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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PLANT HORMONES

II. DETERMINATION OF PLANT HORMONES OF THE INDOLE TYPE

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SUMMARY

A high-performance liquid chromatographic (HPLC) method was used to analyze a mixture of plant hormones —intermediates of the naturally occurring phytohormone, indole-3-acetic acid. The determination was performed on an analytical column (A, 250 × 2 mm) preceded by a short (50 × 2 mm) precolumn, and on a semipreparative column (B, 500 × 8 mm). The columns were filled with LiChrosorb RP-18 and eluted with water–acetic acid–ethanol (79.2:0.8:20) at flow-rates of 40 ml/h (A) and 180 ml/h (B). Detection was done by a UV detector (280 nm) and a fluorometric detector (excitation, 280 nm; emission, 360 nm) connected in series. The detection limit for indole derivatives was 5–20 ng with UV detection and tenths to several ng with the fluorometer. Qualitative analysis was done chromatographically and confirmed by UV spectroscopy and mass spectrometry. The substances were determined by the methods of standard addition and internal normalization. The standard deviation of a determination was $\leqslant 4.2\%$ (relative). The HPLC analysis was used to determine growth hormones of the indole type in extracts from cultures of soil bacteria.

INTRODUCTION

Growth substances of the indole type, auxins, are found in plants and microorganisms in minute concentrations. This biologically and phsyiologically important group of phytohormones is being intensively studied.

High-performance liquid chromatography (HPLC) is a progressive analytical and preparative method which permits a highly effective separation, isolation, identification and determination of mixtures of substances found in biological material. Ion-exchange HPLC with UV- and fluorometric detectors has been used to separate indole derivatives in biological material[1,2]. Reeve and Crozier[3–5] separated a mixture of indoles on Partisil 10 impregnated with 40% of 0.5 *M* formic acid, using a homemade chromatograph and a UV detector. Sweetser and Swartzfager[6,7] used an electrochemical and a fluorometric detector to determine indole-3-acetic acid by HPLC in plant tissue. Berg[8] recommended reversed-phase HPLC separation of indole sub-

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stances using a fluorometric detector, while Durley *et al.*[9] used a UV detector and HPLC in combination with gas chromatography (GC).

Balandrin *et al.*[10] reported the determination of indole substances such as tryptamine in plant tissue and in urine by reversed-phase HPLC, whereas Yamaguchi *et al.*[11] separated indole metabolites in urine. A rapid reversed-phase HPLC method for separation of tryptophan derivatives in biological fluids was developed by Yong and Lau[12]; Anderson *et al.*[13] used fluorometric and amperometric detection, Semerdjian-Ronquier *et al.*[14] and Shum *et al.*[15] used electrochemical detection and Adell *et al.*[16] employed ion-pair separation and fluorometric detection. Studies on the separation of indole substances by HPLC on silica gel have been reported by Svendsen and Greibrokk[17], on reverse phase by Jensen[18], Sandberg *et al.*[19] and Jensen and Junttila[20]. With the use of derivatization, Blakesley *et al.*[21] analyzed indole-3-acetic acid and its derivatives in plant materials by ion-pair HPLC using a fluorometric detection; the HPLC was combined with gas chromatographic–mass spectrometric (GC–MS) methods.

The present paper is based on our preceding study of the separation of plant hormones of the indole type[22]; we aimed at determining indole-3-acetic acid and its derivatives in extracts from soil microorganisms.

EXPERIMENTAL

Chemicals

The mobile phases used for separation of indole substances included ethanol (UV-spectroscopic purity), acetic acid (reagent grade; Lachema, Brno, Czechoslovakia) and doubly distilled water.

