Journal of Chromatography A, 1217 (2010) 1399–1406

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Dual-cloud point extraction and tertiary amine labeling for selective and sensitive capillary electrophoresis-electrochemiluminescent detection of auxins

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ARTICLE INFO

Article history:
Received 15 September 2009
Received in revised form
30 November 2009
Accepted 9 December 2009
Available online 16 December 2009

Keywords:
Auxin
Capillary electrophoresis
Electrochemiluminescence
Dual-cloud point extraction
Tertiary amine labeling

ABSTRACT

The low concentrations of the auxins in samples of plant tissue necessitate the use of selective and sensitive techniques for their quantification. Herein a selective and sensitive method based on dual-cloud point extraction (dCPE) and tertiary amine labeling for the quantification of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) by capillary electrophoresis-electrochemiluminescence (CE-ECL) is proposed. The procedure for dCPE included two cloud point processes with Triton X-114 as the extractant. The two auxins became hydrophobic in an acidic solution and were extracted into surfactant-rich phase after the first cloud point procedure. They were then back-extracted into the alkaline aqueous phase during the second cloud point step. The extracted auxins were reacted with 2-(2-aminoethyl)-1-methylpyrrolidine (AEMP) in acetonitrile that contained N,N′-dicyclohexylcarbodiimide and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine to produce their AEMP-derivatives. The two auxin-AEMP-derivatives were subjected into CE and detected by $Ru(bpy)_3^{2+}$-based ECL. The preconcentration factors for IAA and IBA with dCPE were 40.5 and 43.4, respectively. The on-capillary detection limits (S/N = 3) were 2.5 and 2.8 nM for IAA and IBA. This protocol presents a clear advantage in that it reduces the interference from the matrixes extensively and gives a high sensitivity for the detection of auxins. The proposed method was applied successfully to the detection of the two auxins in acacia tender leaves, buds, and bean sprout.

1. Introduction

Phytohormones, which are typically present at low levels in plant tissues, regulate the growth and development of the plants at their low concentrations [1]. A group of phytohormones termed auxins, which include indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), regulate the division, elongation and differentiation of cells [1,2]. Owing to the low concentration of phytohormones in plant tissues and the complex environment where they are found, the characterization of phytohormone is extremely difficult. Therefore the selective and sensitive technique for their quantification in the samples of plant tissue is required with an efficient sample pretreatment step and a highly sensitive detection technique [2–4].

The sample pretreatment step should concentrate the target analytes and eliminate the potential contaminants that might interfere with the analysis of the trace auxins. At present extraction with methanol/water after adjustment of the pH using various acids (e.g. acetic acid) is the most popular method of extracting auxin from the plant tissues [2–7]. After the tissue sample was ground to a fine powder, acidic methanol/water was added into the powdered tissues for the extraction of the auxins. However, because the methanol/water system has no specificity for auxins, any hydrophobic compounds can be extracted and may interfere with the analysis of the auxins of interest. To improve the efficiency of sample pretreatment and to eliminate the potential contaminating substances, solid-phase extraction or extraction-back-extraction has been used to treat the methanol–water extract [2,4–7]. However, even when a solid-phase extraction is used, some interfering peaks appeared, leading to the quantification of target analytes difficult [2,7]. Ionic liquids have also been tested as extracting solvents for the extraction of IBA [8]. To improve the efficiency of pretreatment, the target analytes were back-extracted into the aqueous phase from the ionic liquid phase by the use of NaOH solution [8].

The detection methods should allow the specific detection of auxins with high sensitivity. Several methods for the quantification of auxins have been developed that eliminate the potential interferences due to their specificity for auxins [5,6,9]. Enzyme immunoassay, which has excellent specificity for the target analytes, has been used as a selective method to estimate auxin levels. However, quantification by immunoassay can be probably misrepresentative because of the potential cross-reactivity of antibodies with the interfering substances. Moreover, enzyme immunoassay requires the synthesis of the antibody for auxins [6]. Calatayud et al. [10] developed a flow injection fluorimetric method for the

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doi:10.1016/j.chroma.2009.12.029

determination of IAA that was based on its native fluorescence. Among the methods available for the quantification of auxins, high performance liquid chromatography (HPLC) in conjunction with various detection techniques, such as mass spectrometry (MS), fluorescence and chemiluminescence, are still considered to be the optimal approach [3,5,8,11–15]. Some capillary electrophoresis (CE)-based methods have been developed for the detection of auxins [2,7,16–18]. To improve the sensitivity of CE for phytohormone determination, a capillary that contains expanded bubble cell and online large volume sample stacking were used [16].

The development of an alternative method to quantify auxins sensitively and selectively is of critical importance. Cloud point extraction (CPE), which is a benign sample pretreatment technique, requires simple instrumentation with low cost and can be performed without the use of dangerous and toxic organic solvents, which are required for the traditional solvent extraction [19–22]. Similarly to solvent extraction, CPE is based primarily on the hydrophobic interaction between the solutes and surfactant. Therefore any hydrophobic species can be extracted into the surfactant-rich phase and interfere possibly with the analysis of the interest analytes [19–22]. Moreover, the injection of high-concentration surfactant into the electrophoretic capillary or chromatographic column may decrease both the efficiency of separation and the reproducibility. In our previous works [21,22], a dual-cloud point extraction technique (dCPE), as a potential solution to the above problems of traditional CPE, was proposed. Speciation analysis of mercury and the analysis of phenols indicated that whereas the target analytes were extracted efficiently using dCPE, the potential interfering species were eliminated to some extent. The effects of the surfactant on injection and separation were also eliminated [21,22].

