Two chromatographic solvent systems, I (hexane–ethyl acetate–methanol–water, 0.6:1.4:1:1) and II (chloroform–acetic acid–water, 2:2:1), were chosen for separation of several indolic compounds by CCC. The partition coefficients (Table I) range from 0.26 to 3.83 in these two solvent systems. The compounds studied either exhibit auxin activity or are the metabolic products of IAA. Because of the inherent differences in their P.C.s in these solvent systems, one would expect these indoles from a mixture to separate into individual components by CCC. If the upper phase is kept

TABLE I

PARTITION COEFFICIENTS OF INDOLES IN TWO DIFFERENT SOLVENT SYSTEMS

Compound	*Abbreviation*	*Concentration (μg/μl)*	*Partition coefficient*			
			Solvent system I		*Solvent system II*	
			Lp/Hp	*Hp/Lp*	*Lp/Hp*	*Hp/Lp*
Indole-3-acetic acid	IAA	20	0.99	1.01	0.94	1.06
Indole-3-acetamide	IA	20	0.26	3.83	1.05	0.95
Indole-3-acetonitrile	IAN	20	3.00	0.33	0.39	2.55
Indole-3-carboxylic acid	ICA	10	0.63	1.61	1.07	0.93
Indole-3-acrylic acid	IAcA	10	0.88	1.44	0.78	1.28
Indole-3-butyric acid	IBA	20	1.74	0.57	0.36	2.81
Indole-3-propionic acid	IPA_3	20	2.02	0.50	0.26	3.80

Solvent system I, hexane–ethyl acetate–methanol–water (0.6:1.4:1:1); solvent system II, chloroform–acetic acid–water (2:2:1). Hp, heavier phase; Lp, lighter phase.

stationary on the column and the lower phase is used to elute the column, the partition coefficients (Hp/Lp) for these compounds (Table I, columns 5 and 7) serve as a useful guide for predicting the order of elution for these compounds in a mixture. When the phases are reversed, one must then use columns 4 and 6 of Table I. As the elution pattern follows the decreasing order of P.C.s, the separation of the mixture begins with the highest P.C.-containing compound followed by compounds with lower P.C.s.

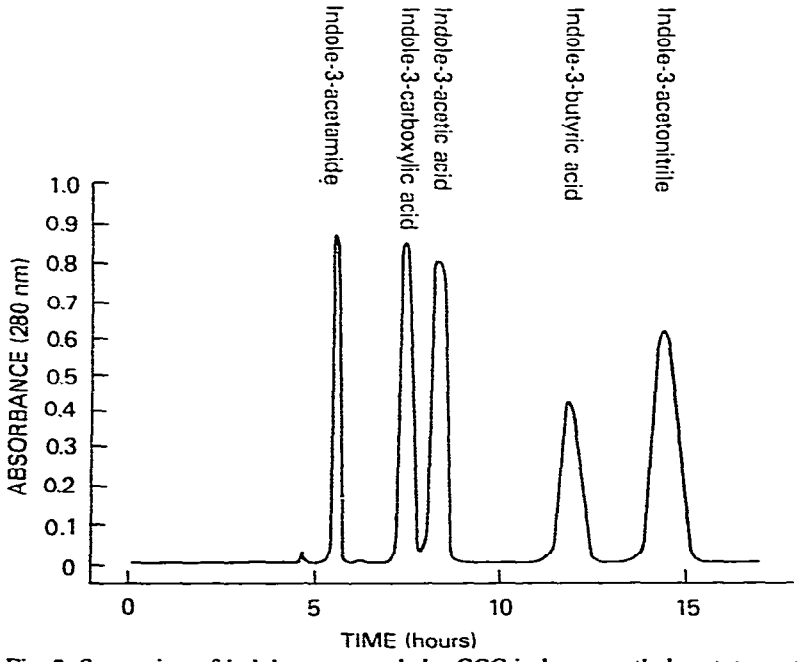

Fig. 2. Separation of indole compounds by CCC in hexane–ethyl acetate–methanol–water (0.6:1.4:1:1); mobile phase: aqueous phase.