Standards of indole derivatives were of the highest available purity and included indole-3-aminopropionic acid, indole-3-acetic acid, indole-3-ethanol, indole-3-aldehyde, indole-3-pyruvic acid (Sigma, St. Louis, MO, U.S.A.), indole-3-lactic acid, 5-hydroxyindole-3-acetic acid (Fluka, Buchs, Switzerland), indole-3-acetamide (Koch-Light, Colnbrook, U.K.), indole-3-acetonitrile (Loba Chemie, Wien, Austria), indole-3-aspartic acid (Calbiochem, San Diego, CA, U.S.A.), N-acetylindole-3-aminopropionic acid, indole-3-propionic acid, indole-3-butyric acid (Lachema).

Microorganisms

A pure culture of the bacterium *Pseudomonas putida* strain K_2 from the collection of the Department of Microbial Ecology, Institute of Microbiology, Czechoslovak Academy of Sciences, was cultivated on a mineral medium containing 0.5% glucose and 0.01% L-tryptophan.

Sample preparation

A 72-h-old culture of *P. putida* was harvested by centrifugation, sodium diethyldithiocarbamate (Lachema) was added to the supernatant to prevent oxidation of indole-3-acetic acid and the solution was filtered through a bacterial filter. It was then concentrated to about half the initial volume on a vacuum evaporator at 35°C and proteins were precipitated by saturating the solution with ammonium sulphate.

Extraction

The solution was made alkaline (pH 8.0) with potassium hydroxide solution and extraction five times with 0.4 volumes of dichloromethane (alkaline extraction). It was then acidified to pH 2.5 with phosphoric acid solution and extracted in the same way (acid extraction). The dichloromethane was then evaporated to dryness *in vacuo* and the residue dissolved in ethanol. The resulting solution was used for HPLC analysis.

High-performance liquid chromatography

Analysis of indole-3-acetic acid and its derivatives was done on a Varian LC 8500 (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with two pumps which enable the use of a concentration gradient of the mobile phase. Separations and determinations were performed on an analytical column (A, 250 × 2 mm) preceded by a short precolumn (50 × 2 mm), and on a semipreparative column (B, 500 × 8 mm), all filled with LiChrosorb RP-18 (10 μm). The columns were eluted with water–acetic acid–ethanol (79.2:0.8:20) at a flow-rates of 40 ml/h (A) and 180 ml/h (B). Column temperature: 25°C. Column pressures: 22 MPa (A) and 23 MPa (B).

Detection was carried out by a variable wavelength UV detector Variscan operated at 280 nm with a flow-through cell (8 μl), connected to a fluorometric detector Varian Fluorichrom (excitation, 280 nm; emission, 360 nm). The amount of sample injected onto the analytical column using a Hamilton syringe was 1–5 μl of ethanolic solution. Samples of 50–100 μl were repeatedly applied on the semipreparative column by a special dosage valve. During the elution, fractions were collected in a 10-ml flask. These were then evaporated *in vacuo* and the residue was used for identification by MS and UV spectroscopy.

Indole derivatives were identified by comparing their retention volumes with those of reference standards, and were confirmed from their UV and MS spectra.

Individual components of the plant hormone mixture were determined by the methods of standard addition and internal normalization. The chromatograms were recorded by a Varian A 25 line plotter and evaluated by a Varian CDS 111 integrator. The reproducibility of analyses was evaluated by their standard deviation.

UV spectrometry

UV spectra of indole standards and samples were measured on a Varian Cary 118 C instrument: wavelength range, 210–350 nm; spectral bandwidth, 0.1 nm; cuvette width, 1 cm. All spectra were measured in water–acetic acid–ethanol (79.2:0.8:20) at 25°C, concentration range 10^{-5}–10^{-6} *M*. Correction factors used in the method of internal normalization were calculated from the values of the absorption coefficients.

Mass spectrometry

Mass spectra of reference indole substances and samples were measured on a Varian MAT 311 instrument at ionization energy 70 eV and current 1 mA. The temperature of ionizing source was 200°C; the temperatures of the direct inlet of individual substances are given in Table I. The measurements had a high resolution power and error of less than 5 ppm.