The $Ru(bpy)_3^{2+}$-based electrochemiluminescence (ECL) is a very sensitive detection technique [23–25]. The ECL reaction can be initiated by a co-reactant (such as the compounds that contain a tertiary amine group), which makes the detection of those analytes possible. To extend ECL to the detection of non-ECL-active compounds, $Ru(bpy)_3^{2+}$ labeling technique has been applied extensively. The synthesis of the $Ru(bpy)_3^{2+}$ derivative labels and the labeling procedure are performed in a light-tight environment at low temperature [23–25]. In principle, if the target analyte is labeled with a compound that contains tertiary amine groups, ECL emission can also be observed when it is in contact with $Ru(bpy)_3^{2+}$ solution and a potential applied. The advantages of tertiary amines over $Ru(bpy)_3^{2+}$ derivatives as labels include high labeling efficiency, good biocompatibility, cheap commercial-availability, and milder labeling conditions [26–31]. Furthermore, the combination of tertiary amine labeling with separation techniques reduces the interference from the excess labels and the disintegrated compounds and allows the detection of multi-analytes [26–30].

In this work, dCPE in combination with 2-(2-aminoethyl)-1-methylpyrrolidine (AEMP) labeling was proposed for analysis of IAA and IBA by CE-ECL for the first time. Triton X-114 was used to extract the two auxins into the surfactant-rich phase and then the auxins were transferred into an alkaline solution by dCPE. The extracted auxins were further labeled with AEMP for the detection by CE-ECL. The factors that affected the sample extractions and detection were investigated in detail.

2. Materials and methods

2.1. Instrumentation

The homemade CE setup with an UV detector used to investigate the extraction efficiency of dCPE was similar to that in the previous work [17]. Briefly, a high-voltage power supply (Tianjin Dongwen High Voltage Power Supply Plant, Tianjin, China) was used to drive the electrophoretic separation in a positive voltage-controlled mode. UV detection was carried out with model 757 UV detector (Beijing Youlian Apparatus Co. Ltd., Beijing, China) using a homemade cartridge with a slice with 150 μm pinhole as slit to cut off the stray light and to fix the separation capillary. UV detection was performed at 217 nm for the detection of two auxins. A chromatographic workstation (Shanghai Junrui Software Co. Ltd., Shanghai, China) was used for data acquisition and treatment. Uncoated fused-silica capillaries (Yongnian Optical Fiber Co. Ltd., Hebei, China) with 60 cm total length, 40 cm effective length, 75 μm i.d., 375 μm o.d. were used as separation column.

The electrochemical measurement for the CE-ECL system was carried out with a Model LK98BII Microcomputer-based Electrochemical Analyzer (Tianjin Lanlike High-Tech Company, Tianjin, China). The ECL emission was detected and recorded with a Model MCDR-A Chemiluminescence Analyzer (Xi'An Remax Science & Technology Co. Ltd., Xi'An, China). The voltage of photomultiplier tube (PMT) in the chemiluminescence analyzer was set at −750 V in the process of detection. The CE-ECL cell and the capillary with porous etched joint were reported previously [32]. Sample was introduced into capillary by a hydrostatic pressure with a height difference of 20 cm between the inlet and outlet of capillary for 20 s in the both two modes.

2.2. Chemicals and reagents

All solutions were prepared with doubly distilled water (DDW). Except otherwise stated, analytical-grade acid, alkali and other chemicals used in this study were obtained from Tianjin Chemicals Co., Tianjin, China. The auxin standard solutions were prepared in DDW by diluting 4.50 mg mL^{-1} alkali solutions of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) (from Dingguo Biotechnology Co., Beijing, China). A stock solution of 2.50% (w/v) Triton X-114 (Sigma–Aldrich, Shanghai, China) was prepared by dissolving 2.50 g of Triton X-114 in 100 mL volumetric flask. HCl and Triton X-114 was used to extract IAA and IBA into surfactant-rich phase at the first cloud point procedure. NaOH solution was used to back-extract the two auxins into aqueous phase from the surfactant-rich phase. 2-(2-Aminoethyl)-1-methylpyrrolidine (AEMP), tris(2,2′-bipyridine) ruthenium dichloride hexahydrate ($Ru(bpy)_3Cl_2{\cdot}6H_2O$) were obtained from Sigma–Aldrich, Shanghai, China. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt) from Shanghai Medpep Co., Ltd., Shanghai, China, were used as the potential coupling agents for labeling two auxins with AEMP. 20 mM $Ru(bpy)_3^{2+}$ solution in DDW was prepared as stock solution and stored in a refrigerator. $Ru(bpy)_3^{2+}$ working solutions were prepared by diluting the stock solution with phosphate buffer solution to the desired concentrations and degassed ultrasonically for 10 min just before use. Sodium dihydrogen phosphate and disodium hydrogen phosphate were used to prepare the detection buffer and running buffer solution, which was adjusted pH with 0.1 M NaOH or 0.1 M HCl. 5 mM phosphate buffer solution at pH 5.5 was used as CE buffer solution to separate IAA- and IBA-AEMP-derivatives for ECL detection.

2.3. Dual-cloud point extraction

For preconcentration and clean-up by pH-mediate dCPE [22], 0.48 mL of 2.50% (w/v) aqueous Triton X-114 solution was added into an aliquot of 10 mL of auxin solution. Then the pH of the mixture was adjusted to 5.0 with HCl solution. The mixture was immersed in a thermostatic bath at 40 °C for 10 min, which triggered the cloud point, resulting in the formation of two phases.

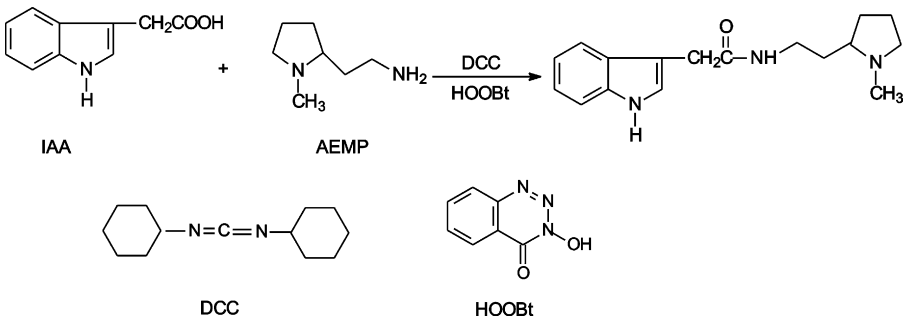

Fig. 1. The coupling of IAA to AEMP with DCC and HOOBt as coupling agents.