A mixture (25 μg) containing five indoles (IAA, IA, IAN, ICA and IBA; 50 μg each) whose P.C.s vary from 0.33 to 3.83 were chromatographed in solvent system I, keeping the lighter (non-aqueous) phase stationary. These compounds eluted in the order that one would predict: IA (P.C. 3.83) eluted first followed by ICA (1.61), IAA (1.01), IBA (0.57) and IAN (0.33) with the lower phase as mobile phase (Fig. 2). In solvent system II, a mixture containing four indoles (ICA, IAcA, IBA and IPA_3) and ABA was analyzed by CCC. IAA was not introduced in this mixture because it would obscure the resolution of ICA and IAcA peaks as it falls between the two peaks (resulting in poor resolution of peaks). Keeping the organic (heavier) phase stationary, all five compounds were separated with the lighter (aqueous) phase solvent (Fig. 3). A small peak just before the ABA peak was observed and was identified as an impurity of the IPA_3 sample. ABA, which has a P.C. (Lp/Hp) of 0.56, eluted after IAcA (0.78). IAA and ABA were easily separated using the same conditions (Fig. 4) because of the differences in their P.C.s in the solvent system II. These results clearly indicate that the indole compounds that are commonly found in plant tissue can be separated with solvent system I and/or II. Although IBA (0.57) and IPA_3 (0.50) could not be separated in solvent system I because of their close P.C.s, they were clearly resolved using solvent system II. ABA present in plant samples could also be detected and separated from IAA by CCC with solvent system II, which is especially useful for the separation of ABA from indoles (because of the difference in their P.C.s). These results suggest that, by a judicious choice of a solvent system, indole compounds as well as other plant metabolites can be readily separated in the presence of ABA.

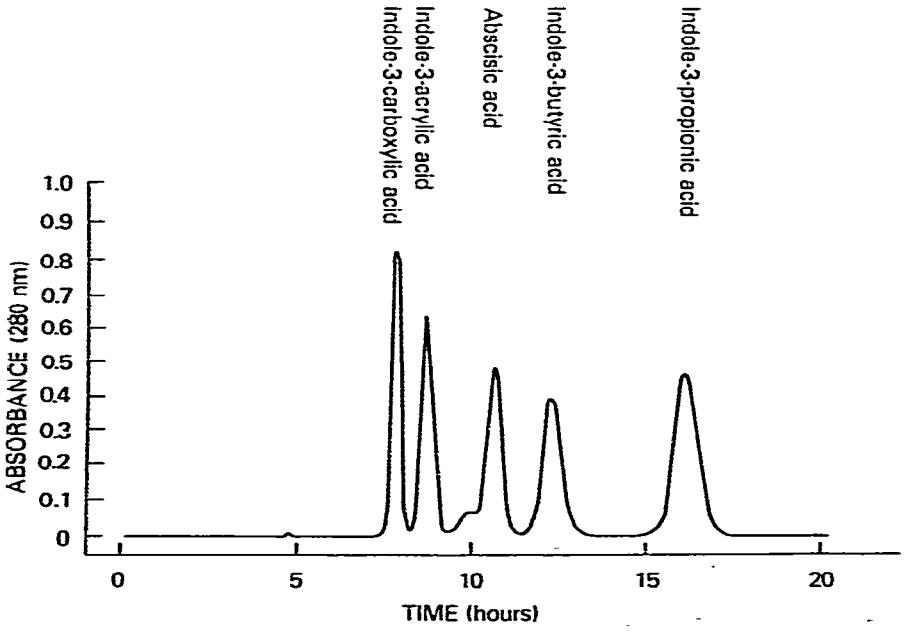

Fig. 3. CCC separation of a mixture of four indolic carboxylic acids and abscisic acid in chloroform–acetic acid–water (2:2:1), retaining organic phase stationary.