TABLE I

TEMPERATURES OF DIRECT INLET OF MEASURED SUBSTANCES

Substance	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
°C	115	185	40	80	80	135	100	80	100	30	70	40	130	80	65

RESULTS AND DISCUSSION

The stationary phase and the composition of the mobile phase used previously for separation of indole substances on several columns[22] were also well suited for the analysis of a mixture of indole-3-acetic acid derivatives by HPLC. The relatively low pH of the mobile phase solution (pH 3.5) suppressed the dissociation of hydrogen atoms from indolecarboxylic acids and had a favourable effect on indole derivatives, buffering to a greater or lesser degree the functionality of individual groups on the indole ring. The aromatic character of the analysed substance was thus more prominent in interactions with the stationary phase. The elution volumes of the separated substances are given in Table II.

TABLE II

RELATIVE RETENTIONS OF PLANT HORMONES OF THE INDOLE TYPE

Precolumn: LiChrosorb RP-18, particle size 10 μm, 50 × 2.0 mm I.D. Columns: LiChrosorb RP-18, particle size 10 μm, 250 × 2.0 mm I.D. (A), 500 × 8.0 mm I.D. (B). Flow-rates: 40 ml/h (A); 180 ml/h (B). Pressures: 22.0 MPa (A); 23.0 MPa (B). Detection: UV detector Variscan LC, wavelength 280 nm; fluorometric detector Varian Fluorichrom (excitation, 280 nm; emission, 360 nm). Mobile phase: water–ethanol–acetic acid (79.2:20:0.8).

Derivative	*A*		*B*	
	$r_{i,s}$	k'	$r_{i,s}$	k'
I 5-Hydroxyindole-3-acetic acid	0.24	1.00	0.26	0.45
II Indole-3-aminopropionic acid	0.45	2.80	0.32	0.80
IIi Indole-3-ethylamine	0.88	6.40	0.66	2.75
IV Indole-3-acetylaspartic acid	0.38	2.25	0.37	1.10
V Indole-3-acetamide	0.43	2.60	0.59	2.35
VI N-Acetylindole-3-aminopropionic acid	0.64	4.40	0.66	2.75
VII Indole-3-lactic acid	0.72	5.00	0.76	3.30
VIII Indole-3-acetic acid	1.00	7.40	1.00	4.56
IX Indole-3-carboxylic acid	1.01	7.50	1.10	5.25
X Indole-3-ethanol	1.02	7.60	1.51	7.55
XI Indole-3-aldehyde	1.07	8.00	1.65	8.30
XII Indole-3-acetonitrile	1.60	12.4	2.03	10.5
XIII Indole-3-pyruvic acid	2.50	20.0	2.12	11.0
XIV Indole-3-propionic acid	2.12	16.8	2.36	12.2
XV Indole-3-butyric acid	4.45	36.4	5.40	29.5
Retention volume of indole-3-acetic acid (ml)	5.54		67.8	