After the two phases were separated by centrifugation for 5 min at 3500 rpm, the centrifuge tube that contained the two phases was cooled in an ice-bath for 5 min to increase the viscosity of the surfactant-rich phase. The aqueous supernatant phase was removed carefully with a pipette. An aliquot of 200 μL of NaOH solution (pH 9.0) was added into the surfactant-rich phase. Subsequently, the mixture of surfactant-rich phase and NaOH solution was shaken vigorously to transfer the two auxins into the alkaline aqueous phase. After it was incubated in a thermostatic bath at 40 °C for 10 min to form new two phases, the mixture was centrifugated again to separate the two resulting phases. The two auxins presented in aqueous phase were injected into CE-UV system to investigate the extraction efficiency or labeled with AEMP for their detection by CE-ECL.

2.4. Synthesis of AEMP-derivatives of IAA and IBA

The AEMP-derivatives of IAA and IBA were prepared by a modification of the method of Morita and Konishi's as shown in Fig. 1 [26,27]. In brief, IAA (4.4 mg), N,N′-dicyclohexylcarbodiimide (DCC; 10.3 mg), and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt; 4.1 mg) were dissolved in 1.0 mL of acetonitrile and then 5.0 μL of AEMP was added into the mixture, which was shaken for several min. The resulting mixture was incubated for 24 h at room temperature. The IBA-derivative was synthesized in the same manner. To obtain the pure IAA- or IBA-AEMP-derivatives, the mixture was filtered and the solvent was removed by evaporation in vacuo. The residue was extracted into ethyl acetate. The combined organic layer was dried with anhydrous Na_2SO_4 and filtered. The filtrate was evaporated in vacuo. The products obtained were used as the standards to validate and quantify IAA and IBA in the experimental samples.

2.5. Sample pretreatments

Samples obtained from mung bean sprout and the acacia buds or tender leaves were used to evaluate the applicability of the method. The acacia buds and tender leaves were collected locally. The mung bean sprout was cultivated in our laboratory. Briefly, mung beans were soaked in tap water for 12 h and germinated in tray with a plastic membrane to give a suitable temperature and humidity. The water was refreshed periodically throughout each day. After 3 days, 2.50 g of shoot apices were collected. The above-mentioned plant tissues were washed with tap water and doubly distilled water (DDW) successively. After being exposed to air for 1 h at room temperature to evaporate the adsorbed water, about 0.50 g (fresh weight) of plant tissue was flash frozen in liquid nitrogen and stored at 4 °C for 3 h for auxin evaluation [33,34].

The prepared plant samples (0.50 g fresh weight) were ground in an agate pestle and mortar. For preconcentration and clean-up by dCPE, 15 mL aqueous alkaline (NaOH, pH 13.0) solution were first added to each sample and the mixture was incubated overnight at 4 °C in the dark to allow two auxins to transfer into the aqueous phase. After centrifugation to remove the residue, 10 mL supernate was selected and quantified and then 0.48 mL of 2.5% (m/v) Triton X-114 solution was added into the supernate. 0.1 M HCl solution was added to adjust pH value to 5.0.

The extraction solution obtained after dCPE was evaporated at room temperature in vacuo for AEMP-labeling as following. The residue was dissolved in 160 μL of acetonitrile, and the resulting solution was added 20 μL of 6×10^{-2} M DCC, 12 μL of 6×10^{-2} M HOOBt, and 10 μL of 3.5×10^{-2} M AEMP in acetonitrile, corresponding to their concentrations of 6.0, 3.6, and 1.8 mM for DCC, HOOBt, and AEMP. The resulting mixture stood for 24 h at room temperature to allow AEMP labeling to occur. The high content of acetonitrile may turn off the electrophoretic separation via formation of bubble due to its high resistance. Therefore, the labeling solution was diluted with the separation buffer to 1 mL and was injected into the capillary for evaluation of the level of IAA and IBA using the CE-ECL system. Methanol–water extraction with HPLC-UV detection of the two auxins was used to validate the possibility of the proposed method. The conditions for methanol–water extraction and HPLC-UV detection were described in Supporting Information.

3. Results and discussion

3.1. pH-mediated dual-cloud point extraction

Because the present dCPE was achieved via the change in sample acidity, it was termed as pH-mediated dCPE [22]. The traditional cloud point procedure was performed twice in the dCPE procedure [21,22]. In the first one, IAA and IBA became hydrophobic and extracted into the surfactant-rich phase after the addition of Triton X-114 and the adjustment of the pH value of sample solution to 5.0. After NaOH solution (pH 9.0) was added to the surfactant-rich phase obtained from the first step, the two auxins formed stable hydrophilic salts and were transferred into the aqueous phase during the second procedure. The factors affecting the efficiency of dCPE included the acidity during the two cloud point procedures, the amounts and type of surfactants, and the incubation temperatures. The investigation of those factors by CE with UV detection was presented in Supporting Information. The optimal conditions for dCPE of the two auxins were presented as following: the solution was adjusted to pH 5.0 with HCl for the first cloud point procedure whereas 200 μL of NaOH solution (pH 9.0) were used for the second cloud point one. Triton X-114 (0.12%, w/v) was selected because it gave a good extraction efficiency and reproducibility for both IAA and IBA. To trigger the cloud point, the mixture that contained Triton X-114 was incubated in a thermostatic bath at 40 °C for 10 min.

