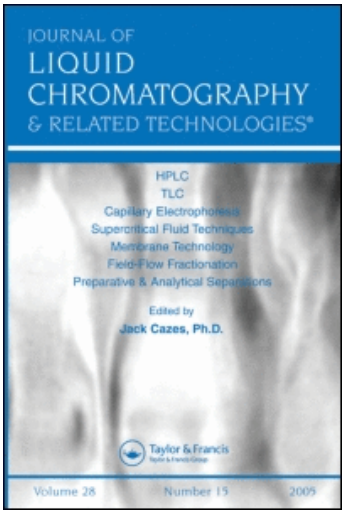

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Separation and Determination of Auxins by Capillary Electrophoresis

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SEPARATION AND DETERMINATION OF AUXINS BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

In this paper, the separation of fifteen plant auxins by capillary electrophoresis was investigated. A simple and efficient method was developed. The migration behaviour of the auxins under different separation conditions was studied. Optimum separation was obtained with cyclodextrin-modified borate-phosphate buffer. This method was applied to the determination of indole acetic acid (IAA), a well known auxin in plant samples, with cinnamic acid as the internal standard. The method is linear over the range of 0.4 - 8.0 mg/mL, with 82.4% recovery and a limit of quantitation of 0.4 mg/mL.

INTRODUCTION

Auxins are a group of plant hormones which play important roles in a variety of diverse plant growth and plant development responses, such as cell elongation, cell division and cell differentiation.[1] Among the auxins, indole-3-acetic acid (IAA) is the most ubiquitous compound in plants.[1]

Other natural auxins include indole acids, such as indole-3-butyric acid (IBA)[2] and indole-3-propionic acid (IPA),[3] various indole acid conjugates, and phenylacetic acid (PAA).[4] Synthetic auxins include 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorphenoxyaceticacid (2,4,5-T), 2,3,5-triiodobenzoic acid (TIBA), α-naphthalenic acid (NAA),[5] etc.

A commonly used method for the detection of novel plant hormones is bioassay, but the choice of suitable bioassays can be problematical, and it is now generally accepted to be unsuitable for quantitative work. In addition, several other methods have been reported for the determination of auxins, including high performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), gas chromatography-mass spectrometry (GC-MS),[6] and electroanalytical techniques.[7] As for the determination of auxins in plant samples, HPLC with fraction collection is generally used to purify the extracted samples before measurements. GC-MS is then used to identify the compounds, and the best method for the quantitive analysis is to use heavy isotope-labelled internal standards.[9-11] Although very precise and accurate results have been obtained by such methods, many special or costly items and chemicals such as HPLC preparative columns, a mass spectrometric detector and radioactive compounds are needed. Furthermore, the column is easily contaminated, and it takes a long time to perform the analysis. To date, there have been few papers concerning the separation of plant hormones by capillary electrophoresis, although this technique is rapidly gaining popularity due to its potential to achieve very high separation efficiencies.[8]

In this paper, the effects of different separation conditions on migration time, separation efficiency and resolution in the analysis of auxins were examined. An efficient and simple method for the separation of fifteen auxins by capillary electrophoresis (CE) was developed. The method was applied in the separation and quantitative analysis of IAA in shoot apices of Vanda Miss Joaquim, a species of orchid.

MATERIALS AND METHODS

Reagents and Materials

The fifteen auxins (See Fig. 1) used were purchased from Sigma Chemical Co. (St. Louis, USA). Shoot apices of Vanda Miss Joaquim were provided by Professor Chong-Jin Goh, Botany Department of National University of Singapore (Singapore). Methanol was purchased from Fisher Scientific (Pittsburgh, USA). Ethyl acetate was purchased from J. T. Baker (Deventer, Holland).

Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare buffers for CE. Other chemicals were obtained from Fluka (Buchs, Switzerland). All chemicals were of analytical reagent grade.

Apparatus and Conditions

CE was carried out with a laboratory-built system and with a commercial instrument. For the laboratory-built CE system, a Spellman power supply (Model RM15P10KD) was used (Plainview, New York, USA). A fused silica capillary of 50 cm effective length and 50 μm I. D. (Polymicro Technologies, Phoenix, AZ, USA) was used as the separation column. The peaks were detected by a Micro-UVis 20 detector (Carlo Erba, Milan, Italy) with wavelength set at 210 nm. The window for the on-column detection cell was made by removing a small section of the polyimide coating on the fused silica capillary. Data processing was performed with a Shimadzu (Kyoto, Japan) Chromatopac C-R6A integrator. Samples were injected into the capillary by gravity feed with an injection time of 5 seconds and an injection height of 10 cm. To obtain more reproducible data in the analysis of the real samples, the HP^{3D} CE system (Hewlett Packard, Palo Alto, CA, USA) was used for quantitative analysis. The same capillary was used in both systems. With the commercial instrument, samples were injected into the capillary by pressure (30 mbar, 20 seconds). The buffer solutions were sodium tetraborate - sodium dihydrogenphosphate solutions, modified by the appropriate cyclodextrin or cyclodextrins. Other conditions are described, where necessary, in the text.