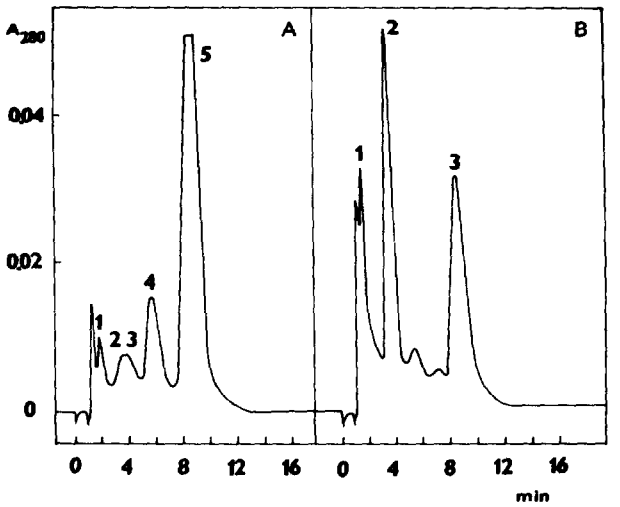

Fig. 1. Chromatogram of extracts from a bacterial culture of *Pseudomonas putida*. Column and precolumn: LiChrosorb RP-18. Mobile phase: ethanol–water–acetic acid (20:79.2:0.8). Flow-rate: 40 ml/h. Pressure: 22.0 MPa. Detection: Varian UV at 280 nm. A, Acid extract: 1 = unknown indole type compound; 2 = indole-3-acetamide; 3 = indole-3-aminopropionic acid; 4 = indole-3-lactic acid; 5 = indole-3-acetic acid. B, Alkaline extract: 1 = unknown indole type compound; 2 = indole-3-acetamide; 3 = indole-3-aldehyde.

TABLE III

MASS SPECTRA OF PLANT HORMONES OF THE INDOLE TYPE

Derivative	*Mol. weight*	*m/z (rel. intensity in %)*
I	191	146(100), 191(37M), 147(13), 91(9), 117(8), 145(7), 118(6), 89(6), 90(5)
II	204	130(100), 131(12), 77(12), 103(10), 204(4M), 129(4), 128(4), 102(4), 51(4)
III	160	130(100), 131(63), 77(19), 160(17M), 103(16), 30(16), 128(9), 132(7), 102(6), 51(5)
IV*	290	–
V	174	130(100), 174(23M), 77(17), 103(12), 131(11), 128(8), 102(7), 129(6), 51(6)
VI	246	130(100), 187(16), 131(12), 77(10), 103(9), 43(8), 246(4M), 129(4), 128(4), 51(2)
VII	205	130(100), 77(13), 205(11M), 131(11), 103(10), 129(6), 102(6), 128(5), 51(3)
VIII	175	130(102), 175(33M), 77(17), 131(12), 103(12), 102(8), 129(7), 51(7)
IX	161	161(100M), 144(97), 116(29), 89(26), 162(11), 145(11)
X	161	130(100), 161(23M), 77(16), 103(13), 131(12), 128(7), 102(6), 129(5), 51(5)
XI	145	144(100), 145(87M), 116(41), 89(32), 146(9)
XII	156	155(100), 156(78M), 130(47), 128(20), 101(17), 77(16), 51(11), 129(10), 102(10)
XIII	203	130(100), 129(29), 203(28M), 77(17), 157(16), 103(11), 102(14), 131(12), 128(9), 51(9)
XIV	189	130(100), 189(31M), 131(11), 77(9), 115(8), 103(8), 143(6), 129(3), 51(2)
XV	203	130(100), 203(21M), 131(12), 77(12), 143(11), 129(10), 103(9), 128(8), 51(4)

* For compound IV only a dicyclohexylammonium salt was available; the spectra yielded only ions due to the dicyclohexylammonium moiety.

An acidic extract from a culture of the soil bacterium *Pseudomonas putida* strain K_2, isolated from wheat rhizosphere, was separated into several fractions on a semipreparative column. The first fraction contained indole-3-aminopropionic acid, the second indole-3-acetamide, the third indole-3-lactic acid, the fourth indole-3-acetic acid and the fifth indole-3-aldehyde. After evaporation of the excess of mobile phase, the collected fractions were analyzed on the analytical column. To assess qualitatively the individual components, alkaline and acidic extracts of the soil bacterial culture were analyzed directly on the analytical column preceded by a precolumn which retained possible biological impurities (Fig. 1). Chromatographic identification of individual substances was confirmed by MS and UV spectroscopy or by biological tests.

The mass spectra of standard indole compounds were compared with those of substances isolated from the culture of *Pseudomonas putida* strain K_2 (Table III). Under the conditions of measurement all compounds formed a molecular ion. They could be divided into three groups: (a) substances with a CH_2 group on C(3) carbon and with no substituents in other positions of the indole ring; (b) substances with a CO group on C(3) and with no functional groups in other positions of the indole ring; (c) substances with a CH_2 group on C(3) and with other substituents on the indole ring.