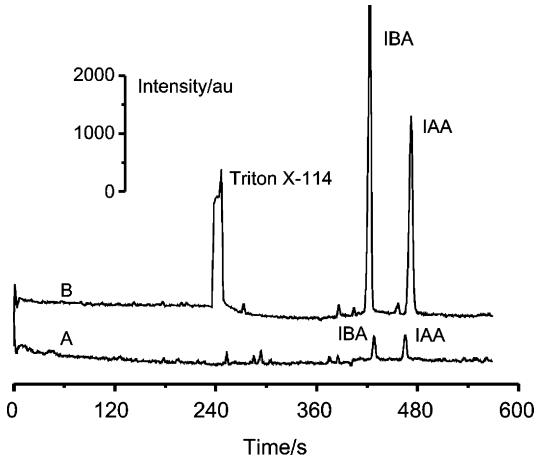

Fig. 2. (A) Electropherogram of the standard solution of IAA and IBA at the concentration level of 5.0×10^{-5} M. (B) Electropherogram of the extract of 2.0×10^{-5} M IAA and IBA after dCPE. Extraction conditions: 0.12% (w/v) Triton X-114, pH 5.0 adjusted with HCl, incubation temperature: 40 °C for the first cloud point procedure. 200 μL NaOH (pH 9.0) was used to back-extract the two analytes into aqueous phase. Capillary: 75 μm i.d. 40 cm effective length and 60 cm total length; running buffer: 10 mM phosphate buffer solution (pH 8.0); UV detection at 217 nm. Sample injection: 20 s with a height difference of 20 cm.

The sample pH plays an important role in pH-mediated dCPE [22]. The extraction of phenols showed that the pH value in the first CPE should in theory be as low as possible because protonated phenols are hydrophobic and can be extracted into the surfactant-rich phase [22]. However, as shown in Fig. S1, the optimal sample acidity for the extraction of IAA and IBA was around pH 5.0. The molecular structures show that IAA and IBA are zwitterions. They were at their most hydrophobic and therefore extracted most efficiently at pH around their isoelectric point (p*I*). It was verified by the acid dissociation constant of IBA, pK_a of 4.8 [8]. In fact, their extraction from plant tissue by the traditional methanol–water method was also based on their hydrophobicity and the mixture of methanol and water at pH 4–5 was often used. At pH higher than 5.0, the extraction efficiency of the two auxins decreased steeply. Therefore, pH 5.0 was selected as the optimal sample pH. Although the extraction of IBA into ionic liquids was characterized previously [8], IAA was found to have similar extraction efficiencies under the tested conditions.

After the first CPE, NaOH solution (pH 9.0) was added into the surfactant-rich phase. The two auxins formed stable hydrophilic salts and were transferred into the aqueous phase. Absalan et al. [8] used NaOH solution to back-extract IBA from the ionic liquid phase. However, their procedure required 50 min for the high extraction efficiency [8]. Because the cloud point procedure of Triton X-114 is reversible, which facilitates the transfer of the analyte from one phase to the other phase, the second cloud point procedure took 15 min in our work. Therefore, faster extraction was achieved using dCPE as compared to that by the use of ionic liquids.

Fig. 2 illustrates the CE-UV electropherograms that were obtained before and after dCPE. A more than 40-fold improvement in signal, based on the peak area, was achieved for the two analytes (Fig. 2a *cf.* b). Moreover, the concentration of Triton X-114 in the aqueous solution after dCPE is around its critical micelle concentration (CMC), which only corresponds to a peak in Fig. 2b. However, in traditional CPE for preconcentration prior to CE separation, the redundant surfactant can be adsorbed onto the inner wall of the capillary, which results in a loss of efficiency and the reduced reproducibility in migration times and electrophoretic peak areas [19,35–37]. To decrease the effect of the surfactants, the surfactant-rich phase was often diluted with organic solvents, such as acetonitrile–methanol–perchloric acid [19], methanol [35], tetrahydrofuran [36], or acetonitirle [37]. The low concentration of Triton X-114 after dCPE significantly decreased the influence of the surfactant on the analysis of the target analytes as compared to the traditional CPE [21,22]. Moreover, the second cloud point procedure removes the potential interfering compounds in the plant samples in a manner similar to traditional solvent back-extraction.

3.2. Labeling of IAA and IBA with AEMP

For ECL labeling, the derivative reagents should possess an active site for coupling to the target analytes and a group to allow highly sensitive detection. A tertiary amine in the derivative reagent is necessary for ECL detection. Given that the two auxins molecules both contain a carboxylic acid group, a primary amine group in the derivative reagent is a good choice because many methods can be used to couple the two groups with high efficiency under mild condition. Therefore, potential derivatization reagents should contain a tertiary amine and a primary amine groups, e.g. N-(3-aminopropyl)pyrrolidine (NAPP) and 2-(2-aminoethyl)-1-methylpyrrolidine (AEMP) [26–28]. AEMP was found to have a high ECL efficiency over NAPP [26], and therefore AEMP was chosen as the derivative reagent for labeling of the two auxins. The molecular structure of AEMP and derivative process is shown in Fig. 1.

Two methods were investigated to label IAA and IBA with AEMP. In the first one, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were used as coupling agents, whereas DCC and HOOBt were selected in the second method. The EDC/NHS system is often used to couple carboxylic and primary amine groups and the synthesis of the IAA-AEMP-derivative is described in Supporting Information. However, as shown in Fig. S4, some of the by-products with high capacity to excite $Ru(bpy)_3^{2+}$ ECL exist and thus could interfere with the detection of the two auxins. Therefore, the DCC/HOOBt system was used. Morita and Konishi [27] used dioxane as a solvent to prepare the AEMP-derivative of ibuprofen, but dioxane is highly toxic. We find that acetonitrile, as a solvent, can also give a high labeling efficiency but it is less toxic than dioxane and is selected for the further experiments.

Fig. 3 shows the effect of the concentration of DCC and HOOBt on the signal of the IAA-AEMP-derivative at a concentration of 1 μM. Using 6.0 mM DCC, the IAA-derivative showed maximum signal intensity at 3.6 mM and the signal then leveled off as the concentration of HOOBt was increased to 7.0 mM. On the other hand, the ECL emission of the IAA-AEMP-derivative increased as the DCC concentration was increased keeping the concentration of HOOBt at 3.6 mM and the maximum signal was obtained at 6.0 mM DCC. Under the tested conditions, equivalent changes in the concentration of either reagent gave a similar ECL profile for IBA-AEMP-derivative. A ratio of HOOBt:DCC:AEMP of 3:5:1.5 gave a high labeling efficiency for IAA and IBA. Consequently, 3.6 mM HOOBt, 6.0 mM DCC and 1.8 mM AEMP were selected as their optimal concentrations for auxin labeling. The labeling procedure for IAA and IBA were performed as follows. AEMP, DCC and HOOBt were added to the acetonitrile solution that contained IAA, IBA or their mixture at a final concentration of 1.8, 6.0, and 3.6 mM, respectively. The resulting mixture was incubated for 24 h at room temperature.