Sample Preparation

Fifteen auxins were dissolved and diluted with methanol to obtain the final concentration of 27 μg/mL. The extraction procedure was modified from that described in a previous study.[12] Each sample of 30 g, fresh weight, was extracted with 80% methanol using a high speed blender maintained at 5°C. The extract was then filtered and the residue further extracted with cold methanol. 250 mL of cinnamic acid solution (200 μg/mL) was added as internal standard for quantification. 125 mL of IAA standard solution (200 μg/mL) was also added when recovery was determined. Methanol was removed from the pooled extract by evaporation, under reduced pressure, in a rotary evaporator. The aqueous concentrate was centrifuged for 10 min at 50,000 g. The supernatant was adjusted to pH 2.5 with 1 M HCl and partitioned 5 times with ethyl acetate. After adding molecular sieves (Type 4A) (Merck, Frankfurt, Germany), the acidic ether phases were kept overnight, then filtered and evaporated to dryness. The residues finally were dissolved in methanol in a 5 mL volumetric flask. The concentration of cinnamic acid in this methanolic solution was 10 μg/mL, and the concentration of the IAA standard was 5 μg/mL, when it was added to check the recovery.

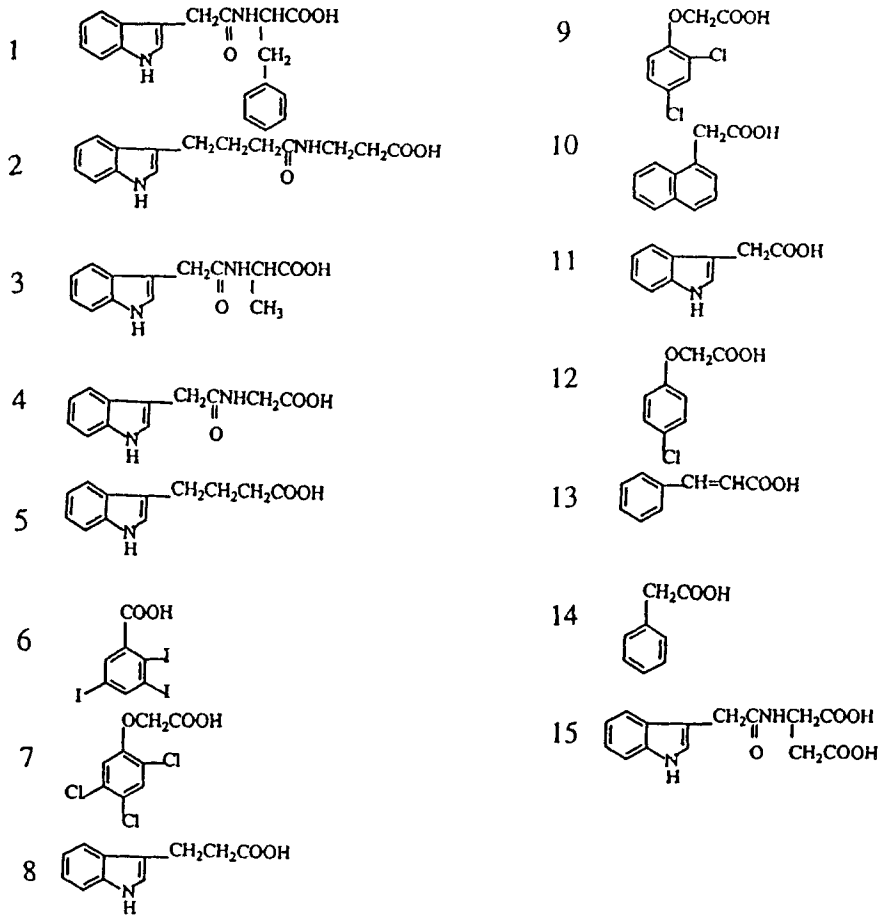

Figure 1. Structures of auxins. 1: Indole-3-acetyl-L-phenylalanine (IAPhe); 2: Indole-3-butyl-β-alanine (IBAla); 3: Indole-3-acetyl-L-alanine (IAAla); 4: Indole-3-acetylglycine (IAGly); 5: Indole-3-butyric acid (IBA); 6: 2,3,5-Triiodobenzoic acid (TIBA); 7: 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T); 8: Indole-3-propionic acid (IPA); 9: 2,4-Dichlorophenoxyacetic acid (2,4-D); 10: α-Naphthaleneacetic acid (NAA); 11: Indole-3-acetic acid (IAA); 12: p-Chlorophenoxyacetic acid (CPA); 13: t-Cinnamic acid (CA); 14: Phenylacetic acid (PAA); 15: Indole-acetyl-aspartic acid (IAAsp).

