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Analysis of plant hormones in tobacco flowers by micellar electrokinetic capillary chromatography coupled with on-line large volume sample stacking

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Abstract

Micellar electrokinetic capillary chromatography was developed to analyze plant hormones including gibberellic acid, abscisic acid, indole-3-acetic acid, α-naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, kinetin-6-furfurylaminopurine and N^6-benzyladenine. The influences of some crucial parameters including buffer concentration, pH value, micelle concentration and applied voltage on electrophoretic separation were investigated. Under optimum conditions (50 m*M* borate as the running buffer containing 50 m*M* sodium dodecylsulfate, pH 8.0; separation voltage: −15 kV; injection: hydrodynamic injection, 5 s at 50 mbar; temperature: 25 °C), a complete separation of seven plant hormones was accomplished within 30 min. Emphasis was placed on improving detection sensitivity in order to detect small amounts of hormones in plant tissue. Multiple wavelength detection and expanded bubble cell capillary were used with enrichment factors of 2 and 3, respectively. In addition, an on-line concentration method of large volume sample stacking was designed. Enrichment factors of up to ~10–600 were achieved for these hormones with detection limits down to 0.306 ng/ml. The method was successfully applied to analyzing abscisic acid in flowers of transgenic tobacco.

Keywords: Tobacco; Sample stacking; Plant hormones

1. Introduction

As signal molecules, plant hormones play crucial roles in mediating plant growth in a whole range of developmental processes [1]. Many of them respond to environmental stimuli such as photoperiod, temperature, water and nutrition supply [2]. Taking gibberellic acid as an example, it promotes seed germination, stem elongation, flowering and cone production, and retards leaf and fruit senescence. It also induces de novo synthesis of numerous enzymes. Thus, it is very important to trace the changes in hormone concentration and tissue sensitivity, and improve understanding of the signal transduction and development of plants. Radioimmunoassay (RIA) [3] and enzyme-linked immunosorbent assay (ELISA) [4] are traditional methods in biology. Although GC/HPLC–MS [5,6] provides a standard method for accurate quantification of plant hormones, the high cost of instruments and the consumption of heavy

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isotope-labeled internal standards hinders its routine use.

Capillary electrophoresis (CE) [7] is now developing rapidly, and has been recognized as a powerful method for analyzing bio-molecules as a result of its high speed, high selectivity, low sample requirement and advanced automation. Several papers have reported the determination of plant hormones by CE [8–11]. Ouyang et al. [10] and Liu and Li [11] developed a capillary zone electrophoresis (CZE) method to analyze auxins in plant, due to the relatively good water solubility of auxins. However, as many of the main hormones in plants are hydrophobic, micellar electrokinetic capillary chromatography (MECC) [12] would be more powerful for resolving these species.

Because plant hormones present in tissue with very low concentrations (nanogram per gram fresh weight) and pool sizes, it is a challenge to analyze these species in a plant sample using CE. A major problem is the low sensitivity of UV detection in CE, which mainly results from the limited path length of the detection window. Although laser induced fluorescence (LIF) detection can provide ultra-high sensitivity [13], it is not practical for these hormones because of the absence of natural fluorescence or a suitable fluorescent tag. A pre-capillary concentration method may be an alternative choice if materials for experiment are abundant. However, it is sometimes impossible when the materials are limited, as, for example, the flower of transgenic plant discussed in this paper. As a consequence, on-capillary concentration procedures for samples are needed for CE analysis. There are many schemes for on-line sample concentration in CE, which have been extensively reviewed by many authors [14–18], such as electrostacking [14,15,19,20], field amplification [16,21–24], sweeping [25,26], pH junction [27,28] and isotachophoresis [29–31], in conjunction with a proper sample preparation step. These concentration methods can offer an enrichment factor ranging from 10 to 10^6 [26]. However, none of them so far has been used for the determination of trace amounts of plant hormones.

In this paper, MECC was used to separate and determine seven main plant hormones: gibberellic acid, abscisic acid, indole-3-acetic acid, α-naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, kinetin-6-furfurylaminopurine and N^6-benzyladenine. An on-line sample concentration procedure, large volume sample stacking was designed to increase the detection sensitivity, which thoroughly fulfilled the requirement of identifying the hormones of low concentration in flowers of tobacco.