Splitting of the molecular ion in the β position relative to the indole ring gives rise to an ion at m/z 130 (relative intensity 100% with the exception of indole-3-acetonitrile, C_9H_8N, XII). Other characteristic peaks are at m/z values 103, 102, 77 and 51. The spectrum of substance XII contains, in addition, an ion M − 1 (relative intensity 100%) and that of indole-3-ethylamine (III) also an ion at m/z 131 (relative intensity 63%, C_9H_9N) and at m/z 30 (relative intensity 16%, CH_4N).

The two possibilities of α cleavage relative to the CO group, *i.e.*, α and β cleavage relative to the indole ring, give rise to ions at m/z 144 and 116, respectively.

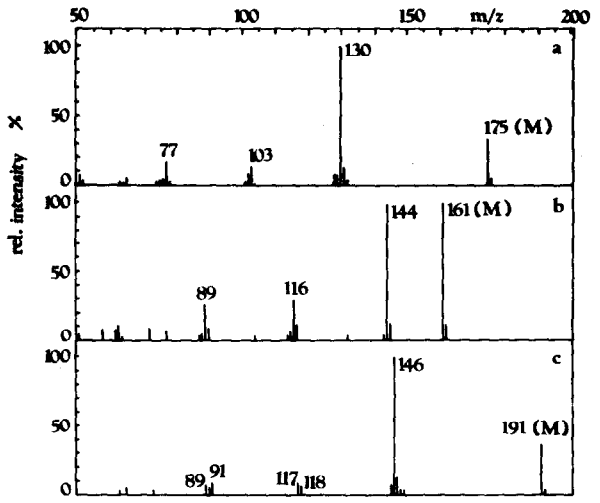

Fig. 2. Mass spectra of plant hormones of the indole type: a, indole-3-acetic acid; b, indole-3-carboxylic acid; c, 5-hydroxyindole-3-acetic acid.

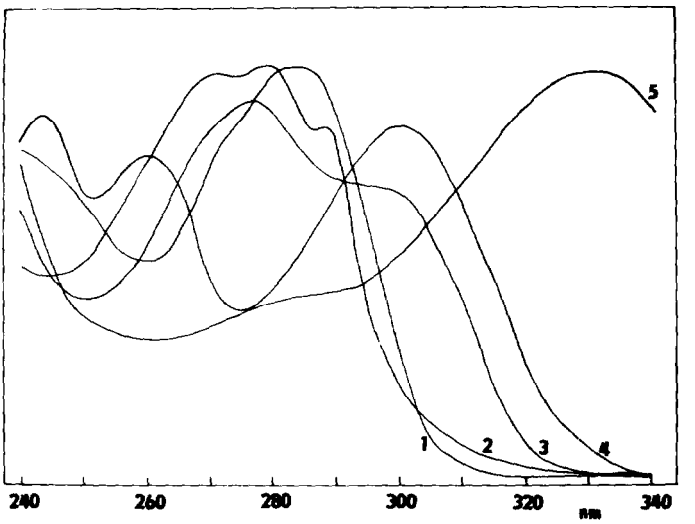

Fig. 3. UV spectra of plant hormones of the indole tyupe. Curves: 1 = indole-3-carboxylic acid; 2 = indole-3-acetic acid; 3 = 5-hydroxyindole-3-acetic acid; 4 = indole-3-aldehyde; 5 = indole-3-pyruvic acid.