All the samples were stored at -4°C, and were passed through a Puradisc™ 25 TF filter (Whatman, UK) before CE analysis.

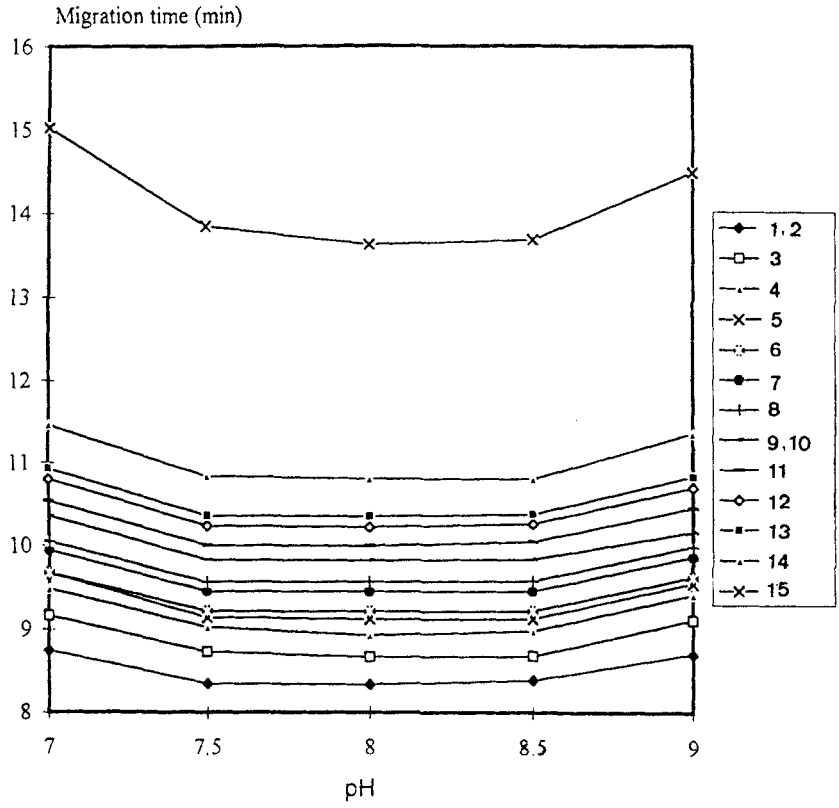

Figure 2. Influence of pH of buffer on migration times of auxins in CE system. Conditions: 25 mM borate-50 mM phosphate buffer; voltage, 15 KV; current, 23; UV (210 nm). Numbers as in Fig. 1.

Solution for Linearity Response

Seven solutions of different concentrations of IAA, which ranged from 0.4 to 6 μg/mL, were prepared from original compounds by dissolving and diluting with methanol. For each sample solution, a known amount of cinnamic acid was added at the concentration of 10 μg/mL.

RESULTS AND DISCUSSION

The names, abbreviations and structures of the auxins used in this paper are

listed in Figure 1. Among these compounds, eight are indole auxins, including IAA, IBA, IPA, and their amino acid conjugates, while others contain phenol groups. Therefore, all of them were amenable to detection in the UV range. All the compounds have low ionization constants (pK< 7) and, therefore, in high pH buffer solutions, they would exist as anionic compounds and are expected to be more easily separated by CE .

Effect of pH

Sodium borate and sodium dihydrohydrogen phosphate solution were used to prepare buffer solutions of pH 7 to 9. The effects of pH on migration times and resolution of auxins are shown in Figure 2 and Table 1, respectively. It was noted that, at pH >7, most of the auxins could be resolved using the borate-phosphate buffer except for two pairs of peaks, i.e. IAPhe and IBAla, and 2,4-D and NAA. However, increasing pH resulted in little change in the migration times and migration orders of the auxins.

Satisfractory separation was not obtained by varying pH alone. Nevertheless, slightly better separation and faster migration were observed in the pH range of 8.0-8.5. Therefore pH 8.4 was chosen to be the buffer pH in subsequent experiments.

The electrophoregram of auxins in 25 mM borate - 50 mM phosphate system at pH 8.4 is shown in Fig. 3. The migration behavior of the auxins could be attributed to differences in the electrophoretic flows of the anionic compounds, which would be in opposite direction to that of the electroosmotic flow, i.e. the ones with higher negative charges migrated slower.[13] The negative charges of the anionic compounds could be explained by inductive effects. IAAsp with two carboxyl groups possesses a higher negative charge than others, and migrated last.

The amino groups in other indole conjugates reduce their negative charges, and these compounds migrated faster than their indole acids. Among the indole acids, the migration order was based on the chain length of the side chains, i.e. less negative charge with longer carbon chains.

