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Micellar liquid chromatography of plant growth regulators detected by derivative fluorometry[1]

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Abstract

A micellar liquid chromatographic method with fluorimetric detection was developed for the determination of the plant growth regulators indol 3-yl acetic acid (IAA), 2-(1-naphthyl) acetic acid (1-NAA), indol 3-yl propionic acid (IPA), 2-(2-naphthyl) acetic acid (2-NAA), indol 3-yl butyric acid (IBA), 2-(1-naphthyl) acetamide (1-Namide) and indol 3-yl acetic acid ethyl ester (IAA ethyl ester). Extraction of plant hormones was performed with methanol, followed by purification with ethyl acetate, solid-phase extraction and again partitioning against diethyl ether. Sodium dodecylsulphate (10 m*M*) is used as mobile phase to elute the compounds in a maximum run-time of 23 min. Partially or unresolved peaks are separated by calculation of the 1st derivative chromatogram. Detection limits are between 0.30 $\mu g\ g^{-1}$ and 1.10 $\mu g\ g^{-1}$.

Keywords: Indoles; Plant hormones

1. Introduction

Molecules involved in plant growth regulation and development have received increasing attention over the past decade. Collectively these molecules are referred to as plant growth regulators or plant hormones. Plant hormones are secondary metabolites that are generally segregated into five groups, i.e. auxins, abscisins, cytokinins, gibberellins and ethylene [1,2]. These plant hormones are present in tissues at the ng g^{-1} level and the difficulties associated with the analysis of extracts are severe because it is essential to distinguish the compound of interest from a vast array of impurities; thus, purification of extracts prior to analysis is essential.

Physicochemical assays, particularly liquid chromatographic (LC) [1–10] and gas chromatographic (GC) [1–3,11,12] methods, and immunoassays [1–3,13] are used for quantitative determination. LC is used with highly selective detectors (e.g. fluorimetric detection) or extensively purified samples. LC is important for IAA and related indoles, the natural fluorescence of which allows for sensitive and selective detection.

Usual LC methods use an aqueous–organic mobile phase, but they may lack sufficient specificity for accurate quantitation unless extensive sample purification is performed prior to analysis. Recently micellar liquid chromatography has expanded the scope of LC. Liquid chromatographic secondary chemical equilibria, e.g. solute–micelle association,

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provide an additional mechanism to control retention and selectivity and can be used to optimize a separation. Partitioning is a dynamic process which is characterized by various rate constants. The first partitioning theory for a solute between a micellar and an aqueous phase in liquid chromatography is accredited to Herries et al. [14]. The partitioning theory was applied to LC by Armstrong and Nome [15] who derived equations which account for the chromatographic behavior of the solutes eluted with a micellar mobile phase. Arunyanart and Cline-Love [16] expanded the model to obtain the equilibrium constant for a solute between the bulk aqueous phase and the micellar aggregate; Foley and May [17] presented a general model for secondary chemical equilibria.

In the present paper we report a liquid chromatographic method with fluorimetric detection using a micellar mobile phase for the determination of the plant growth regulators shown in Fig. 1, i.e. indol 3-yl acetic acid (IAA), 2-(1-naphthyl) acetic acid (1-NAA), indol 3-yl propionic acid (IPA), 2-(2-naphthyl) acetic acid (2-NAA), indol 3-yl butyric acid (IBA), 2-(1-naphthyl) acetamide (1-Namide) and indol 3-yl acetic acid ethyl ester (IAA ethyl ester).

