Quick and Concise Nonaqueous Capillary Electrophoresis Approach for Simultaneous Separation and Determining Five Phytohormones

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Summary: A new, quick and concise nonaqueous capillary electrophoresis approach has been matured to separate phytohormones involving indobutyric acid, indoleacetic acid, abscisic acid, 1-naphthaleneacetic acid and gibberellin. Using a running buffer composed of 85% methyl cyanide, 0.8% 1.0 M caustic soda and 20.0 mM methanol ammonium acetate, satisfactory separation of five phytohormone standards was achieved within 4 mins. The lowest detection limit was obtained for 1-naphthaleneacetic-acid (0.06 µg/ml) and the highest for gibberellin (1.71 µg/ml). The approach has been utilized for wheat bud samples with spiked 5 phytohormones using a solid phase extraction procedure, acquiring recovery percentage ranging from 95 to 102%.

Keywords: Nonaqueous capillary electrophoresis, Phytohormones, Buffer, Solid phase extraction.

Introduction

Phytohormones are compounds that make a chain of vital plant physiological reactions at various phases of plant evolution. Thus, it is very important to simultaneously determine plant phytohormone concentrations. So far, a number of analytical techniques for the determination of some phytohormones have been developed including radioimmunoassay (RIA) [1, 2], enzyme-linked immunosorbent assay (ELISA) [3], gas chromatography (GC) [4-6] and liquid chromatography (LC) [7-10]. Capillary electrophoresis (CE) was also applied to analyze some phytohormones [11-13] due to short analysis time, highly-effective separation, inexpensive analysis, and cheap sample and reagent consumption. Nonaqueous capillary electrophoresis (NACE) can provide sundry selectivities owing to various physicochemical characteristics of organic solvents [14-16].

Organic solvents present many amusing characteristics, such as various relative dielectric constant, as well as acid-base and solvation features, contributing to a vital adaptation on the ionisation & protonation extent. The pKa & zeta potential of capillary are greatly influenced by the background electrolyte property, ending in various electrophoretic and electroosmotic flow (EOF) mobilities, causing separation selectivity & efficiency. The lower polarity of organic solvents improves the hydrophobic analyte solubility, permits the compound separation with like electrophoretic mobility in aqueous media, and makes sure better detection-sensitivity in comparison with aqueous electrophoresis system. The most amusing property of nonaqueous capillary electrophoresis (NACE) covers widely organic solvents, whether in pure or mixture forms, which can lead to the development of a suitable buffering electrolyte system. NACE produces a lower electric current in comparison with aqueous capillary electrophoresis (CE) and a high electric field can be used.

As abscisic acid, indoleacetic acid, indobutyric acid, 1-naphthaleneacetic acid, as well as gibberellin are hydrophobic, insoluble in water, and easily soluble in organic solvents. NACE would be more favorable for their separation.

As far as we know, the detection of phytohormones has never been studied by NACE. A concise and quick NACE approach to the detection of abscisic acid, gibberellin, indoleacetic acid, indobutyric acid and 1-naphthaleneacetic acid has been developed.

Experimental

Reagents

Indobutyric acid, indoleacetic acid, abscisic acid, 1-naphthaleneacetic acid and gibberellin were

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bought from Beijing Biodee Biotechnology in China. methyl cyanide, methanol, caustic soda and ammonium acetate were bought from Beijing Chemical Factory in China. All reagents belonged to analytical grade. Plant samples were gotten by breeding wheat seeds in the circular tray coated with clean paper for 3 days. A sustaining dampness with tap water at 25°C and a restrained light circumstance of sixteen-hour brightness and eight-hour darkness were preserved. Wheat-buds were cut into tissues aground to be analyzed. Stock solutions of indobutyric acid (1280.00 μg/ml), indoleacetic acid (1080.00 μg/ml), abscisic acid (1180.00 μg/ml), 1-naphthaleneacetic acid (464.00 μg /ml) & gibberellin (1050.00 μg/ml) were presented in methanol and subsequently diluted with methanol to prepare the required concentrations. The operating buffers were concocted with blended 200 mM ammonium acetate, 1.0 M caustic soda, methyl cyanide and methanol.