Among the conjugates, the migration times increased with the decrease of the isoelectric points of their amino acid groups.[14] As for the series of phenoxyacetic acids, their migration times decreased with the increase of the number of chloro substituents. TIBA, with iodine substituents, migrated faster than 2,4,5-T with chloro substituents. With conjugation of the double bond, cinnamic acid formed stable anions and migrated slower.

Table 1

Effect of pH on Resolution

Peak No.	pH				
	7	7.5	8	8.5	9
1,2	0	0	0	0	0
2,3	3.64	3.59	3.29	2.68	3.32
3,4	3.01	3.08	2.73	2.64	2.79
4,5	1.30	1.15	1.08	1.75	1.21
5,6	0.00	0.74	0.71	0.82	0.65
6,7	1.79	2.21	2.03	1.88	2.00
7,8	0.84	0.98	0.93	0.98	0.96
8,9	2.34	2.25	2.00	2.05	2.13
9,10	0.00	0.00	0.00	0.00	0.00
10,11	1.31	1.42	1.25	1.37	1.41
11,12	2.00	1.92	1.87	1.76	1.85
12,13	0.97	1.04	1.04	0.96	0.92
13,14	3.81	3.65	3.31	3.54	3.52
14,15	17.79	19.72	18.25	16.95	18.47

Effect of Cyclodextrins

Cylodextrins have been used extensively as modifiers in CE.[15-17] Enhancement of selectivity is attributable to differences in the stability of the cyclodextrin inclusion compounds, which could be influenced by many factors, such as hydrogen bonding, hydrophobic interaction, solvation power and the ability of the molecule to fill the cavity of the cyclodextrin.[13] In Fig. 4, the electrophoregrams obtained by adding 10 mM each of α-, β- and γ-cyclodextrins into the borate-phosphate buffer at pH 8.4 are shown.

It can be observed that the migration order and resolution for the auxins changed significantly when different cyclodextrins were used. It was, therefore, expected that, by varying the type and concentration of the cyclodextrins used as modifier, it might be possible to obtain satisfactory separation of the auxins.

Subsequently, the effects of varying the concentrations of α-, β- and γ-cyclodextrins on the migration times of auxins were investigated. The results obtained confirmed that the migration times of each auxins differed significantly when different cyclodextrins were used. However, in the cases of α- and β-cyclodextrins, the migration times remained almost constant when different concentrations of the cyclodextrins were used.

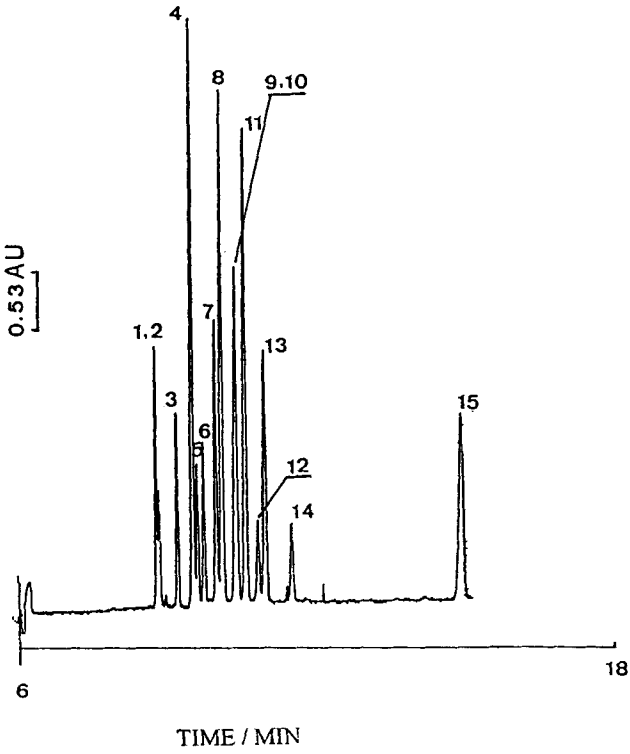

Figure 3. Capillary electropherogram of fifteen auxins without cyclodextrins. Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

For γ-cyclodextrin, the migration times were found to increase when higher concentrations of the cyclodextrins were employed. Among the three cyclodextrins, α- and γ-cyclodextrins provided better resolution than β-cyclodextrin. Since β-cyclodextrin is relatively less soluble in aqueous solutions than the other two cyclodextrins,[18] it is possible that the inclusion complexes between the auxins and β-cyclodextrin were not as stable as those for the other two cyclodextrins and, thus, the effect of β-cyclodextrin on migration behaviour was less significant. In Figures 5 and 6, the effects of different concentrations of α- and γ-cyclodextrins on the migration times are shown. In Tables 2 and 3, the resolutions between adjacent pairs of peaks, determined from the migration time data for the two cyclodextrins, are given. For α-cyclodextrin, it was noted that relatively better resolutions between adjacent pairs of peaks observed at