2. Experimental

2.1. Chemicals

All the hormones (gibberellic acid (GA), abscisic acid (ABA), indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and N^6-benzyladenine (BA) (Gibco Brl, USA)) were accurately weighted, and dissolved in a solution containing 10% (v/v) CH_3OH and 0.1 *M* NaOH, at a concentration of ~10 mg/ml. Aliquots were then diluted with pure water as needed. Sodium dodecylsulfate (SDS) was purchased from Sigma (USA), and was prepared in solution at a concentration of 0.2 *M*. All the reagents were of analytical grade. Double distilled water was used for the preparation of all the solutions. The stock solutions of carrier electrolytes including NaH_2PO_4–Na_2HPO_4 (pH 7.0, 7.5, 8.0), HBO_3–$NaBO_4$ (pH 8.5, 9.0, 9.3) and $NaBO_4$–NaOH (pH 10.0) were prepared at a concentration of 0.2 *M* according to the manufacturer's instructions. The running buffer was prepared by mixing the correct ratio of SDS solution and the stock solution of carrier electrolyte daily and centrifuging at 12 000 rpm for 5 min prior to use.

2.2. Instruments

Analyses were carried out on a commercially available CE system (Agilent, CA, USA) equipped with an effectively air-cooling system. On-line UV detection was performed at 210 nm or by multiple wavelength scanning at 200, 210, 220, 225 and 246 nm with diode array detection (DAD). Data collection and processing were performed on a Hewlett-Packard ChemStation (CA, USA). Capillaries including a normal capillary (48.5 cm (40 cm effective length)×360 μm O.D.×50 μm I.D.) and a bubble detection cell (64.5 cm (56 cm effective length)×360 μm O.D.×50 μm I.D., bubble factor: 3) were

purchased from Agilent. A new capillary was pretreated with 1 *M* NaOH for 60 min at 60 °C followed by pure water for 60 min at room temperature. Prior to use, the capillary was flushed with 0.1 *M* NaOH and pure water for 5 min at room temperature, followed by preconditioning with running buffer for 10 min at a chosen separation temperature as stated in next section. Every five injections, the above flushing cycle was repeated to ensure the separation reproducibility.

2.3. Procedures

Tobacco was grown in a greenhouse (Wuhan University). Flowering plants were selected and treated with 10% (v/v) poly(ethylene glycol) (PEG). After subjecting a tobacco plant to 3-h drought stress, the flowers were immediately used to isolate the hormones.

Hormones were extracted as described by Chen et al. [32] with some modifications. Briefly, accurately weighed fresh tobacco flowers were ground to powder with liquid N_2, hydrogenized in 5 ml 70% (v/v) aqueous methanol using a high-speed blender, and then maintained overnight at 4 °C. The extraction was centrifuged at 4 °C (5000 *g* for 10 min). The supernatant was collected, and the residue was further extracted with cold 70% (v/v) aqueous methanol. A 1-ml extract was dried by blowing with N_2 gas, and then dissolved in 200 μl Na_2HPO_4 (pH 9.2) buffer. The solution was extracted three times with equal volume of ethyl acetate. It was further extracted with ethyl acetate, after adjusting solution acidity to pH 2.5. All aliquots of ethyl acetate phase were collected, dried with N_2 gas, and finally dissolved in 1 ml 70% (v/v) aqueous methanol.

3. Results and discussion

3.1. Effect of buffer concentration on separation

In MECC, a high speed and stable electroosmotic flow (EOF) in the capillary is required as the separation engine when a bare capillary is used. Consequently, buffer concentration has great influence on separation selectivity by regulating EOF. The effect of borate concentration on the separation was investigated. With the increase in buffer concentration, the migration time of analytes increased rapidly due to the decrease of EOF. The increase in ionic strength of the buffer decreased the thickness of the double layer of the inner capillary wall which further led to the compression of EOF. Although the separation selectivity was enhanced at the same time by increasing buffer concentration, the separation time of BA was too long, while the concentration of borate was over 50 m*M*. For considerations of separation speed, 50 m*M* borate was chosen for further investigation.

