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Normal-Phase Liquid Chromatography of Plant Hormones Using Reversed Cholic Acid Micelles as the Mobile Phase

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When several plant hormones with similar structures are present in a sample, conventional analytical methods are not sufficiently selective to effectively differentiate them. However, the excellent selectiviy of micellar liquid chromatography allows similarly structured compounds to be easily differentiated. We used reversed cholic acid micelles dissolved in tetrahydrofuran as the mobile phase in normal-phase chromatography of seven plant hormones. Our results conformed closely to those predicted by the pseudophase micellar liquid chromatography theory. The chromatographic efficiencies were calculated from the peak width and the retention times, and we compared these values with those obtained by reversed-phase chromatography using sodium dodecyl sulfate mobile phase in water. To test the efficiency of this selective method for the analysis of plant tissues that may also contain contaminants, we applied it to homogeneous suspensions of spiked maize roots. Recoveries ranged from 91 to 110%, the relative standard deviations were between 0.46 and 10.03%, and the detection limits were between 0.08 and 0.80 μg g^{-1}.

Molecules that regulate plant growth and plant development are also known as plant growth regulators or plant hormones.[1,2] Hormones occur in plant material at very low concentrations (ng g^{-1}), together with many other compounds, from which they must be separated to permit their accurate analysis. Plant hormones are classified into five groups: auxins, abcisins, cytokinins, gibberellins, and ethylene. The most important hormone is an auxin, indol-3-ylacetic acid (IAA), and its concentration in the tissues varies with the plant species and the different tissues of the plant. The concentration of IAA in the tissues is regulated by the rate of synthesis, tissue concentrations, and deactivation mechanisms. The inactivation of IAA gives ester or peptide conjugates. There are also several synthetic compounds with structure similar to that of IAA that elicit auxin-like physiological responses. These synthetic compounds are called plant growth regulators, the most common of which naphthylacetic acid, indol-3-ylbutyric acid, 2,4-dichlorophenoxyacetic acid, and their derivatives.

The most commonly used physicochemical methods for measuring plant hormones involve a high-resolution chromatographic step, usually liquid chromatography (LC) or capillary gas chromatography, plus on-line detection of high selectivity.[3] Quantitative analysis by LC with fluorometric detection is particularly useful to measure IAA and related indoles, because their natural fluorescence permits sensitive detection. In this way, picogram concentrations of IAA can be measured in crude plant extracts.[4] In addition, the sensitivity of fluorometric methods for plant hormone analysis is exploited by creating fluorescent derivatives,[5−7] but the poor selectivity of this method is still a problem for structurally similar compounds.

Bile salts appear to be more effective[8] than synthetic surfactants or cyclodextrins and greatly improve luminescence analysis.[9,10] In addition, because bile compounds increase separations,[11−13] they have been used in reversed-phase micellar liquid chromatography[14−16] and as ion-pair reagents for liquid chromatography.[17] However, the polar nature of plant hormones suggests the use of a normal-phase separation of a polar stationary phase with reversed micelles as the mobile phase.

Almost all of the analytical applications that use micellar solutions have also utilized normal micelles, i.e., micelles formed in polar solvents. The behavior of reversed micelles is more complex, less studied, and less understood than that of normal micelles,[18] but reversed micelles offer the same potential advantages for analysis as normal micelles because they also solubilize polar species, which normal micelles cannot do. At the present time, the use of reversed micelle mobile phase is the only solution to the problem caused by water content for normal-phase liquid

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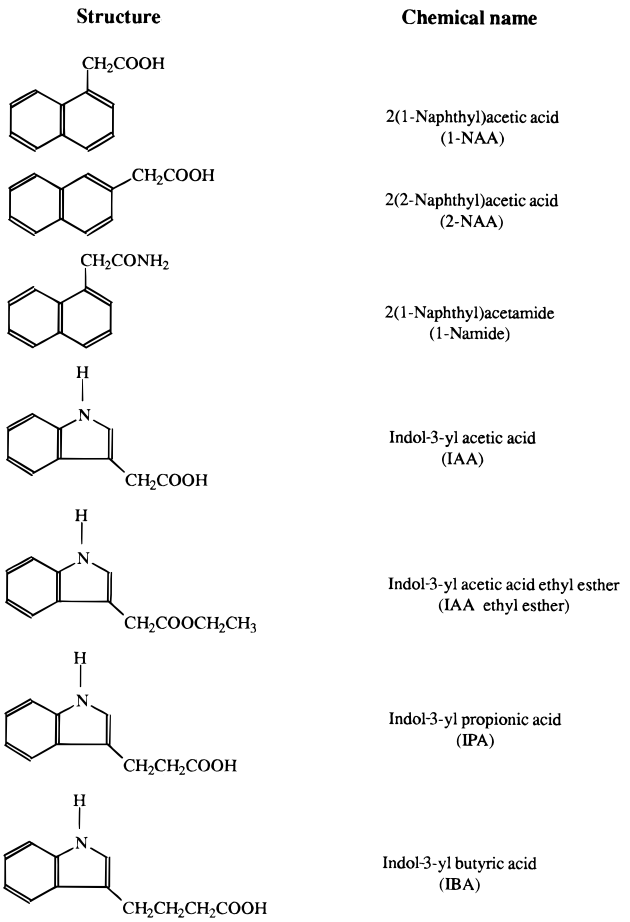