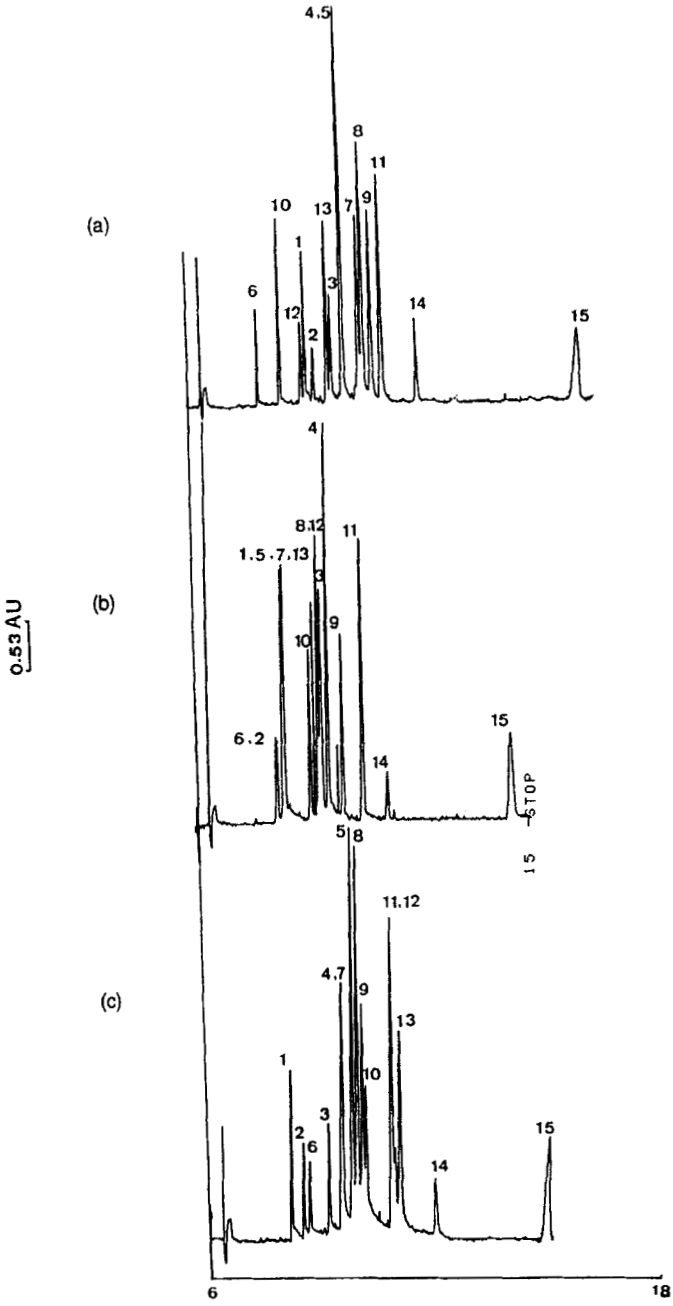

Figure 4. Capillary electropherograms of fifteen auxins using cyclodextrins as modifiers. a) with 10 mM α-CD; b) with 10 mM β-CD; c) with 10 mM γ-CD; Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