Capillary zone electrophoresis system

All experiments were conducted on a CL2001 high-performance capillary electrophoresis (HPCE) apparatus (Beijing Cailu, China) allocated with UV detectors. The HPCE system was equipped with power supply (not more than voltage 30 kV) and N2000 chromatography workstation. Separation was performed on 48.5 cm (40 cm between inlet and detector)×75 μm I.D. fused silica capillary (Hebei Yongnian Optical Fiber Factory, China). Before first utilization, new capillary was pretreated through washing with 1 M caustic soda with 40 mins. Per day, capillary was flushed with 1.0 M caustic soda for 2 mins, succeeded using clean water for 2 mins, then separation buffer for 2 mins. Between two runs, capillary was flushed with 0.1 M caustic soda 2 mins, clean water 2 mins, separation buffer 2 mins. When not being used, capillary was rinsed with clean water 5 mins and dry-stored. Hydrodynamic injections (20cm high, 30s) were chosen to load samples. Wavelength for detection was set to 214 nm.

Sample preparation

The procedure for phytohormone solid-phase extraction out of wheat germs was revised from the approach presented by Hou and others [9] with minor modifications. Briefly, wheat germs were cut and turned into powder with liquid nitrogen, and then 3.0 g was accurately weighed. The weighed samples were being creamed off at 4°C for 12 hours with clean water. Extraction was being centrifuged at 4°C at 3000 rpm for 15 mins. The supernate got run immediately through a C_{18} cartridge preconditioned with 3 mL deionized water, succeeded with 3 mL methanol. Cartridge was rinsed with 1 mL 20% methanol with 0.1% (v/v) formic acid and the checked phytohormones were removed with 1mL 80% methanol. The eluent was vacuum-dried at room temperature and solved in 1.0 mL methanol to further analysis.

Results and discussion

Separation circumstances

Indobutyric acid, indoleacetic acid, abscisic acid,1-naphthaleneacetic acid and gibberellin are hydrophobic compounds, which were not easily soluble in water, but easily solved in organic solvents (Fig.1). Thus, nonaqueous capillary electrophoresis system was of great advantage to segregation. The limited detection in capillary electrophoresis is usually obtained at an extremely low wavelength, which will greatly limit to choose the organic solvent to serve as an electrophoretic medium. methyl cyanide and methanol are the best choice for UV detection because of their relatively low UV absorbance. After certain initial investigations, it was found that using a buffer with 20.0 mM ammonium acetate, 0.8% 1.0 M caustic soda and 85% methyl cyanide in methanol, all analytes were well resolved. The effects of some experimental parameters, like acetontrile percentage, 1.0M caustic soda percentage, ammonium acetate concentration and using voltage on separation, were comprehensively studied.

Firstly, the activity of methyl cyanide percentage was studied. Pure methanol or methyl cyanide was excluded as the former would result in long separation times and the latter would result in low ammonium acetate solubility. Fig. 2 generalized the activity of methyl cyanide percentage in methanol (75~87.5%) comprising 20.0 mM ammonium acetate and 0.8% 1.0 M caustic soda on the migration of the analytes. The migration times of analytes lessened with the rise of methyl cyanide percentage, but resolution ratio at two adjacent peaks would lessen when methyl cyanide percentage grew 75~87.5%. However, at the methyl cyanide percentage over 85%, the migration time would change a little. In addition, methyl cyanide percentage over 90% may cause current breakdown in separation owing to the precipitation of ammonium acetate in running buffer. Therefore, 85% methyl cyanide was selected.

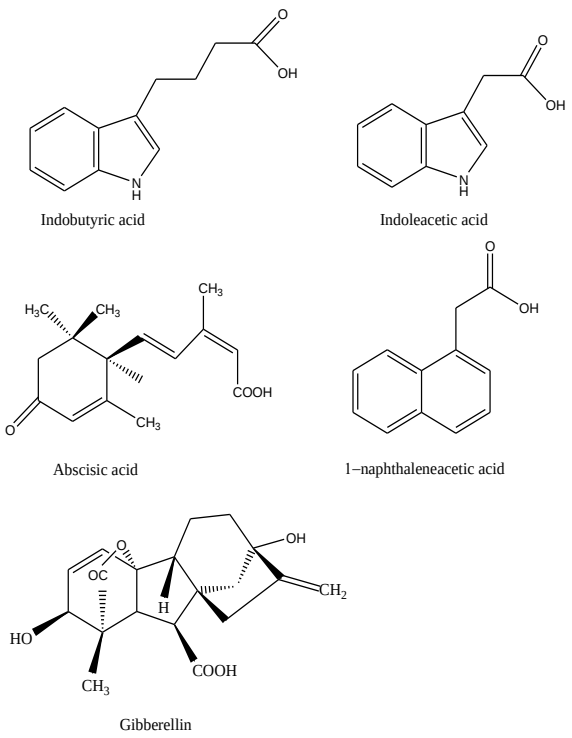

Fig. 1: Structure of the five phytohormones.