3.3. Validation of the IAA- and IBA-AEMP-derivatives

The IAA- and IBA-AEMP-derivatives were validated using the migration times obtained during electrophoretic procedure. Fig. 4 shows the electropherograms of IAA or IBA alone after labeling with AEMP (Fig. 4a), the mixture of IAA and IBA (Fig. 4b) after labeling with AEMP, and the individual standard IAA- and IBA-

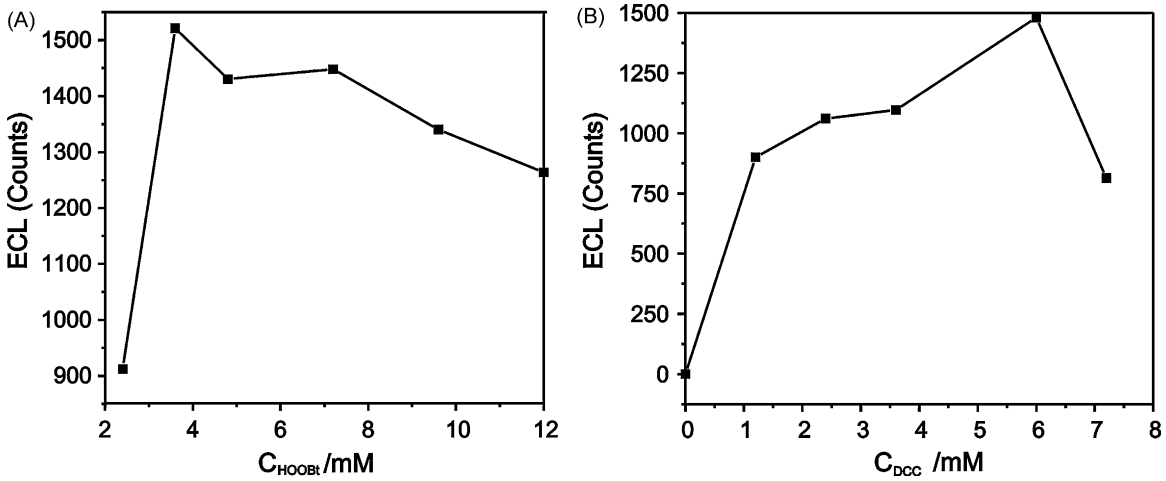

Fig. 3. The effect of the concentration of HOOBt (A) and DCC (B) on the signal of 1 μM IAA labeled with AEMP. Capillary: 75 μm i.d. 60 cm capillary; separation buffer: 5.0 mM phosphate buffer solution (pH 5.5); detection buffer: 50.0 mM phosphate buffer solution (pH 7.0) containing 5.0 mM $Ru(bpy)_3^{2+}$; applied detection potential: 1.25 V; sample injection: 20 s with a height difference of 20 cm.

AEMP-derivatives (Fig. 4c). The migration times are 561 and 587 s for IAA- and IBA-derivatives, respectively. Based on the peak area, the IAA- and IBA-AEMP-derivatives showed approximately 95.0% of the intensity of AEMP at the same concentrations, which indicated that the tertiary amine group retained its ECL activity even when AEMP was coupled to either of the two auxins. With the signal intensity at the same concentration, the labeling efficiency for IAA and IBA using HOOBt and DCC as coupling agents was 85 and 83%, respectively.

3.4. Separation of the two auxin-AEMP-derivatives by capillary electrophoresis

CE separation of AEMP, and the IAA- and IBA-AEMP-derivatives was first optimized by the use of standard solutions. Among the factors that affect CE separation, the pH of the electrolyte is considered to be one of the most important parameters. It influences the separation efficiency by affecting the electroosmotic flow (EOF) and the acidic–alkaline equilibrium of the analytes. Over the range of pH value tested from 4.0 to 8.0, AEMP was separated easily from the two auxin-derivatives and no interference with the target analytes was observed, as shown in Fig. 4.

The resolution between the IAA- and IBA-AEMP-derivatives and the migration time of the IBA-AEMP-derivative are shown in Fig. 5A. The resolution R was calculated as following: $R = 2(t_2 - t_1)/(W_1 + W_2)$, where t_1 and t_2 are the migration times of the two AEMP-derivatives and W_1 and W_2 are their peak widths at the baseline. It was difficult to separate the two derivatives baseline when the pH of the buffer solution was higher than 6.0. Although they were separated from each other within the pH ranged from 4.0 to 5.5, the separation time increased as the buffer pH decreased due to the decreased EOF. Fig. 5B shows the effect of the pH of the running buffer on the ECL emission of the two auxin-AEMP-derivatives. As the pH value was increased from 4.0 to 5.5, the ECL emission of the two derivatives remained constant. When the pH of the buffer was increased from 5.5 to 8.0, their ECL intensity first improved obviously and then leveled off. To allow baseline-separation of the two AEMP-derivatives with lowest possible separation time, pH 5.5 was chosen as the optimal pH for separation buffer, although some of the ECL signal intensity was sacrificed.