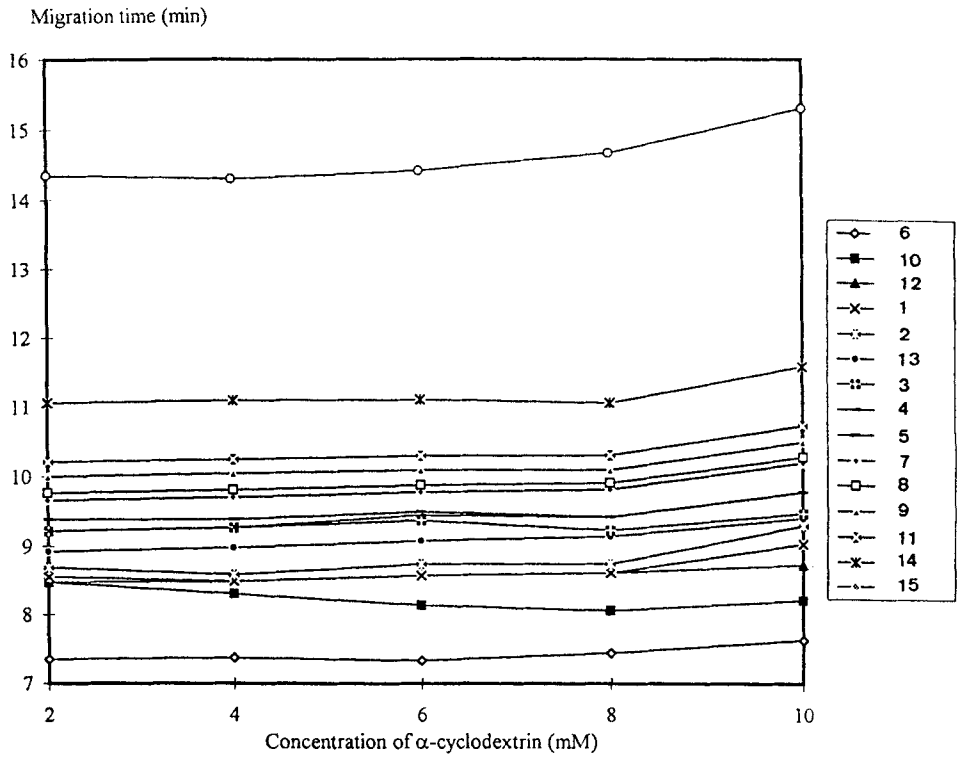

Figure 5. Influence of α-CD concentration on migration times of fifteen auxins in CE system. Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

around 6 mM of α-cyclodextrin whereas, for γ-cyclodextrin, a concentration of around 2 mM was found to provide better separation of the auxins. However, it was found that, when each of the cyclodextrins was used individually, satisfactory separation of the fifteen auxins could not be obtained.

In view of the significant differences in migration times when different cyclodextrins were used, modifier systems consisting of mixtures of the cyclodextrins were considered as potentially more capable of enhancing selectivity in the separation of the auxins. Borate-phosphate buffer, modified with different mixtures of α- and γ-cyclodextrins were thus evaluated for the separation of auxins by CE. Satisfactory separation of all the fifteen peaks could be obtained using buffers containing 5.8 mM of α-cyclodextrin and 1.8 mM of γ-cyclodextrin.

Table 2

Effect of α-CD Concentration (mM) on Resolution

Peak No.	Concentration of α-CD				
	2	4	6	8	10
6,10	12.50	11.85	8.31	8.54	7.96
10,12	0.00	2.09	4.65	6.69	6.27
12,1	0.96	0.00	0.00	0.00	1.50
1,2	1.51	1.11	1.12	1.49	1.89
2,13	2.47	3.63	2.21	3.86	3.55
13,3	2.98	2.50	2.31	0.81	0.72
3,4	0.00	0.00	0.86	1.59	2.75
4,5	1.38	1.08	0.35	0.00	0.00
5,7	2.06	2.23	2.95	2.95	3.27
7,8	0.98	0.76	0.77	0.74	0.69
8,9	1.59	2.03	1.74	1.65	1.84
9,11	1.39	1.65	1.59	1.82	1.81
11,14	6.70	6.78	5.95	6.06	6.46
14,15	18.37	16.70	14.88	10.11	14.56

Other modifiers such as bile salts and methanols were also investigated. However, none of these modifiers provided better separation of the auxins than that obtained with the above mixture of α- and γ-cyclodextrins.

In summary, the optimum conditions for the separation of the auxins were determined as: applied voltage of 15 kV, a buffer solution containing 25 mM sodium tetraborate - 50 mM sodium dihydrogen phosphate (pH 8.4), 5.8 mM α-CD, and 1.8 mM γ-CD. A typical electropherogram of the auxins obtained using these conditions are shown in Fig. 7.

Determination of IAA in Vanda Miss Joaquim

Vanda Miss Joaquim (Vanda Hookeriana Rchb.f. x V. teres) is a terete, monopodial ground orchid. The inflorescence is axillary, usually develops at the second node from apex.[12] IAA percentage in the shoot apices of this plant was determined by CE using the optimum conditions. Cinnamic acid (No cinnamic acid was detected in this plant) was chosen to be the internal standard.