Figure 1. Structures of plant hormones.

chromatography, because reversed micelles solubilize water within their micelle structure.[19]

We report the use of cholic acid as the reversed micellar mobile phase for normal-phase chromatography to determine concentrations of the following seven plant growth regulators in spiked crude plant extracts (Figure 1): indol-3-ylacetic acid (IAA), indol-3-ylacetic acid ethyl ester (IAA ethyl ester), indol-3-ylbutyric acid (IBA), indol-3-ylpropionic acid (IPA), 2-(1-naphthyl)acetic acid (1-NAA), 2-(2-naphthyl)acetic acid (2-NAA), and 2-(1-naphthyl)-acetamide (1-Namide). The chromatographic parameters were compared with those obtained by using sodium dodecyl sulfate (SDS) with normal micelles as the mobile phase in reversed-phase liquid chromatography.

EXPERIMENTAL SECTION

Chemical and Solutions. Cholic acid was obtained from Sigma (St. Louis, MO), and dodecyl sulfate sodium salt (SDS) and salicylic acid were from Merck (Darmstadt, Germany). Tetrahydrofuran, methanol, ethanol, gradient grade Lichrosolv, ethyl acetate, diethyl ether, and acetic acid were also from Merck. The plant growth regulators indol-3-ylacetic acid (IAA), 2-(1-naphthyl)acetic acid (1-NAA), indol-3-ylpropionic acid (IPA), 2-(2-naphthyl)acetic acid (2-NAA), indol-3-ylbutyric acid (IBA), and indol-3-ylacetic acid ethyl esther (IAA ethyl esther) were all of standard purity, purchased from Sigma, and 2-(1-naphthyl)-acetamide (1-Namide, standard purity) was purchased from Aldrich. The stock standard solutions of 1-Namide (5.40×10^{-3} M), NAA (5.37×10^{-3} M), IBA (4.92×10^{-3} M), IPA (5.26×10^{-3} M), IAA (5.71×10^{-3} M), and IAA ethyl esther (4.94×10^{-3} M) were prepared by dissolving in methanol; they were then stored at 4 °C. Working solutions were prepared by diluting the stock solutions with tetrahydrofuran. The cholic acid stock solutions (0.1 M) were prepared fresh on the day of use by dissolving the powder in tetrahydrofuran. Lower concentrations of cholic acid were obtained by preparing serial dilutions of the stock solution in tetrahydrofuran. Salicylic acid (0.1 M) solution was prepared by dissolving the powder in tetrahydrofuran. All solutions were filtered through 0.2 μm Nylon membrane filters.

Extraction. Caryopses of *Zea mays* L. were germinated between layers of moist cotton-wool for 8 days. The harvesting was carried out when the apex zone, the center zone, and the root reached 1, 0.5 and 2.5 cm, respectively, and were then stored at −40 °C before trituration and analysis. The extraction procedure and bulk purification of plant material were described in a previous work.[20]

LC Operating Conditions. The measurements were made with a Merck-Hitachi liquid chromatograph (Darmstadt, Germany) that consisted of an L-6200 pump, an AS-4000 autosampler, an L-4250 UV−visible detector and a D-6000 interface. A Perkin-Elmer LS-50 fluorescence detector (Beaconsfield, UK), with a 15 μL cell volume and a xenon discharge lamp with two monochromators, was placed in series with and after the UV−visible detector. The instrumental parameters were controlled by a fluorescence data manager (FLDM) using LC Program software.