The effect of the concentration of phosphate on the separation was examined over the concentration range of 2–15 mM. Increase in the phosphate concentration in the buffer improved slightly the resolution of the two derivatives but it also increased their migra-

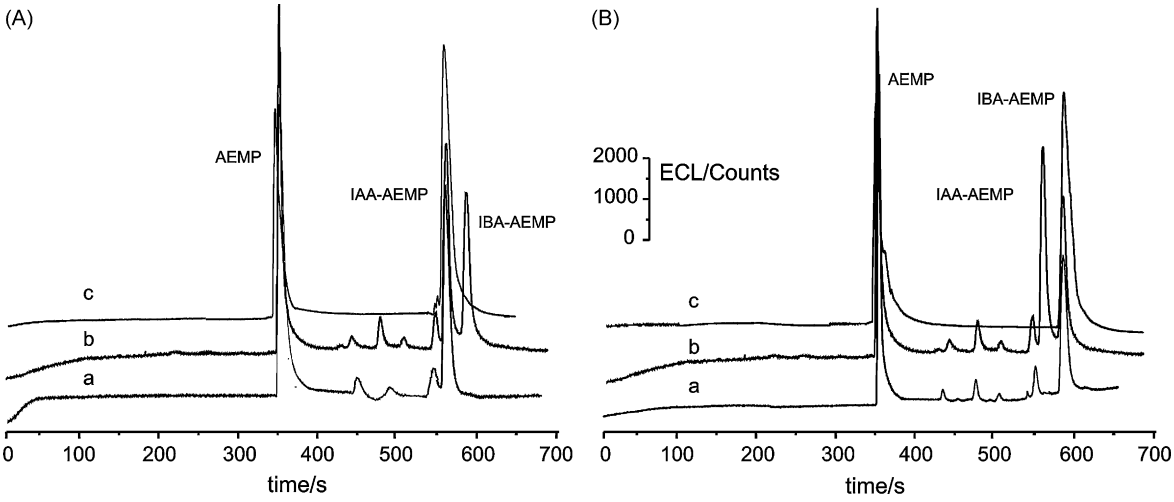

Fig. 4. Identification of IAA-AEMP-derivative (A) and IBA-AEMP-derivative (B). (a) The electropherogram of IAA or IBA (2.50 μM) after being labeled with AEMP; (b) the electropherogram of the mixture of IAA and IBA (2.50 μM for each analytes) after being labeled with AEMP; and (c) electropherogram of pure IAA- or IBA-AEMP (4.0 μM for each analytes) spiked with AEMP. The other conditions as shown in Fig. 3.

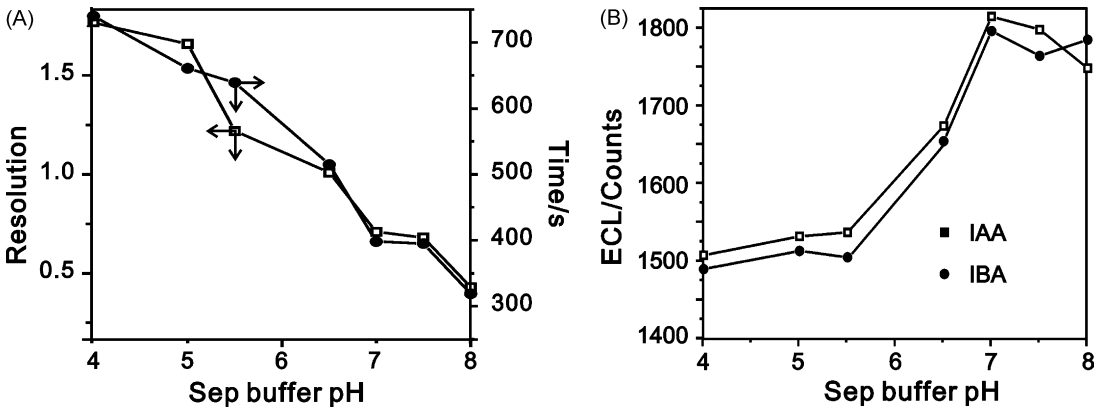

Fig. 5. The influence of separation buffer pH on the resolution of IAA- and IBA-AEMP-derivatives, migration time of IBA-AEMP-derivative (A), and the influence of separation buffer pH on the signal intensity of the two auxin-AEMP-derivatives (B). The other conditions as shown in Fig. 3.

tion times and electrophoretic current. Therefore, 5 mM phosphate buffer at pH 5.5 was used as a compromise to ensure a baseline-separation in the minimal time with a reasonable electrophoretic current (~20 μA).

The voltage applied for electrophoresis influences the separation efficiency because it drives the analytes through the capillary and changes the electrophoretic EOF. The influences of the applied voltage varying between 8 and 20 kV was investigated. The migration time decreased as the applied voltage was increased. The AEMP-derivatives of IAA and IBA separated well when the voltage was lower than 13 kV but the separation time increased at the lower separation voltages. A voltage greater than 13 kV resulted in a decreased resolution between the IAA- and IBA-AEMP-derivatives. To give good separation with the shortest separation time, 12 kV was selected as the optimal voltage and used for the subsequent analyses.

After IAA and IBA had been tagged with AEMP, they showed a totally different electrophoretic behavior to that of the unlabeled forms. As shown in Fig. 2 and Fig. S5, free IAA and IBA can be separated easily from each other with high resolution over a wide pH range. The labeling of IAA and IBA with AEMP reversed their order of migration, and, in addition, separation of the IAA- and IBA-AEMP-derivatives was much difficult. The results indicated that the difference in the molecular charges of the two auxins was critical to the separation of their free forms. In the auxin molecules that were tagged with AEMP, the negative charge on the carboxylic group was suppressed. Therefore, their molecular sizes played an important role in their separation, and this lead to a reversal in the order of migration as compared to their free forms.