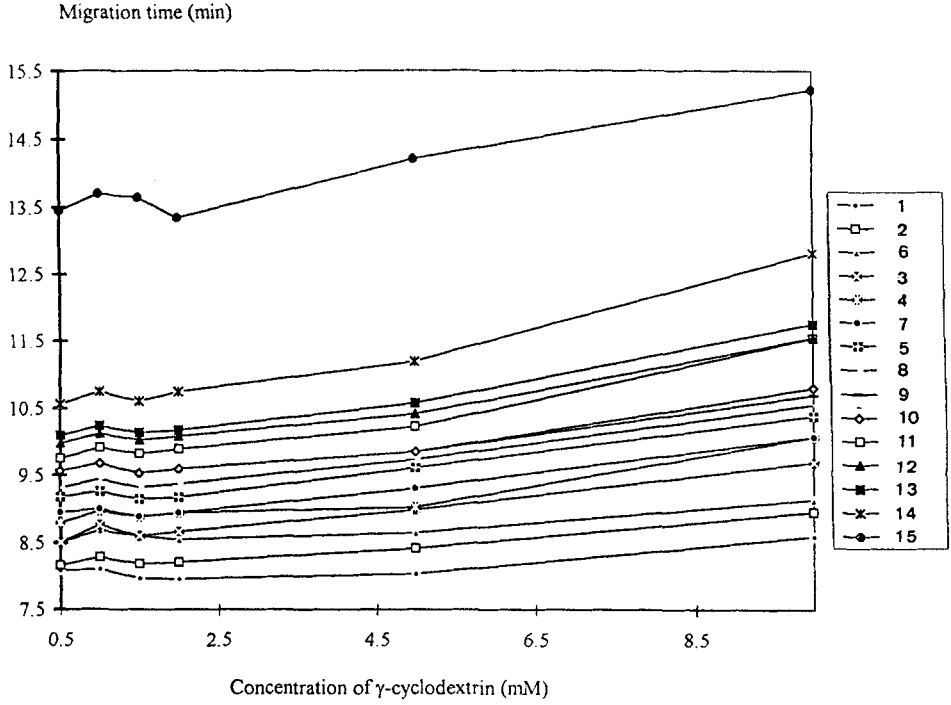

Figure 6. Influence of γ-CD concentration on migration times of fifteen auxins in CE system. Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

Calibration graphs for IAA and cinnamic acid (peak-area ratio, y, vs concentration, x μg/mL) were constructed in the range 0.40-6.00 μg/mL (The limit of detection of the CE system was 0.40 μg/mL for IAA). The regression equation of the plot and its correlation coefficients were determined as follows:

$$y = 0.140x - 0.066 \qquad r = 0.9934$$

The electrophoregram of the plant extract is illustrated in Figure 8. Using the photodiode array detector in the HP^{3D} CE system, IAA and cinnamic acid were identified by their migration time and UV spectra (shown in Fig. 9). By substituting the area ratios of these peaks for y in the above equations, the concentrations of IAA in the final extract solutions were obtained (see Table 4). The content of IAA in fresh weight samples was determined and the result is shown in Table 4. In addition, the recovery of IAA, determined using the procedure described in the sample preparation section, is also listed in this table.

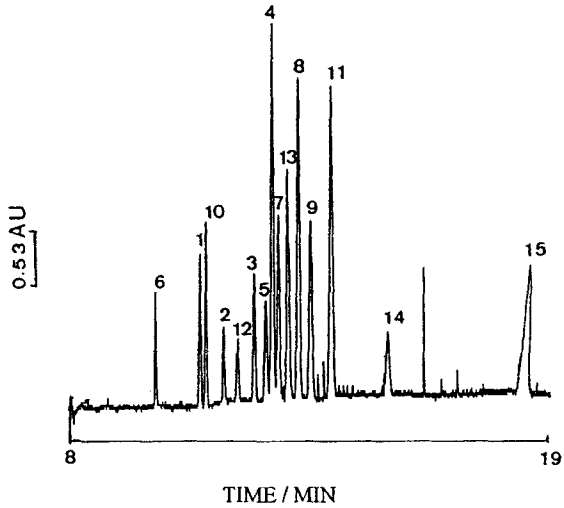

Figure 7. Capillary electropherograms of fifteen auxins obtained using the optimum conditions. Conditions: Buffer, 25 mM sodium tetraborate-50 mM sodium dihydrogenphosphate (pH 8.4), 5.8 mM α-CD and 1.8 mM γ-CD; UV detection at 210 nm; voltage, 15 KV; temperature, ambient. Numbers as in Fig. 1.

Table 3

Effect of γ-CD Concentration (mM) on Resolution

Peak No.	Concentration of γ-CD					
	0.5	**1**	**1.5**	**2**	**5**	**10**
1,2	1.00	2.10	2.67	3.16	3.17	3.26
2,6	3.33	4.28	4.08	3.79	0.00	1.49
6,3	0.00	0.79	0.00	1.20	3.79	4.52
3,4	2.13	1.94	2.38	2.64	2.45	2.88
4,7	1.34	0.33	0.00	0.00	0.00	0.00
7,5	2.23	2.38	2.19	2.03	2.13	2.15
5,8	1.17	1.61	1.55	1.82	1.12	1.06
8,9	2.22	2.06	1.75	1.97	1.45	0.92
9,10	0.00	0.00	0.00	0.00	0.34	0.61
10,11	1.58	2.04	2.48	2.45	2.98	3.96
11,12	1.87	1.68	1.62	1.67	0.00	0.00
12,13	1.02	0.95	0.84	1.03	1.24	1.11
13,14	3.75	4.18	5.49	4.32	6.02	6.13
14,15	15.27	16.44	19.99	15.77	15.89	17.82