The plant hormones were analyzed using a Spherisorb S5-NH_2 analytical column (25 cm × 4.6 mm; 5 μm particle size) from Phase Separations (Deesire, UK). The injected volume was 20 μL for both standard and sample solutions; the flow rate was 1 mL min^{-1}, and, for fluorometric detection, the emission wavelength was 340 nm (excitation 280 nm). Mobile phase composition was maintained at 100% 0.01 M cholic acid between 0 and 16 min, from 16 to 27 min the composition was 90% 0.01 M cholic acid−10% 10^{-4} salicylic acid, and finally from 27 to 40 min it was 100% 0.01 M cholic acid. The peak areas were measured by noting the retention times of IAA ethyl ester (3.63 min), 1-Namide (6.43 min), IBA (10.1 min), IPA (13.87 min), 1-NAA (19.8 min), 2-NAA (22.6 min), and IAA (27.23 min). The calibration graphs were constructed using peak areas.

RESULTS AND DISCUSSION

The critical micelle concentration (cmc) of inverted micelles formed by cholic acid in tetrahydrofuran was determined previously by fluorescence[21] and gave cmc's at cholic acid concentrations between 5 and 7 mM. Liquid chromatography, on the other hand, gave cmc's of around 5 mM. Figure 2 shows the plot of the capacity factor (log k) of the solutes IAA, IBA, IPA, 1-NAA, and 2-NAA versus different cholic acid concentrations in the tetrahydrofuran mobile phase when an amino-bonded stationary phase was used. When cholic acid concentration in the mobile phase passed through the cmc, we noted significant changes in retention times of the test solutes. The plots showed that the slope of the curve decreased close to the cmc, and if the two linear components of the curve were extrapolated, they would intersect near the cmc of the system under investigation. The average value was 5 mM cholic acid in tetrahydrofuran. The results for the cmc of cholic acid in tetrahydrofuran agree with published data on

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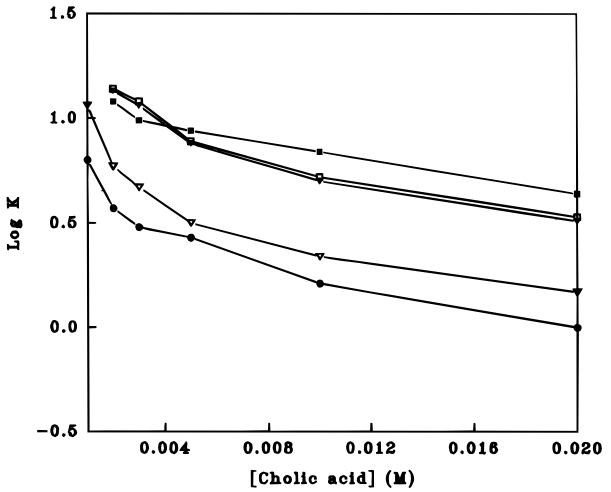

Figure 2. Log k vs cholic acid concentration. (●) IBA, (△) IPA, (▼) IAA, (□) 1-NAA, and (■) 2-NAA.

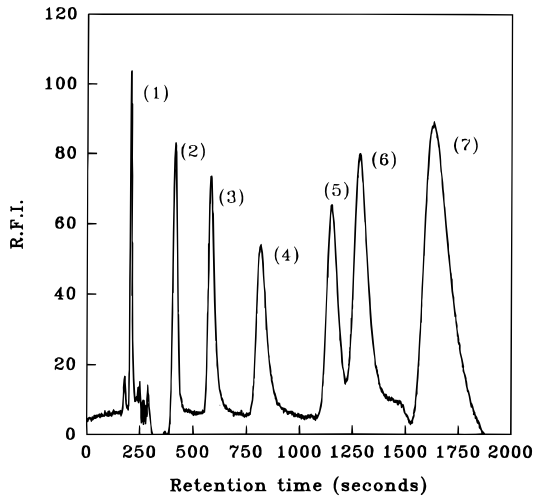

Figure 3. Chromatogram of maize root spiked with (1) 0.4 mg L^{-1} of IAA ethyl esther, (2) 0.5 mg L^{-1} of 1-Namide, (3) 0.5 mg L^{-1} of IBA, (4) 0.5 mg L^{-1} of IPA, (5) 2 mg L^{-1} of 1-NAA, (6) 2 mg L^{-1} of 2-NAA, and (7) 2 mg L^{-1} of IAA.

micellar bile salt solutions, which indicate significant polydispersity of aggregate size and structure.[22]