3.5. ECL detection

$Ru(bpy)_3^{2+}$-based ECL emission is dependent on the pH at which the reaction was carried out. The dependence of the ECL intensity on the detection pH was examined for AEMP, and the IAA- and IBA-AEMP-derivatives. As shown in Fig. 6, the three species showed their maximum peak intensity at pH 7–7.5. Although a high pH was considered to facilitate the ECL activity of $Ru(bpy)_3^{2+}$/tertiary amine systems [38], pH 7–7.5 would be more suitable for sensitive and stable detection for the IAA- and IBA-AEMP-derivatives. The above results were similar to those observed by Morita and Konishi [26,27]. They found that an increase in electrolyte pH beyond pH 7.0 resulted in a decreased ECL emission for the AEMP-derivative of ibuprofen [26]. Consequently, pH 7.0 was selected as the detection pH. $Ru(bpy)_3^{2+}$-based ECL is generated by forming the excited-state $Ru(bpy)_3^{2+*}$ at the electrode surface, and thus the applied potential has a significant effect on the emission efficiency. Therefore, the ECL intensity was investigated at the applied potentials from 1.00 to 1.30 V. No emission signal was observed when the potential was below 1.00 V. Similar to the results of the previous work [26,27], the highest emission levels were obtained at 1.20–1.25 V for both AEMP-derivatives, 1.25 V was chosen as the optimal potential.

3.6. Interference

A number of molecules that are found in plant tissues could interfere with the detection of the two auxins. Three amino acids, cysteine (Cys), arginine (Arg), and histidine (His), were chosen to evaluate the potential interference in the developed method. These amino acids contain a carboxylic group, which enables them to be coupled to AEMP. However, no interference was observed with 1 μM of individual Cys, Arg, and His on the determination of the two auxins due to their low efficiency of extraction under the optimal dCPE condition for IAA and IBA, because they are highly soluble in aqueous solution. Even some species are extracted after dCPE, they can only be detected when they are ECL-active molecules or can be labeled with AEMP. Therefore, both tertiary amine labeling and ECL detection contributes to the high selectivity of the proposed method. No interference peaks were detected in Fig. 7, which indicated that no interference from the sample matrix was observed.

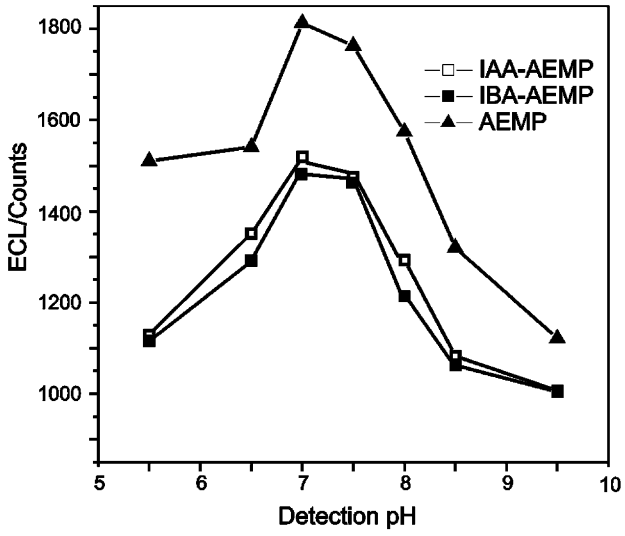

Fig. 6. The effects of detection buffer pH on the ECL intensity of AEMP, IAA- and IBA-AEMP at their 1 μM level. The other conditions as shown in Fig. 3.

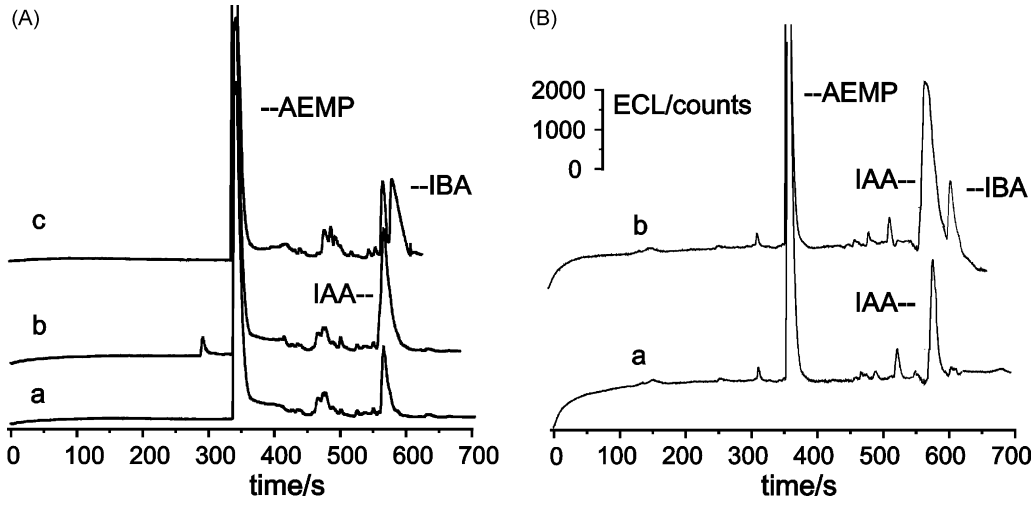

Fig. 7. (A) The CE-ECL electropherogram of the acacia leaf after dual-cloud point extraction and AEMP labeling (a); the electropherogram of (a) spiked with IAA at 0.5 μg g^{-1} level (b); and the electropherogram of (a) spiked with IBA at 1.0 μg g^{-1} level (c). (B) The CE-ECL electropherogram of the bean sprout after dual-cloud point extraction and AEMP labeling (a); the electropherogram of (a) spiked with 0.5 μg g^{-1} IAA and 1.0 μg g^{-1} IBA (b); the other conditions as shown in Fig. 3.

3.7. Analytical performance of the dCPE and AEMP labeling for the detection of IAA and IBA by CE-ECL

Characteristic analytical data for the dCPE and AEMP labeling to detect the two auxins are summarized in Table 1. The preconcentration factors were calculated by determining the ratio of the peak area obtained after dCPE to that obtained from direct injection of the initial sample using CE-UV detection. Preconcentration factors of 40.5 and 43.4 were obtained for IAA and IBA, respectively. The precision (relative standard deviation, RSD) of the migration time, the peak area, and the peak height for five replicate injections was 1.4–1.5, 2.1–3.4, and 3.6–5.3%, respectively. In combination with the AEMP labeling, the detection limits (3σ) of IAA and IBA were 2.5 and 2.8 nM. The method described here was highly sensitive with respect to auxin detection and also gave improved anti-interference capacity as compared to methanol–water extraction with HPLC-UV detection (Fig. 7Aa *cf.* Fig. 8A).