3.2. Effect of buffer acidity on separation

Buffer pH value is considered one of the most important parameters in CE separation. It can improve separation selectivity by adjusting the velocity of EOF and the acidic/alkaline equilibrium of analytes. This further affects the intrinsic mobility and water-solubility of the analytes and the partition coefficients into the micellar phase in the MECC separation mode. Thus, different hormones exhibited quite divergent behaviors as pH value was varied from 7.0 to 10.0 as shown in Fig. 1. BA is a highly hydrophobic hormone in a weakly alkaline environment. As a result, it appeared very late in the electropherograms using buffers of pH 7.0–9.3. However, when using pH 10.0 buffer, the band of BA migrated faster than IAA, NAA, 2,4-D or KT, due to an increase in water-solubility in a more alkaline solution. In experiments, three different buffer compositions were employed for the consideration of buffer capacity, including NaH_2PO_4–Na_2HPO_4 (pH 7.0, 7.5, 8.0), HBO_3–$NaBO_4$ (pH 8.5, 9.0, 9.3) and $NaBO_4$–NaOH (pH 10.0). Increasing buffer pH apparently led to the shortening of separation time due to the enhancement of EOF, which was marked by methanol peak. But the selectivity of separation decreased when using a buffer of pH 10.0; the peaks of IAA, NAA and 2,4-D overlapped. After comparing separation selectivity shown in Fig. 1, a pH value of 9.3 was chosen as the best buffer acidity.

3.3. Effect of concentration of SDS on separation

In this work, the effect of SDS concentration on

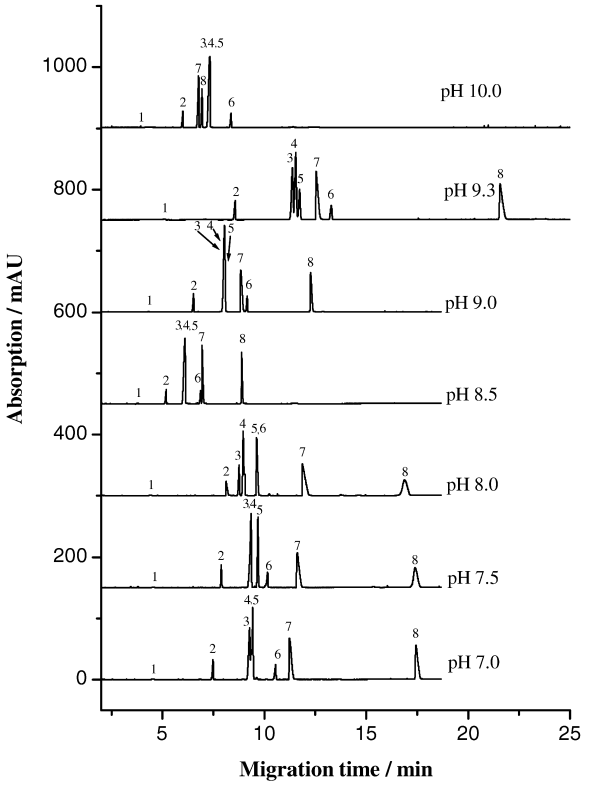

Fig. 1. Effect of buffer pH on separation. Conditions: buffer: 50 m*M* borate solution with varied pH, containing 50 m*M* SDS; capillary: 48.5 cm (40 cm effective length)×360 μm O.D.×50 μm I.D.; detection wavelength: 210 nm; injection: hydrodynamic injection, 5 s at 50 mbar; applied voltage: 15 kV; temperature: 25 °C. Peak identity: 1, EOF; 2, ABA; 3, IAA; 4, NAA; 5, 2,4-D; 6, KT; 7, GA; 8, BA.