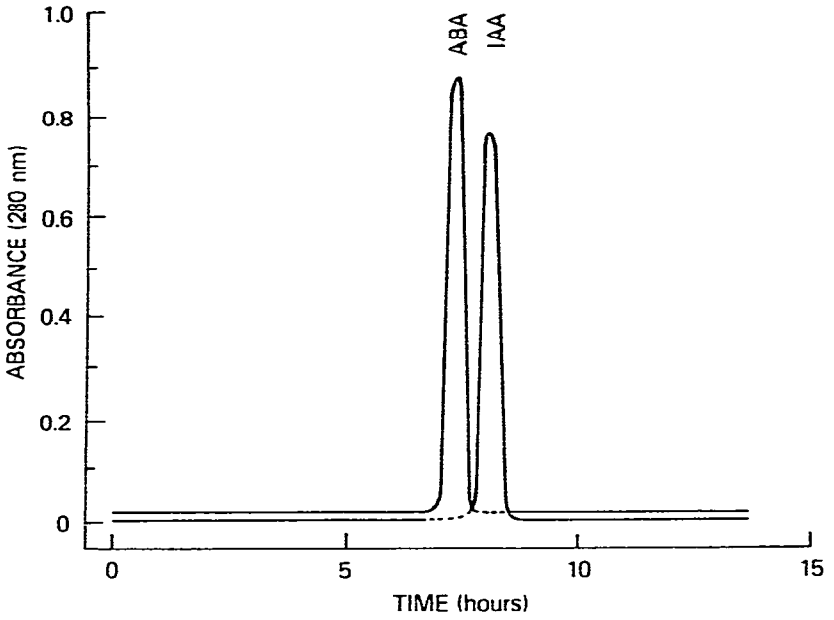

Fig. 4. CCC separation of indole-3-acetic acid and abscisic acid in chloroform–acetic acid–water (2:2:1), retaining organic phase stationary.

Gibberellins

As in HPLC, the separation of this group of plant hormones presented problems, mainly because of the lack of a proper detection system. None of the gibberellins separated here showed UV absorption between 220 and 360 nm. However, all of them had strong end absorption around 210 nm, which was used to monitor them with a UV detector. The choice of solvents was limited because gibberellins do not partition very well in the ordinarily employed solvent systems. A solvent system containing ethyl acetate–phosphate buffer (pH 7) was reported previously to be an ideal system in the countercurrent distribution of gibberellin[11]. As ethyl acetate (UV cut-off at 230 nm) interferes with UV monitoring at 210 nm, diethyl ether was used in its place. The use of the diethyl ether–0.50 *M* phosphate buffer system had the disadvantage that it has a very high interfacial tension, which creates a high column pressure.

This difficulty was eliminated by introducing methanol into the system. A solvent system containing diethyl ether–methanol–0.5 *M* phosphate buffer (pH 5.9) (3:1:2) was suitable for gibberellin separation. A GA_3 and GA_4–GA_7 mixture was used for separation work. With the lighter (organic) phase mobile, GA separation was achieved (Fig. 5). Under these conditions, GA_3 eluted first, followed by GA_7 and finally GA_4. The total run took less than 8 h, although we allowed the chromatograph to run for 24 h. To confirm that the chromatographic peaks contain gibberellins, all the fractions were bioassayed in a bean second internode test[12], which showed activity for those fractions that represented the peaks. Thus, the correlation of the peaks with activity was made for the separated gibberellins.