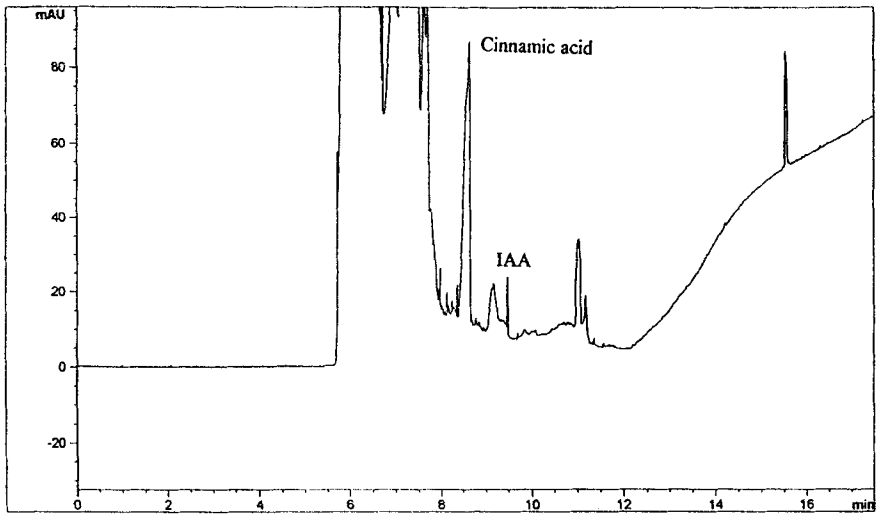

Figure 8. Capillary electropherogram of the extract of Vanda Miss Joaquim. Separation conditions as in Fig.7.

Table 4

Content of IAA in Real Sample (n = 3)

Sample	Area Ratio (IAA/Cinnamic Acid)	Concentration (μg/mL)	IAA Content (μg/g)	Recovery
Extract	0.1443	0.561	0.093	
Extract + 5 μg/mL IAA	0.7184	4.673	0.779	82.40%

IAA content in this kind of plant was previously determined by bioassay.[12] However, only a crude range of IAA concentration was obtained. The CE method described in this work is relatively more rapid and precise and, therefore, should be useful for quantitative analysis of auxins in plant samples.

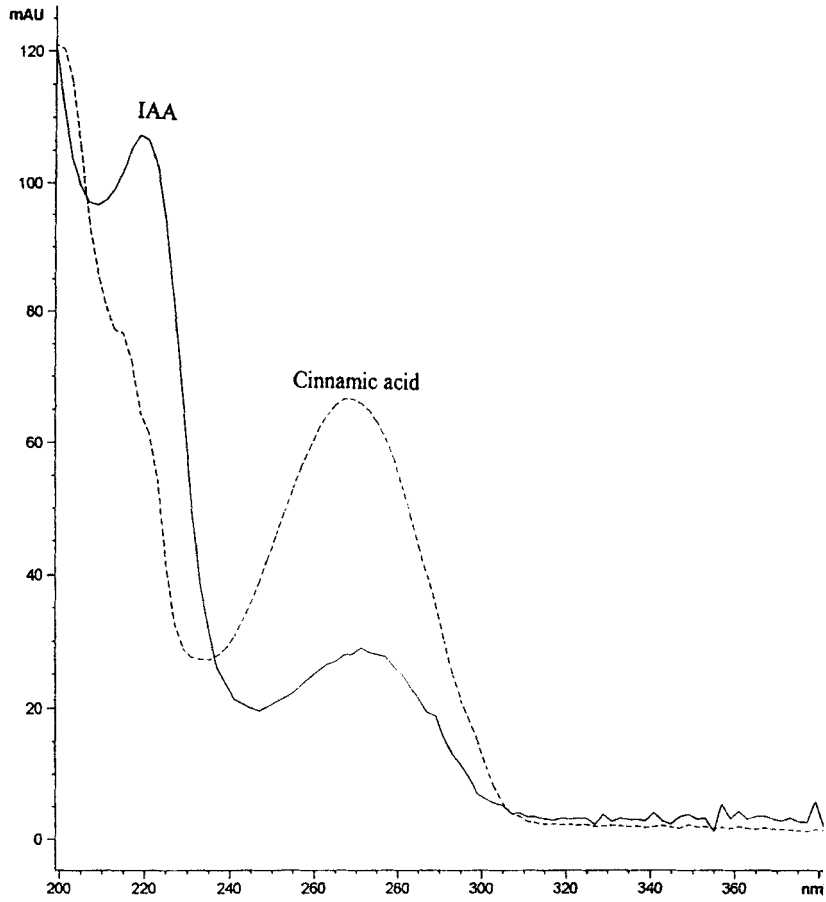

Figure 9. UV spectra of IAA and cinnamic acid in CE system. Wavelength: 190-380 nm.

CONCLUSION

In this study, a method for the separation and determination of auxins by capillary electrophoresis was developed. Optimum conditions for the separation of auxins were obtained.

A simple and reliable analytical procedure was described, which was demonstrated successfully as a suitable method for the determination of IAA in a real plant extract sample.

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