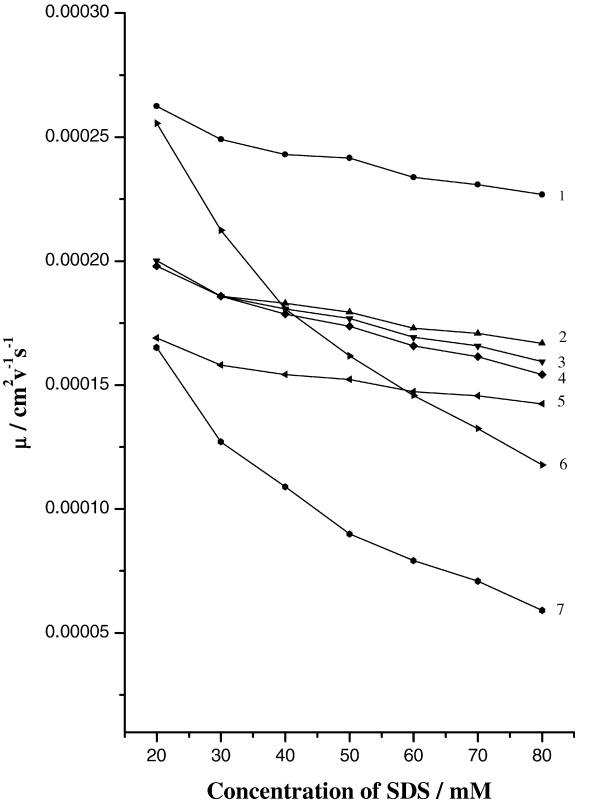

Fig. 2. Effect of concentration of SDS on separation. Conditions: buffer: 50 m*M* borate containing various concentrations SDS (pH 9.3). Other conditions as in Fig. 1. Curve identity: 1, EOF; 2, ABA; 3, IAA; 4, NAA; 5, 2,4-D; 6, KT; 7, GA; 8, BA.

separation was investigated. It was found that the selectivity of the separation was enhanced by increasing SDS concentration as shown in Fig. 2, while using 50 m*M* borate buffer (pH 9.3). The variation in SDS concentration changed the partition fractions of hormones in SDS phase, which further caused the changes in the observed mobility. Consequently, the increase in SDS concentration increased the difference in electrophoretic migration velocity among analytes resulted from the difference partition coefficient. Of seven plant hormones, the migrations of GA and BA were very sensitive to the variation in SDS concentration due to their highly hydrophobic nature. The peak of BA overlapped the peak of KT peak with 20 m*M* SDS as micellar additive in the running buffer. However, if the SDS concentration was increased to 30 m*M*, the co-eluted peaks could be completely separated. Similarly with IAA, NAA and 2,4-D, the three species could not be identified in a buffer containing relatively low concentration of SDS due to their similar mobility. An SDS concentration above 50 m*M*, 60 m*M* for example, improved their resolutions. Nevertheless, the peak of GA overlapped that of KT at such a SDS concentration by virtue of the mobility shift of GA and KT. An SDS concentration of 70 m*M* was finally selected as optimized parameter for further investigation, at which concentration all the hormones were thoroughly resolved. Although a higher SDS concentration would lead to better resolution, the separation time would increased accompanied by a loss of separation efficiency.

3.4. Effect of applied voltage on separation

Due to the use of capillary in the electrophoretic method, high voltage up to 30 kV could be employed with high separation performance. However, ultra high voltage would cause apparent Joule heating effect if the produced heat could not be effectively dissipated by the capillary. With the increase in voltage, the separation resolution was improved at first. However, it became worse when the separation voltage increased above 15 kV. The separation efficiency calculated as the theoretical plate number also decreased, due to the Joule heating effect. Accumulated heat in capillary disimproved the separation quality. Although higher voltage could speed up separation, 15 kV was chosen as the best separation voltage.

Based on the optimized conditions mentioned above, seven hormones were successfully separated in 30 min at a detection wavelength of 210 nm as shown in Fig. 3.

3.5. Effect of detection wavelength on sensitivity

Table 1 lists the detection limits of the hormones at 210 nm, calculated on the basis of a peak height three times the baseline noise ($S/N=3$). Because the UV spectra of seven plant hormones are quite different (data not shown), it is impossible to find a wavelength at which all the analytes got the largest absorption if a single detection wavelength is employed. For improving detection sensitivity, multi-wavelength detection was used and an approximate 2-fold improvement for ABA (246 nm), IAA (220 nm), NAA (225 nm), 2,4-D (200 nm) and KT (200 nm) was found as indicated in Table 1. Furthermore, the UV spectra obtained could facilitate the qualitative identification of separated species in the electropherogram according to their intrinsic UV spectra.