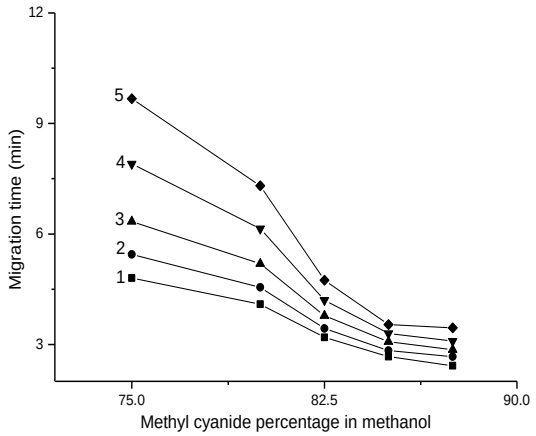

Fig. 2: Impact of methyl cyanide percentage on separation.

Peaks: 1= indobutyric acid, 2 = indoleacetic acid, 3=abascisic acid, 4=1-naphthaleneacetic acid and 5 = gibberellin. Capillary: uncoating fused silica capillary 48.5 cm (40 cm between inlet and detector) ×75 μm I.D.; UV detection at 214 nm; Hydrodynamics injection: 20 cm high, 30 s; Applied voltage at 25 kV; capillary temperature at 25℃. Buffer: 20 mM ammonium acetate and 0.8% 1.0 M NaOH with various percentage of methyl cyanide (from 75 to 87.5%).

The effect of changing the percentage of 1.0M caustic soda was also investigated. The acidity of the electrophoretic medium is a controlling factor as well as the separation of the ionizable analytes, as it ascertains the degree of ionization of each separate analyte in the nonaqueous capillary electrophoresis. In Fig. 3, when the percentage of 1.0 M caustic soda increased, the migration times of all analytes and resolutions among peaks increased due to the extent of ionization of analytes. In experiments, it was found that it may cause current breakdown due to bubble formation when 1.0 M caustic soda percentage remained over 2.0%. Therefore, 0.8% 1.0 M caustic soda was selected as the running buffer based on the test educts.

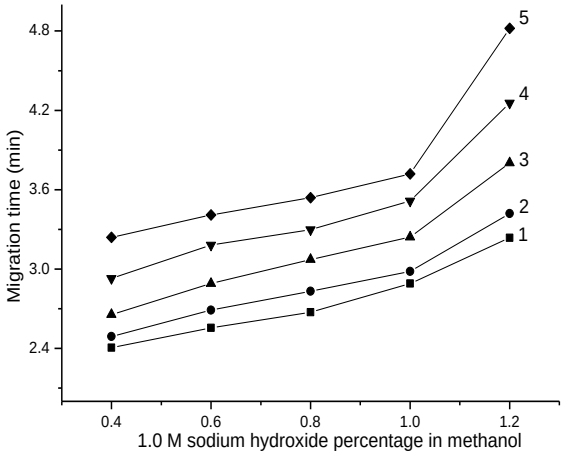

Fig. 3: Effect of 1.0 M NaOH percentage on the separation.

Peaks: 1=indobutyric acid, 2=indoleacetic acid, 3=abascisic acid, 4=1-naphthaleneacetic acid and 5 = gibberellin. Buffer: 20.0 mM ammonium acetate and 85% methyl cyanide with different percentage of 1.0 M NaOH (from 0.4 to 1.2%). Other conditions as in Fig. 2.

Since electrolytes have a large effect on separation parameters such as Joule heating, running current, and migration time of analytes, the effect of ammonium acetate concentrations of 15.0~25.0 mM on analyte separation was investigated. Fig. 4 shows that as the ammonium acetate concentration was increased, the migration times of analytes would rise. Ammonium acetate buffer with concentration of 20.0 mM was chosen because the peak shape could be maintained, a lower current (about 15 uA) and good resolutions among peaks.