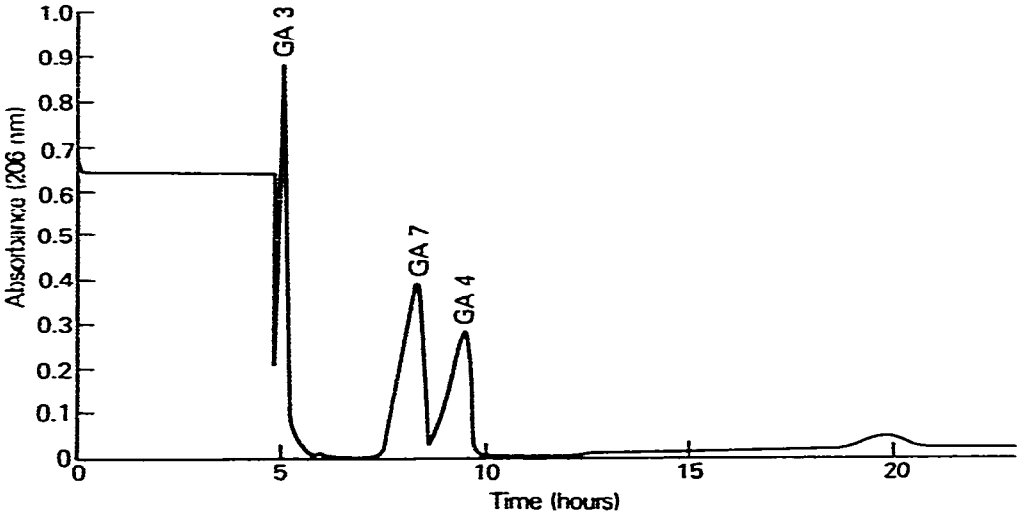

Fig. 5. Separation of gibberellins A_3, A_4/A_7 by CCC; solvent system: ethyl acetate–methanol–0.5 *M* phosphate buffer, pH 5.9 (3:1:2); mobile phase: organic phase.

Cytokinins

This method is especially useful for separation of cytokinins, namely for separating a mixture of Z, ZR, IP and IPA, as they were well resolved in a solvent system containing ethyl acetate, methanol and phosphate buffer. A change of pH from 5 to 9 affected their partition coefficients considerably when they were partitioned between ethyl acetate and 0.5 *M* phosphate buffer[13]. For example, ZR showed a P.C. (C_{aq}/C_{org}) range of 8–10 whereas IPA has P.C.s of 0.08–0.1 in this solvent system. The solvent combination of our choice was ethyl acetate–methanol–0.5 *M* phosphate buffer (pH 7) (3:1:3) in which the desired range of P.C.s (0.2–5.0) for these compounds was accomplished (Table II). The cytokinin mixture, when subjected to CCC separation keeping the organic phase stationary, was completely resolved, according to the decreasing order of their P.C.s. (C_{aq}/C_{org}); *i.e.*, ZR which has the highest P.C., eluted first, followed by Z, IPA and IP (Fig. 6). However, the Z and ZR peaks appeared too close, whereas IP and its riboside (IPA) were separated farther apart (because of the long retention times). The commercial samples of Z and ZR contained

TABLE II

PARTITION COEFFICIENTS OF CYTOKININS IN ETHYL ACETATE–METHANOL–0.5 *M* PHOSPHATE BUFFER (pH 7) (3:1:3)

Cytokinin	*Abbreviation*	*Partition coefficient*	
		C_{aq}/C_{org}	C_{org}/C_{aq}
Zeatin riboside	ZR	4.25	0.24
Zeatin	Z	2.26	0.44
6-Isopentenyladenosine	IPA	0.73	1.37
6-Isopentenyladenine	IP	0.20	5.00

impurities which were found in the chromatogram before, after and between these peaks. In order to make the Z and ZR peaks well resolved one should adapt by either simply reversing the phase in the CCC run or choosing an entirely different solvent system, so that the P.C.s for these compounds appear below 1.0 and preferably around 0.5. We have chosen to use the same solvent system but simply to reverse the phase, whereby the change in P.C.s (C_{aq}/C_{org}) reverses the order of the elution pattern for these compounds (Fig. 7). In this way, IP, which had the longest retention time in the above situation (see Fig. 6), is now eluted with the shortest retention time whereas ZR had the longest retention time (Fig. 7). Further, Z and ZR were well separated ($\Delta R_t = 5$ h) and thereby the impurities in the mixture did not interfere with the Z and ZR peaks.