3.6. Employment of bubble cell

The poor sensitivity of UV detection is due to the short optical path of the detection cell. Much work has been done to overcome this limitation by using expanded detection cells, such as bubble cells [33], ''Z'' shape cells [34], rectangular cells [35] and multireflection cells [36]. Here, the bubble cell was used to further enhance separation sensitivity. Because the use of a bubble cell would lead in practice to a decrease in separation resolution, a capillary of

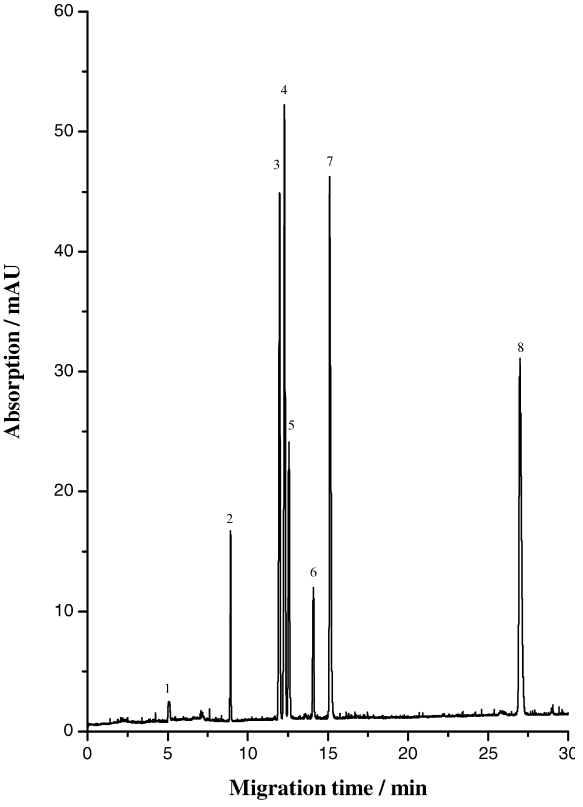

Fig. 3. Electropherogram of separating seven plant hormones. Conditions: buffer: 50 m*M* borate containing 70 m*M* SDS (pH 9.3). Other conditions as in Fig. 1. Peak identity: 1, EOF; 2, ABA; 3, IAA; 4, NAA; 5, 2,4-D; 6, KT; 7, GA; 8, BA.

Table 1
Comparison of detection limits

Species	Detection limit ($\mu g\ ml^{-1}$)			
	210 nm	MWD	BCC	Stacking
ABA	0.31	0.17	0.069	0.00101
IAA	0.12	0.11	0.038	0.00133
NAA	0.077	0.046	0.029	0.000306
2,4-D	0.18	0.086	0.039	Not included
KT	1.37	0.35	0.212	0.00242
GA	0.12	0.12	0.065	0.00820
BA	0.13	0.13	0.070	0.00668

BCC, bubble cell capillary; MWD, multi-wavelength detection.

longer effective length was used accompanied by higher applied voltage (25 kV) to keep the electric field strength of separation similar to that in normal capillary. A factor of ~2–3 was obtained for all the analytes as shown in Table 1. However, the actual amount of hormones present in the plant (nanogram per gram fresh tissue) still could not be determined. As a result, other approaches should be developed to further improve separation sensitivity.

3.7. Application of on-line concentration procedure

Two techniques, the electrostacking scheme, termed the normal stacking (NS) mode and the large volume sample stacking (LVSS) mode, are often used. NS is the simplest sample concentration method. It is achieved by hydrodynamically injecting a sample dissolved in a low conductivity matrix. The stacking effect for the sample occurs at the interface between sample and buffer zones due to the non-uniform electric field distribution. The limitation of NS is its low enrichment factor (~10) because of the short length of optimum sample zone [14]. LVSS [18] overcomes such limitation. A larger sample zone than that in NS is introduced into the capillary. To facilitate subsequent separation, the sample matrix should be pumped out of the capillary by EOF [18] or external pressure [19]. An enrichment factor of more than 100 can be accomplished with LVSS. Its apparent limitation is that only negative or positive species can be effectively concentrated in a single run. Because the plant hormones included in this work are all anionic species, a reverse electrode polarity-stacking (REPSM) mode [37,38] in LVSS was selected for on-line sample concentration. The same capillary was used with longer length than that employed for bubble cell detection.