3.8. Validation of the proposed method for the detection of IAA and IBA

Due to the lack of availability of suitable reference materials for the auxin detection, the accuracy of the proposed method for the detection of IAA and IBA (Fig. 7Aa) was verified by comparing the results with those obtained by methanol–water extraction and HPLC-UV detection (Fig. 8A). The analytical results are shown in Table 2. No IBA was found in the acacia tender leaves using either of the two methods. The IAA levels determined by the two methods showed a good agreement. Although extraction-back-extraction was performed twice for the methanol–water extract, there were some unidentified peaks in the chromatogram (Fig. 8A). As a comparison, the results described in Fig. 7Aa show the high anti-interference capacity of the proposed method for auxin detection. The accuracy of the present technique was demonstrated further by the results obtained for the recovery of IAA and IBA from

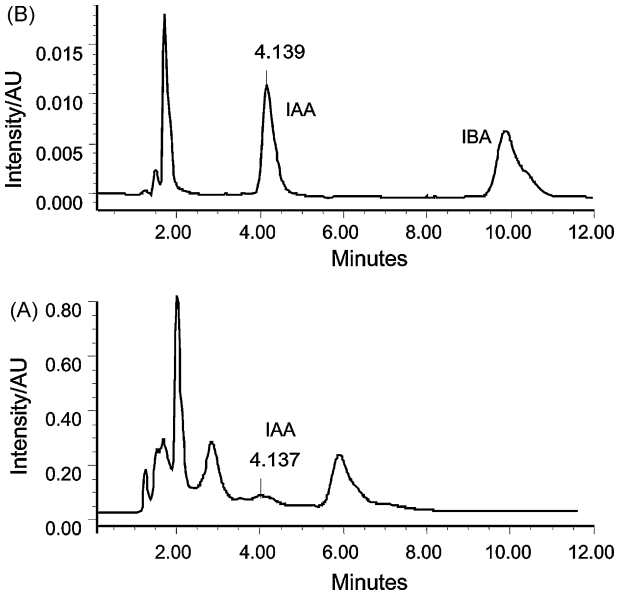

Fig. 8. (A) The HPLC chromatograms of the acacia leaf after methanol–water extraction; (B) the HPLC chromatograms of the standard mixture of IAA and IBA at 2.0 μg g^{-1} level. The pretreatment with methanol–water extraction and the chromatographic conditions as described in Supporting Information.

Table 1
The analytical performance of dCPE and AEMP labeling for CE-ECL detection of IAA and IBA.

	IAA	IBA
Preconcentration factors	40.5	43.4
Precision (RSD, n = 4) (%)		
Migration time	1.5	1.4
Peak height	3.4	2.1
Peak area	5.3	3.6
Detection limits (3σ) (nM)	2.5	2.8
Calibration function[a]	I = 1566C + 48	I = 1476C + 39
Calibration ranges (M)	1.0×10^{-8} to 5.0×10^{-5}	1.0×10^{-8} to 5.0×10^{-5}
Corr. coeff.	0.9987	0.9981

[a] I, ECL intensity (counts); C, concentration (μM).

Table 2
The analysis results for IAA and IBA in acacia leaves.

Method employed	Content determined (μg g^{-1})	
	IAA	IBA
The present method	0.6884	n.d.[a]
Methanol–water extraction and HPLC-UV detection	0.7268	n.d.[a]

[a] n.d., not detectable.

Table 3
Sample analysis results obtained by using the present method.

	IAA	IBA
Found ($\mu g\,g^{-1}$)		
Acacia leaves	0.6884	n.d.[a]
Acacia burgeon	1.032	n.d.[a]
Bean sprout	0.9642	n.d.[a]
Recovery (%)		
Acacia leaf	108[b]	106
	101[c]	90.5
Acacia burgeon	89.7[b]	92.5
	93.3[c]	97.6
Bean sprout	111[b]	103
	99.2[c]	93.1

[a] n.d., not detectable.
[b] Recovery for spiking with 0.5000 $\mu g\,g^{-1}$ of each auxin.
[c] Recovery for spiking with 1.000 $\mu g\,g^{-1}$ of each auxin.

auxin-spiked plant samples. As shown in Table 3, the amount of recovery results is ranged from 90.5 to 111%.

3.9. Application to the analysis of real samples

The developed method was successfully applied to the determination of the two auxins in acacia tender leaves, buds and mung bean sprouts. The sample pretreatment and AEMP labeling of the samples were described in Experimental section. Fig. 7 shows the electropherograms of the real samples spiked with and without IAA or IBA. We can find the method based on dCPE and AEMP labeling can eliminate the interferences from the substances in the real samples. The analytical results obtained for the two auxins with the present method are given in Table 3. No IBA was detected in acacia tender leaves, buds and mung bean sprouts and the levels of IAA in the three samples were 0.6884, 1.032, and 0.9642 $\mu g\,g^{-1}$, respectively. For the acacia samples, the different levels of IAA in leaves and buds showed that the auxin content varied between its different growth periods.

4. Conclusion

Using dual-cloud point extraction (dCPE) and tertiary amine labeling, two auxins were detected selectively and sensitively by using capillary electrophoresis-electrochemiluminescence (CE-ECL). The results have demonstrated the feasibility of the proposed protocol for auxin detection. The ability to preconcentrate target analytes and to eliminate the potential interference makes dCPE attractive as a sample pretreatment method for the detection of analytes in a complex matrix. This work also demonstrates the feasibility of using tertiary amine derivatives as ECL labels to allow the ECL detection of the two non-ECL-active analytes. The detection limits (S/N = 3) were 2.5 and 2.8 nM for IAA and IBA, respectively. The proposed method was applied successfully to the detection of the two auxins in real samples.

Acknowledgments

This work is supported by National Nature Science Funding of China (No. 90717104) and the Program for New Century Excellent Talents in University (NCET-06-0214), Chinese Ministry of Education.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.12.029.

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