Chromatographic micellar systems can be described by a three-phase equilibrium model showing the interactions of the solute with the micelle and the stationary phase.[23,24] There are two main equilibria: a reversible equilibrium of the solute in the bulk solvent mobile phase with the stationary phase sites and a reversible equilibrium of the solute in the bulk solvent mobile phase with cholic acid within the micelle of the mobile phase. The model relates capacity factor to micellar mobile phase concentration and is expressed as

$$1/k = K_{SCh}/k_S[Ch] + 1/k_S$$

in which k is the capacity factor, [Ch] is the cholic acid concentration in the mobile phase, K_{SCh} is the association constant of the solute−cholic acid, and k_S is the retention factor of the free solute. A plot of $1/k$ vs [Ch] gave the slope K_{SCh}/k_S and an intercept $1/k_S$, whose quotient is the solute micelle association constant (K_{SCh}). The reciprocal of the intercept is the retention factor of the free solute, k_S.

Increases of cholic acid concentration give decreases of retention times; consequently, the capacity factors of all solutes, based on the pseudophase MLC chromatography,[25] were as expected. The curve that expresses the reciprocal of the capacity factor and the cholic acid concentration was linear.

The optimum concentration of cholic acid, $[Ch]_{opt}$, for the resolution of the compounds was obtained from K_{SCh} and k_S by using the equation $pCh_{opt} = \log K_{SCh} + {}^1/_2 \log(k/k_S)$.[24] We calculated the mean cholic acid concentration of 10.4 mM from this data. Experimentally, the mobile phase concentration of cholic acid of 10.4 mM was found to be the optimum for resolving five of the plant hormone compounds, but not for 1-NAA and 2-NAA. The resolution of structural isomers of NAA was achieved by varying the acidity of the mobile phase by adding 10^{-4} M salicylic acid. We chose salicylic acid because it is soluble in tetrahydrofuran. Varying the salicylic acid in the mobile phase within a range of dilutions of the 10^{-4} M stock solution from 2 to 10% increased the retention of both 1-NAA and 2-NAA; at higher percentages, however, retention progressively decreased. Consequently, we chose the 10% dilution of salicylic acid stock solution to resolve these compounds.

Table 1. Retention time (t_R, s.), Peak Width ($W_{0.1}$, s), Plate Counts (N), Plate Heights (H, mm), Reduced Plate Height (h), and Asymmetry Factor (B/A) for the Plant Growth Regulators in 10 mM Cholic Acid in THF and 10 mM SDS in Water

Cholic Acid/Spherisorb S5-NH_2

	IAA ethyl ester	1-Namide	IBA	IPA	1-NAA	2-NAA	IAA
t_R	205	240	475	550	1100	1075	1330
$W_{0.1}$	10	25	50	50	75	100	75
N	7789	1708	1673	2242	3987	2142	2973
H	0.003	0.015	0.015	0.011	0.006	0.012	0.008
h	0.007	0.032	0.033	0.025	0.014	0.026	0.019

SDS/R-CN Lichrospher

	IAA	1-NAA	IPA	2-NAA	IBA	1-Namide	IAA ethyl ester
t_R	275	475	590	600	800	820	1390
$W_{0.1}$	50	50	80	65	100	100	175
N	5.61	1673	1008	1579	1186	1246	1169
H	0.022	0.007	0.012	0.008	0.010	0.010	0.011
h	0.056	0.019	0.031	0.020	0.026	0.025	0.027

On the basis of these results, we selected the optimum conditions for both qualitative and quantitative analyses of the seven plant growth regulators. Figure 3 displays the chromatogram. All the calibration curves were linear between 0.09 and 40 ng for IAA ethyl ester, between 0.59 and100 ng for 1-Namide, between 0.67 and 100 ng for IBA, between 0.30 and 100 ng for IPA, between 1.28 and 200 ng for 1-NAA, between 0.52 and 200 ng for 2-NAA, and between 0.45 and 200 ng for IAA.

Efficiency. We studied the efficiency of normal-phase LC using reversed cholic acid micelles as the mobile phase. Table 1 shows the retention times, peak widths, plate counts, plate heights, and reduced plate heights for the 10 mM cholic acid in tetrahydrofuran reversed micelle mobile phase and for the 10 mM SDS

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normal micelle mobile phase. The reverse order of the retentions of the seven plant hormones obtained by the two chromatographic systems supports well the hypothesis that reversed cholic acid micelles exist in the mobile phase. Consider, for example, the data in Table 1 that refer to IAA with the amino-bonded stationary phase. The retention time of IAA was longest, because it has high affinity for the stationary phase and low affinity for the mobile phase ($K_{SCh} = 3.05$). On the contrary, when alkane nitrile was the stationary phase and SDS the mobile phase, the retention time of IAA was shortest because affinity was low for the stationary phase and high for the mobile phase. The efficiency of cholic acid mobile phase systems in this study was higher than that of SDS normal micelle mobile phase systems.