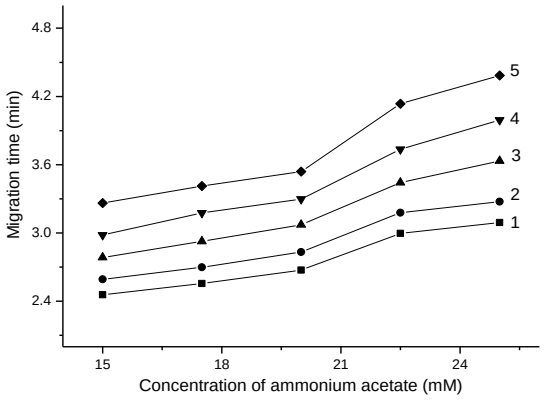

Fig. 4: Impact of ammonium acetate concentration on separation.

Peaks: 1= indobutyric acid, 2= indoleacetic acid, 3=abascisic acid, 4=1-naphthaleneacetic acid and 5=gibberellin. Buffer: 85% methyl cyanide and 0.8% 1.0 M NaOH with various ammonium acetate concentration (from 15.0 to 25.0 mM). Other circumstance seen in Fig. 2.

The impact of voltage (15~30 kV) on separation was researched in the experiment. High voltage was needed in capillary electrophoresis to lessen time for analysis, yet the voltage heightened the signal-to-noise ratio and lessen resolution between peaks.

At the low separation voltage, the peak width would increase. On the basis of the experiment, 25kV was selected as the best voltage, and a favorable result was achieved.

According to the above test educts, the best segregation was gotten with a running buffer solution including 85% methyl cyanide, 0.8% 1.0 M caustic soda and 20.0 mM ammonium acetate in the methanol at 25 kV. The specific electrophoregrams obtained under the optimal separation conditions of the standard mixed solution are shown in Fig. 5a. It is obvious that the analytes were liquated in less than 4 mins.

Approach validation

In optimal separation conditions, the concentration of the analyte is linearly related to the corresponding peak area. Three separate injections were performed at each calibration point. The educts achieved were generalized in Table 1. The calibration curve showed a sound linear relationship with the measured concentration. LOD was considered the minimum analyte concentration that produced a signal-to-noise ratio equal to 3. The test results were listed in Table 1. The minimum limit for detection was obtained for 1-naphthlcetic acid (0.06 µg/ml) and the highest for gibberellin (1.71 µg/ml). The detection limits of phytohormones (except gibberellin, partly due to its good water-solubility) of nonaqueous capillary electrophoresis method is slightly lower other capillary electrophoresis methods [12, 14] with direct UV detection and without on-line sample concentration.

The repetition rate for the migration time of all analytes in the optimum condition was studied by reduplicative injections (n = 6) of mixed standards at a concentration of 6.42 µg ml-1 for Indobutyric acid, 5.86 µg ml-1 for indoleacetic acid, 4.97 µg ml-1 for abscisic acid, 2.33 µg ml-1for 1-naphthlcetic acid and 38.01 µg ml-1 for gibberellin. The RSD of the migration times for Indobutyric acid, indoleacetic acid, abscisic, 1-naphthaleneacetic and gibberellins was 1.7, 1.9, 2.2, 2.1, and 2.6%, respectively. The repetition rate of peak areas gotten for Indobutyric acid, indoleacetic acid, abscisic, 1-naphthlcetic and gibberellins with RSD values (n=6) was 2.0, 2.2, 2.6, 2.5, and 3.1%, respectively.

Table-1: Regression data and limit of detection (LOD).

Chemical compound	Regression equation [a)]	Correlation coefficient	Linear range(µg/ml)	LOD [b)](µg/ml)
Indobutyric acid	**Y = 6307.2 + 18990.5 X**	**0.9996**	**25.68 - 0.4**	**0.13**
Indoleacetic acid	**Y = 3756.2 +20223.0 X**	**0.9993**	**24.43 - 0.37**	**0.10**
Abscisic acid	**Y = 17053.8 + 6378.8 X**	**0.9995**	**39.75 - 0.62**	**0.16**
1-Naphthaleneacetic acid	**Y = 4411.3 + 35661.4 X**	**0.9992**	**9.30 - 0.15**	**0.06**
Gibberellin	**Y = -3558.5 + 3020.9 X**	**0.9993**	**152.0 - 2.38**	**1.71**

a) Y and X referred to peak area and density (µg/ml) of analytes, separately.

b) LOD was reaped on the basis of the signal-to-noise rate of 3.