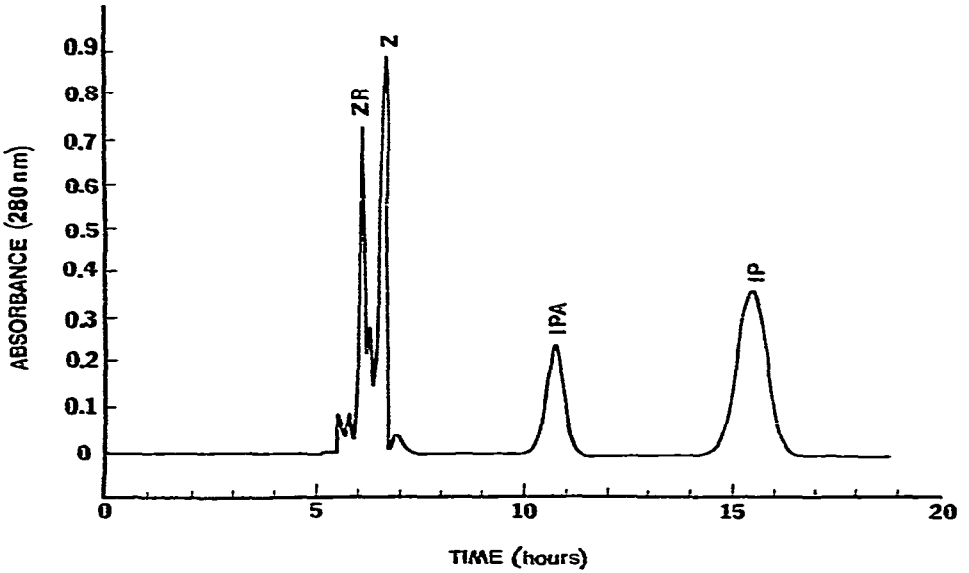

Fig. 6. Separation of zeatin and 6-isopentenyl adenine and their ribosides in ethyl acetate–methanol–0.5 *M* phosphate buffer, pH 7 (3:1:3); stationary phase: non-aqueous phase.

This approach demonstrates that (1) the partition coefficients either directly or inversely relate to the retention times measured from the solvent front and (2) reversal of the choice for the mobile phase gives the reciprocal values of the P.C.s and therefore affects the retention times of the solute molecules. The approach has the following advantages. (1) Changing the phase from stationary to mobile is very easy to perform in CCC. (2) If particular compound takes several hours to elute, changing the phase reduces the retention time considerably. (3) In plant sample mixtures (containing cytokinins), one could first separate the hormones from other undesired compounds by a process where they can be collected first (with shorter or long retention times) and later, by reversal of the phase in CCC, one could obtain a pure compound.