The relatively poor efficiency of reversed-phase micellar liquid chromatography is caused by poor mass transfer properties of the stationary phase, due mainly to poor wetting of the stationary phase.[26] In systems that use conventional reversed micelles in normal phase, the polar solutes are localized in the hydrophilic core, and the inefficiency observed is caused by the slow transfer step out of the micelle.[19] On the contrary, the cholic acid structure is different from that of conventional detergent monomers that have a hydrophilic head group and a long hydrophobic tail. The molecular conformation of bile acid gives a hydrophobic surface on one side and a hydrophilic surface on the other. Consequently, cholic acid molecules in nonpolar solvents form reversed micelles, stable amphiphilic assemblies that are characterized by having a polar core region with grouped heads and a hydrophilic tail that also has a proximal polar core region of grouped heads, a distal hydrophilic tail segment, and a nonpolar continuous phase. There are no binding sites analogous to those of SDS in the smaller, more rigid cholic acid micelles and their polar compounds solubulized by favorable interactions with the hydrophilic surfaces of the cholic acid micelles that facilitate the mass-transfer step into the interior of the reversed micelles and increase MLC efficiency. Normal micelles of the SDS mobile phase, on the other hand, reduce efficiency.

Extraction and Analysis of Maize Root Samples. Plants produce a vast array of secondary metabolites; consequently, plant extracts, the starting points for plant hormone analyses, are exceedingly complex multicomponent mixtures. Plant hormones occur only in trace amounts; for analysis, they are usually separated from organic extracts of homogeneized plant tissues. They are usually partitioned chemically before analysis by classical methods or before being purified by modern chromatographic techniques.

In this present work, maize root samples were spiked prior to extraction by immersing them in a methanolic solution of the seven plant growth regulators. The extraction by methanol is described by several authors.[1,2,27−29] The initial purification step after extraction of plant tissues partitions the extracts between an aqueous phase and an immiscible organic solvent such as ethyl acetate. The compounds separate according to their different solubilities. The separation technique used organic solvents and aqueous buffers with different pH values. The seven plant hormones were finally purified by combining solid-phase extraction with a new partition using diethyl ether. Finally, the samples were subjected to the LC procedure. Table 2 displays the recoveries, together with the analytical characteristics of the method for the plant hormone-spiked plant samples.

Table 2. Analytical Characteristics and Recovery of Plant Hormones from Spiked Plants

compound	D_L[a] (μg/g)	C_Q[b] (μg/g)	taken (μg/g)	recovery (%)	%RSD[b]
IAA ethyl ester	0.11	0.38	4.0	97.75	0.78
			2.0	93.0	2.15
			1.0	93.0	3.22
1-Namide	0.32	1.08	9.0	93.0	4.30
			6.0	95.15	1.94
			3.5	92.85	4.58
IBA	0.10	0.32	10.0	112.7	8.25
			4.0	116.7	4.92
			1.0	99.0	1.01
IPA	0.17	0.55	10.0	108.5	4.15
			4.0	109.2	3.89
			1.0	104.0	1.92
1-NAA	0.26	0.88	20.0	87.25	10.03
			10.0	92.5	2.70
			4.0	97.5	2.56
2-NAA	0.80	2.00	20.0	84.55	7.75
			10.0	91.5	1.64
			4.0	101.25	1.23
IAA	0.08	0.28	10.0	108.5	0.46
			4.0	110.0	4.54
			1.0	105.0	4.76

[a] Detection limit (signal-to-noise ratio, $n = 3$). [b] Quantification limit (signal-to-noise ratio, $n = 10$). [c] $n = 3$.

CONCLUSION

This application of reverse micelles of cholic acid for normal-phase chromatography demonstrated the usefulness of cholic acid as mobile phase for chromatographic separation of plant hormone samples. The inverted order of the retention times of the seven plant hormones, given by analytical systems that employed cholic acid as the mobile phase, when compared with the retention times given by similar analytical systems that employed SDS normal micelles as the mobile phase, supports well the hypothesis that reversed cholic acid micelles form in the mobile phase. Cholic acid micelle systems gave higher efficiencies than those obtained when normal SDS micelles were used as the mobile phase in similar systems.

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