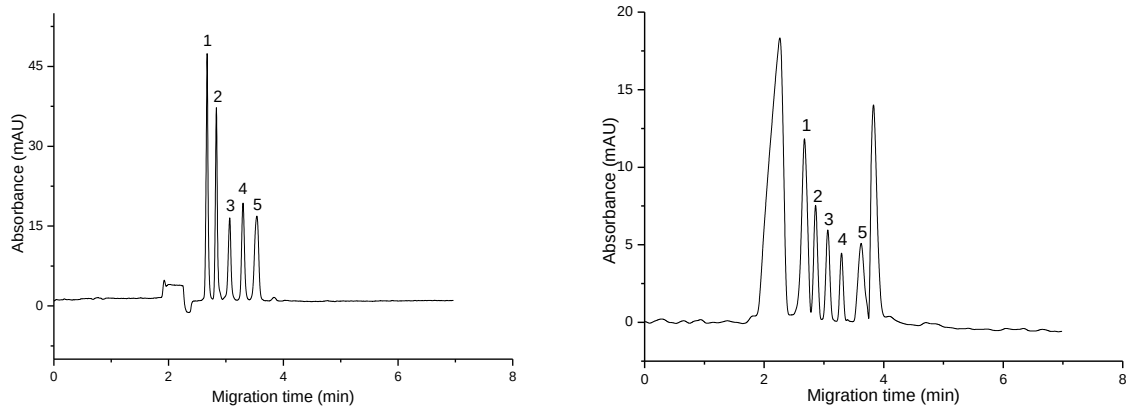

Fig. 5: Electropherograms of the standard mixtures & spiked wheat bud extracts.

(a) The standard mixture, (b) Spiked wheat bud extracts by the five phytohormones
Peaks: 1=indobutyric acid, 2=indoleacetic acid, 3=abascisic acid, 4=1-naphthaleneacetic acid and 5=gibberellin. Peaks were identified by standard addition method. Buffer: 85% methyl cyanide, 20.0 mM ammonium acetate and 0.8% 1.0 M NaOH. Other conditions as in Fig. 2.

Sample analysis

Five plant hormones in wheat germ after solid phase extraction (SPE) were analyzed by nonaqueous Capillary electrophoresis approach in optimum separation prerequisites. The new approach was detected by a standard addition method. The five phytohormones could not be detected in the wheat buds after solid-phase extraction due to their content beyond the limit of detection of this nonaqueous capillary electrophoresis method. An electropherogram of the spiked wheat bud samples extracts was shown in Fig. 5b. Five plant hormones were isolated from other interfering substances and discerned by migration time and standard addition. The average recoveries of wheat germ samples after SPE were determined by standard additive approach. The outcomes were shown in Table 2. Satisfactory average recoveries (in the range of 95~102%) demonstrate that nonaqueous methods are feasible approaches for the isolation and analysis of these five phytohormones.

Table-2: The average recoveries and RSD in the wheat bud (n=3).

Compound	Added amount (μg/ml)			Recovery (%)	RSD (%)
Idole-3-butyric acid	**12.84**	**6.42**	**3.21**	**97**	**2.1**
indoleacetic acid	**11.71**	**5.86**	**2.93**	**101**	**2.4**
Abscisic acid	**19.88**	**9.94**	**4.97**	**98**	**2.3**
1-Naphthaleneacetic acid	**4.65**	**2.33**	**1.16**	**102**	**2.3**
gibberellin	**76.01**	**38.01**	**19.0**	**95**	**3.1**

Conclusions

As we know, a fresh, quick and concise approach for the separation of phytohormones by nonaqueous capillary column has been conducted and satisfactorily adapted to the segregation of phytohormones from purified wheat samples. The principal superiorities of the current approach were less time (within 4 mins) in comparison with other reported approaches and its MS compatibility due to low levels of non-volatile additives, usually applied to enhance the selectivity of closely linked compounds in aqueous medium. For our future task, combining non-aqueous capillary electrophoresis methods with direct analysis of plant hormones in biological samples to compensate for current UV detection methods will be an amusing approach to mass spectrometry.

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