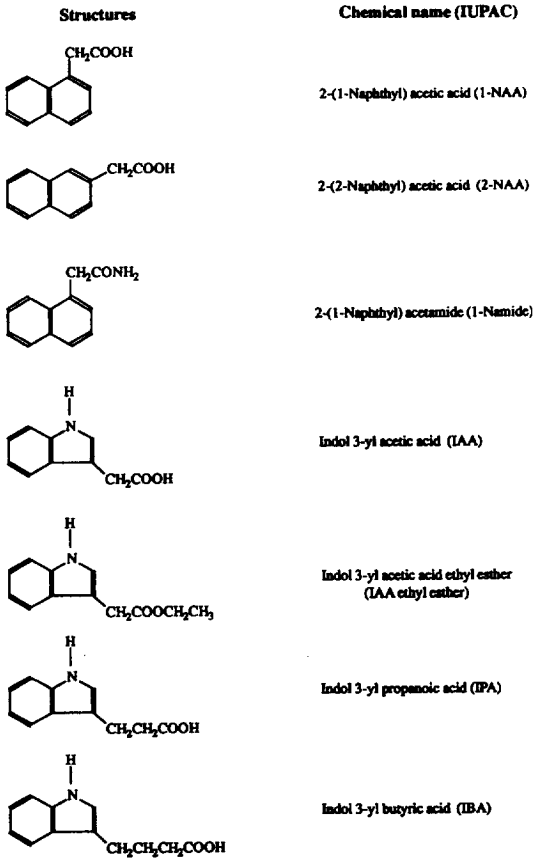

Fig. 1. Structures of the plant growth regulators.

2. Experimental

2.1. Chemicals and solutions

Sodium dodecyl sulphate (SDS) was obtained from Merck (Darmstadt, Germany). Methanol was gradient grade Lichrosolv (Merck), and ethyl acetate, diethyl ether, acetic acid from Merck (Darmstadt, Germany). The plant growth regulators indol 3-yl acetic acid (IAA), 2-(1-naphthyl) acetic acid (1-NAA), indol 3-yl propionic acid (IPA), 2-(2-naphthyl) acetic acid (2-NAA), indol 3-yl butyric acid (IBA) and indol 3-yl acetic acid ethyl ester (IAA ethyl ester), all of purity standard, were purchased from Sigma; 2-(1-naphthyl) acetamide (1-Namide) (purity standard) was purchased from Aldrich. The stock standard solution 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) was prepared by dissolving in methanol and stored at 4°C. Working solutions were prepared by dilution with methanol.

Solutions of 0.010 M SDS, 0.2 M acetate buffer pH 4.30 and pH 3.50 were prepared in doubly deionized water. The solutions were filtered through 0.2-μm Nylon membrane filters.

2.2. Plant material

Caryopses of *Zea mays L.* were germinated between cotton for eight days. Collection was at day nine when the apical, central and root parts were 1, 0.5 and 2.5 cm long, respectively. The samples were stored at −40°C until they were analyzed.

2.3. Extraction procedure

The plant material was homogenized using an ultrasonic probe (Ultra-Turrax T-25, Janke and Kun-

kel IKA-Labortiechnik) for 2 min with 30 ml of methanol–water (80:20, v/v). The homogenate was filtrated through a fritted glass Buchner funnel (coarse porosity) under reduced pressure, and the residues on the filter were rinsed with 20 ml of extraction solvent. The filtrate was concentrated by rotary evaporation below 40°C until the methanol has disappeared. The aqueous solution was adjusted to pH 2 with 1% aqueous acetic acid and partitioned against 20 ml ethyl acetate (10 min, under stirring). The organic layer was evaporated (below 40°C) to dryness in a rotary evaporator. The residue was dissolved in 1 ml of methanol–1% acetic acid (40:60, v/v) and applied on a Sep-Pak C_{18} cartridge.

Cartridges were preconditioned by washing with 1 ml of methanol (100%) followed by 1 ml of 1% acetic acid. The residue (1 ml, obtained as mentioned above) was then loaded onto the column and washed twice with 1-ml portions of methanol–1% acetic acid (30:70, v/v). The plant grown regulators were eluted with six 1-ml portions of methanol–1% acetic acid (65:35, v/v). The methanolic fraction was evaporated (below 40°C) and the aqueous fraction was adjusted at 3 ml with acetic acid 1% before partitioning (10 min, rotative stirring) against 3 ml of diethyl ether. The organic layer was evaporated (below 40°C) and the residue was redissolved in 2 ml of methanol.