Abscisic acid analysis

In connection with our work on seed germination, we have investigated Zoysia

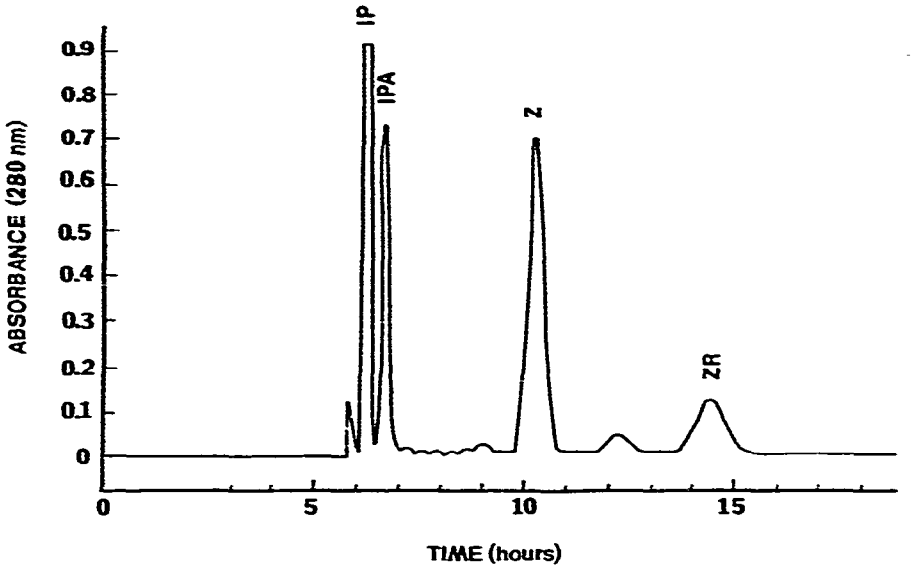

Fig. 7. Separation of cytokinins in ethyl acetate–methanol–0.5 *M* phosphate buffer, pH 7 (3:1:3); stationary phase: aqueous phase.

grass seed, which has a tendency to remain dormant but scarification with alkali for a few minutes[14] allowed the seed to germinate easily. We extracted the scarified seed with diethyl ether–ethyl acetate and chromatographed the extract by HPLC. A fraction corresponding to the retention of standard ABA was collected and subjected to CCC separation. This fraction showed a single peak that corresponded to the retention time of the standard ABA. Thus the confirmation for ABA in the plant extract was obtained by comparing it in two chromatographic systems followed by MS analysis of the corresponding methyl ester.

Advantages of CCC over CCD and LC

In comparison with CCD, CCC offers the following advantages: (1) much higher partition efficiency; (2) shorter separation times; (3) separation of small amounts of samples without much dilution; (4) little or no risk of emulsification; (5) a wide selection of solvents which are required in relatively small amounts; (6) separation of compounds whose P.C.s fall between 0.2 and 5; (7) molecules of any size can be separated; (8) the apparatus is much smaller and less expensive (*ca.* $5000); (9) samples are not exposed to the air and hence there is no deterioration of sensitive compounds; and (10) continuous monitoring of the eluate is possible with a conventional UV detector. The main disadvantage of CCC compared with CCD is that the sample sizes are limited to a few hundred milligrams, unless larger bore coils are employed in CCC.

A few obvious advantages over LC are (1) sample loss is minimal and even the samples precipitated in the column can be recovered easily; (2) sample deterioration is minimal; (3) there is no tailing of the solute peaks due to the adsorption effects of the solid support; (4) there is no contamination due to the use of solid supports; (5) high reproducibility; (6) prediction of the location of the solute peaks is possible simply by

measuring the P.C.s of the sample; (7) the system provides the choice of the mobile phase, either aqueous or non-aqueous and either upper or lower phase; (8) the large amount of the stationary phase allows a large sample loading capacity; and (9) the column is easily cleaned and can be reused almost indefinitely. One major disadvantage of CCC over LC is the separation time, which is very long; it takes at least 10 h to yield several thousand theoretical plates, whereas HPLC can produce comparable separations in less than 1 h. Despite the limitation of long separation times, CCC appears to be useful for the separation of polar compounds from the crude mixtures. It will not replace conventional chromatographic methods such as TLC, GC and HPLC, but is complementary. When used alone or in conjunction with these methods, CCC is of great help for the separation of plant hormones.

CONCLUSIONS

The limited work presented in this paper demonstrates that CCC appears to have great potential for the separation of plant hormones and other growth substances in an efficient manner. Neither expensive columns nor costly instruments are required in this type of separation. With the availability of different CCC prototypes, separation can be carried on on either an analytical or a preparative scale. When desired, gradient elution can be performed in a manner similar to HPLC. In retrospect, this method may have wide applicability for the total hormonal analysis of plant samples after conventional extraction methods give acidic, basic and neutral fractions, each of which could be successfully analyzed following the procedures outlined above.

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