TABLE IV

SPECIFIC ABSORPTION COEFFICIENTS OF PLANT HORMONES OF THE INDOLE TYPE

Derivative	*Absorption coefficient (l mol^{-1} cm^{-1})*			
	300 nm	*280 nm*	*260 nm*	*243 nm*
I 5-Hydroxyindole-3-acetic acid	4068	5369	3298	3217
II Indole-3-aminopropionic acid	1467	6716	4168	2080
III Indole-3-ethylamine	817	5309	3560	1821
IV Indole-3-acetylaspartic acid	408	3570	2580	1458
V Indole-3-acetamide	703	5588	3830	1977
VI N-Acetylindole-3-aminopropionic acid	770	4438	2696	1255
VII Indole-3-lactic acid	831	4862	3314	2134
VIII Indole-3-acetic acid	1285	6130	4640	3136
IX Indole-3-carboxylic acid	1763	4161	2314	3495
X Indole-3-ethanol	955	6228	4630	2104
XI Indole-3-aldehyde	14,199	8309	14,326	17,095
XII Indole-3-acetonitrile	306	4141	3485	1508
XIII Indole-3-pyruvic acid	3388 6327*	2782	2192	3550
XIV Indole-3-propionic acid	1166	4543	3072	1852
XV Indole-3-butyric acid	4481	6175	2559	2604
XIV Indole	130	5243	4999	2041

* Absorption at 330 nm.

TABLE V

DETERMINATION OF A MIXTURE OF PLANT HORMONES OF THE INDOLE TYPE

Derivative	*Amount (wt.%)*		*Standard deviation*	
	Weighed	*Determined**	*Absolute*	*Relative*
5-Hydroxyindole-3-acetic acid	6.65	6.3	0.27	4.2
Indole-3-aminopropionic acid	18.86	19.0	0.70	3.7
Indole-3-lactic acid	16.17	16.5	0.42	2.5
Indole-3-acetic acid	34.44	34.2	0.71	2.1
Indole-3-acetonitrile	23.88	24.0	0.55	2.3

* A total of eleven determinations was performed.

Elimination of CHN from the ion at *m/z* 116 yields an ion at *m/z* 89 in the case of indole-3-carboxylic acid (IX) and indole-3-aldehyde (XI).

The molecular ion of N-acetylindole-3-aminopropionic acid (VI) yielded, via elimination of ketene from the acetyl and β cleavage relative to the indole ring, an ion at *m/z* 130. The molecular ion of 5-hydroxyindole-3-acetic acid (I) gave rise, through β cleavage relative to the indole ring, to an ion at *m/z* 146 (relative intensity 100%, C_9H_8NO).

The mass spectra of compounds I, III, VII, IX and XIV have already been described[23]; characteristic spectra for individual groups are shown in Fig. 2.

The qualitative and quantitative analysis of indole-3-acetic acid derivatives also included the measurement of UV spectra of pure reference indole compounds. Quantitative analysis was performed by detecting the sample at several wavelengths. Fig. 3 shows the UV spectra of several indole substances. In the region of 300 nm, indole-3-acetic acid and indole-3-carboxylic acid have a very low absorption, whereas indole-3-aldehyde has a maximum absorption. Indole-3-pyruvic acid has a maximum absorption at 330 nm, whilst all other derivatives have practically no absorption in this region. At 265–270 nm indole-3-pyruvic acid has a low absorption, whereas indole-3-acetic and indole-3-carboxylic acids have a nearly maximal absorption. Table IV gives the values of the absorption coefficients used for the calculation of correction factors necessary for determination of a mixture of indole-3-acetic acid derivatives.

The analysis of a mixture of indole substances was verified by use of an artificially prepared mixture of some pure reference compounds. The results of the analysis by the internal normalization method are given in Table V. The reproducibility was evaluated by the standard deviations of measured values, which for individual compounds, were in the range currently encountered in liquid chromatography. To determine the sensitivity limit and minority substances in the mixture, we used the method of a standard addition. The detection limit for indole substances determined by the UV detector at 280 nm was 5–20 ng. The fluorometric detector, which operates with filters only, had a sensitivity limit 10–15 times higher, *i.e.*, in tenths to units of ng.

CONCLUSIONS

The HPLC method combined with UV and MS detection is suitable for determining and analysing a mixture of indole substances in extracts from bacterial cultures and in pre-purified extracts from plant material.

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