2.4. Liquid chromatography

Measurements were performed with a Merck-Hitachi liquid chromatograph (Darmstadt, Germany) consisting of a L-6200 pump, AS-4000 autosampler, L-4250 UV–Vis detector and D-6000 interface. Integration was made with a PC/AT computer and the instrumental parameters were controlled by Hitachi-Merck HM software. A Perkin-Elmer LS-50 fluorescence detector (Beaconsfield, UK) placed in series with and after the UV–Vis spectrophotometer, was equipped with a xenon discharge lamp and two monochromators. Software Fluorescence Data Manager (FLDM) (LC program) and an RS232C interface were used to send the data to an external computer. For graphical recording, a NEC Silenwriter 2 S60P laser printer was connected to the computer.

The plant growth regulators were analyzed using an analytical column R-CN Lichrospher (Merck) (12.5 cm×4.6 mm; 5 μm alkyl nitrile bonded-phase packing material). The injection volume was 10 μl and the flow-rate was 1 ml min^{-1}. For fluorimetric detection the excitation wavelength was 281 nm and the emission 340 nm. The mobile-phase composition is detailed in Table 1. The peak-area response was measured at retention times of IAA (4.73 min), 1-NAA (8.60 min), IPA (10.35 min), 2-NAA (11.90 min), IBA (13.93 min), 1-Namide (15.30), and IAA ethyl ester (23.22 min). A linear regression curve of concentration versus peak area was calculated.

3. Results and discussion

Micellar liquid chromatography (MLC) provides an additional mechanism for the control of retention and selectivity sand can be used to optimize a separation. Chromatographic selectivity and eluent strength can be controlled by micelle concentration and surfactant type in the mobile phase. The negatively charged surfactant SDS was selected as mobile phase to increase the separation of the ionic solutes.

In terms of stationary-phase selection in MLC, it is important to select a phase which provides sufficient but not excessive retention for a given sample and surfactant. From previous experiences a relative

Table 1
Mobile phase composition

Time (min)	% SDS 0.010 *M*	% Acetate buffer 0.2 *M*, pH 4.30	% Acetate buffer 0.2 *M*, pH 3.50
0.0	80	20	0
8.0	80	20	0
8.5	80	0	20
30.0	80	0	20
32.0	80	20	0

lack of interaction of the compounds with a C_{18}-bonded stationary phase was observed when SDS was used as the aqueous mobile phase. Thus, an alkyl nitrile column, which has a different polarity, was selected.

The concentration of surfactant plays a key role in separations and the optimum surfactant concentration is largely determined by the magnitude of the solute–micellar interactions, i.e. by the solute–micelle association constant or related parameters. Solute retention in micellar liquid chromatography is given by [18]:

$$\frac{1}{k} = \frac{K_{sm}}{k_S}[M]\frac{1}{k_S}$$

A plot of $1/k$ vs. [M] yields a slope K_{sm}/k_S and intercept $1/k_S$ whose quotient is the solute–micelle association constant (K_{sm}). The reciprocal of the intercept is the retention factor of the free solute, k_s. In the equation [M] may represent either the concentration of surfactant, [surf] or the concentration of micelle, [micelle]. The resulting constant, K_{sm}, is then understood to be per surfactant molecule or per micelle, respectively.

The retention data of the compounds were obtained at different sodium dodecyl sulphate concentrations in the aqueous mobile phase. The experimental capacity factors, k, for each solute were calculated in the usual way, from the retention data. The reciprocal of the capacity factors were plotted as a function of the surfactant concentration in the micelles, giving a linear relationship. In Table 2 are given the solute–micelle association constant, K_{sm}, and the free solute constant, k_S.

The solute–micelle data in Table 2 are used to predict the optimum surfactant concentration. The optimum concentration of the surfactant $[Surf]_{opt}$ was calculated from equation: $pM_{opt} = \log K_{sm} + 1/2\log(k/k_S)$ and the identity $[Surf]_{opt}$ = (aggregation number) $\times 10^{-pMopt}$ + CMC, where CMC is the critical micelle concentration, the CMC value for SDS was 8.1×10^{-3} M and aggregation number 62 [19]. Values of $[Surf]_{opt}$ are reported in Table 2, from these data a mean SDS concentration of 10 mM was obtained. This surfactant concentration is optimum with respect to selectivity, but not necessarily optimum with respect to retention.

The pH effect of the bulk micellar solution was found to be of fundamental importance in determining the retention. With neutral or basic mobile-phases the compounds elute at 100 s with exception of IAA ethyl esther and 1-Namide which elute at longer times. At neutral and basic pH the less hydrophobic and negatively-charged acidic compounds elute very quickly (unretained) from the cyano column because of repulsion from the micelle and the negatively-charged modified stationary phase. In contrast, the largest values for the capacity factor are found in acidic solutions; as the analytes are non-dissociated their retention times are higher and the plant hormones can be resolved. A gradient elution, as indicated in Table 1, with acetate buffers of pH 4.30 and pH 3.50 was selected for the separation.

Fig. 2A illustrates the separation of plant hormones. As can be seen 1-NAA and IPA are

Table 2
Constants of the solute–micelle association and free solute

Compound	$K_{sm(micelle)}$	$K_{sm(monomer)}$ [a]	k_S	$[Surfactant]_{opt}$ (mM)
IAA	998	16.10	3.60	11.82
1-NAA	2531	40.82	7.27	9.40
IPA	3807	61.41	10.94	9.00
2-NAA	3300	53.23	9.70	9.10
IBA	5084	82.00	14.01	8.70
1-Namide	4764	76.83	13.97	8.90
IAA ethyl esther	10874	17.40	29.15	8.40

[a] K_{sm}(monomer) = K_{sm}(micelle)/aggregation number.

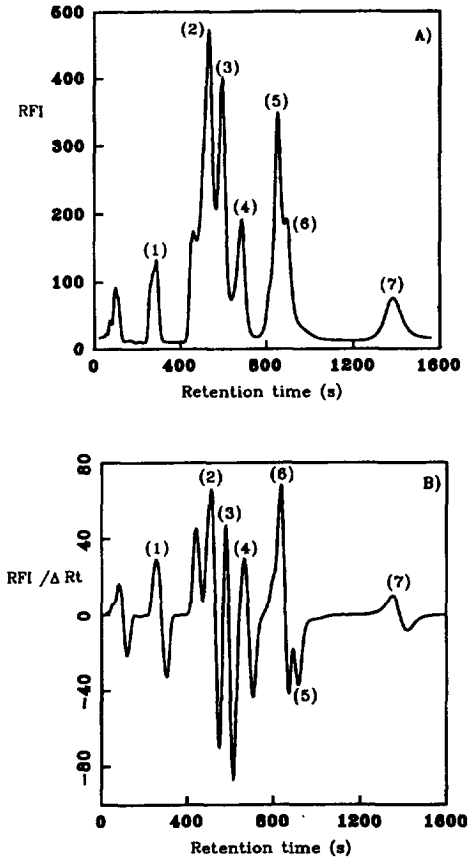

Fig. 2. (A) Chromatogram of maize root spiked with (1) 2 mg l^{-1} of IAA, (2) 4 mg l^{-1} of 1-NAA, (3) 5 mg l^{-1} of IPA, (4) 5 mg l^{-1} of 2-NAA, (5) 5 mg l^{-1} of IBA, (6) 5 mg l^{-1} of 1-Namide, (7) 5 mg l^{-1} of IAA ethyl ester; (B) 1st derivative chromatogram, concentrations as in Fig. 1A.

only partially resolved while IBA and 1-Namide co-elute. However, this problem can be overcome by using data treatment: the 1st derivative chromatogram was obtained [20,21] and the resultant is shown in Fig. 2B. The 1st derivative chromatogram totally resolves the 1-NAA and IPA peaks and the strongly overlapping IBA and 1-Namide peaks.

On the basis of the results the optimum conditions for qualitative and quantitative analysis of the plant growth regulators were selected. Calibration curves are linear in the range 0.01–4 μg/ml for IAA, 0.01–8 μg/ml for 1-NAA, 0.01–8 μg/ml for IPA, 0.02–8 μg/ml for 2-NAA, 0.01–20 μg/ml for IBA, 0.02–20 μg/ml μg/ml for 1-Namide and 0.05–20 μg/ml for IAA ethyl ester.

3.1. Extraction and bulk purification

Maize root samples were spiked prior to extraction with a methanolic solution of the plant growth regulators. Extraction was essentially as previously described [1,22,23]. Methanol is among the most commonly used extractants for IAA and related compounds. The initial purification step after the extraction of the plant tissues involves partitioning between an aqueous phase and an immiscible organic solvent such as ethyl acetate. The distribution of ionizable molecules, however, is influenced by their pK_a and the pH of the aqueous phase and they migrate into the organic phase when they are in an uncharged form. Amphoteric compounds tend to remain in the aqueous phase because they exist as dissociated structures regardless of pH. Acidic indoles are extracted by partitioning with ethyl acetate at pH 2.0.

A large number of methods is available for the purification of indoles in plant extracts. In practice it is often necessary to use more than one procedure to achieve an adequate degree of sample purity prior to analysis. The use of solid phases (C_{18}-coated silica support) packed into small disposable columns led to rapid sample preparation, good recoveries, and the requirement of only small solvent volumes. We used a combination of solid-phase extraction and a new partition against diethyl ether, giving optimum results.

After extraction and purification the samples were subjected to the LC procedure. Table 3 shows the results obtained for the plant hormone analysis. Recoveries range from 84 to 110% in maize root samples. The precision deduced from the R.S.D. values is consistently good, although the results have been affected by the high R.S.D. for the repeatability at high levels of IAA, 1-NAA and IPA. The detection limits, as defined by the lowest amount that gave a signal three

Table 3
Analytical characteristics and recovery of plant growth from spiked plants

Compounds	D_L^a (μg/g)	C_Q^b (μg/g)	Taken (μg/g)	Recovery (%)	R.S.D.[c]
IAA	0.30	0.90	10.0	98.57	5.79
			4.0	91.70	9.09
			1.0	87.00	3.45
1-NAA	0.80	2.60	20.0	97.51	5.32
			10.0	96.00	9.90
			4.0	93.85	1.76
IPA	0.30	0.90	10.0	93.31	7.20
			4.0	83.75	9.70
			1.0	90.00	0.00
2-NAA	1.00	3.40	20.0	89.50	5.90
			10.0	100.0	6.56
			4.0	90.00	0.00
IBA	0.30	0.90	10.0	96.40	0.00
			4.0	86.50	2.02
			1.0	110.0	0.00
1-Namide	1.10	3.60	20.0	102.70	2.54
			10.0	83.50	0.00
			5.0	92.50	0.00
IAA ethyl ester	1.10	3.80	40.0	100.0	0.00
			20.0	100.0	0.00
			5.0	100.0	0.00

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

times higher than the baseline noise, ranged from 0.03 μg/ml for IAA and IBA to 0.11 μg/ml for 1-Namide and IAA ethyl ester.

4. Conclusions

The combination of micellar chromatography with derivative fluorescence detection offers the possibility of separation and quantitative determination with adequate precision of seven plant growth regulators at the levels usually found in plant extracts.

Acknowledgments

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