As described in Fig. 4, a large volume sample zone was introduced into the capillary (step 1). To avoid sample leakage, a short buffer zone was then injected (step 2). Consequently, the sample zone was sandwiched between two buffer zones. The EOF was used as a pump to clear the sample matrix out of the capillary by applying negative voltage, so-called stacking voltage, across the capillary (steps 3 and 4). The stacking voltage is −10 kV, lower than that used in separation (25 kV), so as to have sufficient stacking time to thoroughly remove the sample

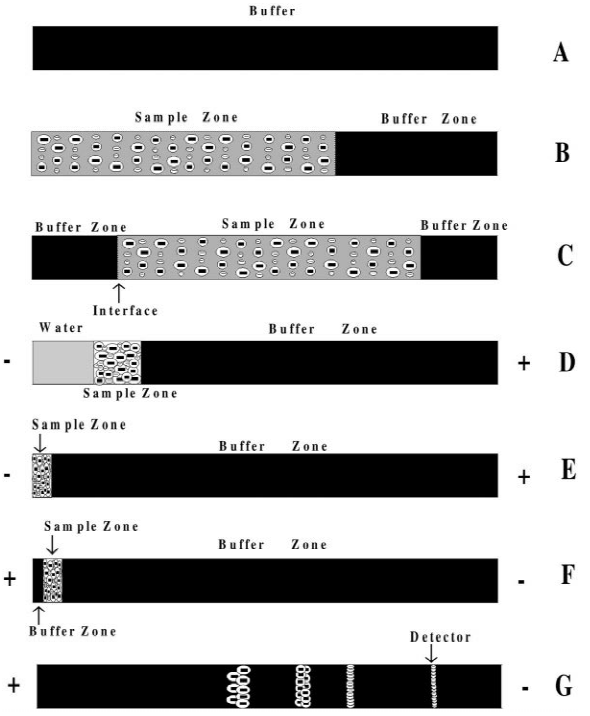

Fig. 4. Schematic description of LVSS procedures.

matrix from the capillary without any loss of the analytes. With REPSM, the current shift in stacking step should be monitored in real time. The current was first gradually increased with the removal of the sample matrix, instead of the running buffer. When it reached 95% of the actual current that was obtained by filling the capillary thoroughly with the running buffer, polarity was switched to start the subsequent MECC separation (step 5). Due to the high performance of MECC and the high enrichment factor of LVSS, analytes were completely separated with high sensitivity by following the separation conditions discussed above (step 6).

In theory, the maximum fraction (α_{max}) of the column filled with sample zone is given by following equation [38]:

$$\alpha_{max} = \mu_{ep}(mc)/\mu_{eof}$$

where $\mu_{ep}(mc)$ and μ_{eof} represent electrophoretic mobility of micelle and EOF, respectively. Furthermore, an increase in sample zone would cause loss

of analytes into the buffer vial [16]. In this work, the injection plug of sample was optimized by experimentally varying the injection time at a fixed injection pressure (950 mbar). An injection time of 0.6 min was chosen for achieving the best detection sensitivity.

Based on the optimizing on-line concentration procedures, six endogeneous plant hormones were completely separated with high sensitivity (data shown in Table 1). 2,4-D was not included here, because it was an exogeneous hormone. Fig. 5 compares the MECC separations of samples with LVSS concentration (A), bubble cell capillary (B) and normal procedure (C). The enhancement in detection sensitivity is obvious. Together with on-line LVSS, the MECC method described in this work could be applied to the analysis of hormones in plant.

3.8. Quantification

Calibration curves were established from seven concentration levels of mixed samples, which showed very good linearity ranged within three orders of magnitude between peak area and sample concentration. Relevant data are given in Table 2. Relative standard deviations (RSDs) of peak area were calculated based on five duplicate injections of a diluted sample. RSDs of peak height were calculated from injections of samples with different concentrations used to evaluate calibration curves. The high reproducibility of the method indicated that MECC is reliable for analyzing plant hormones. It was very important to designate the species from the extractions in plant tissues. The separation efficiency, calculated as theoretical plate number, was obtained by averaging the data from electropherograms for establishing calibration curves.

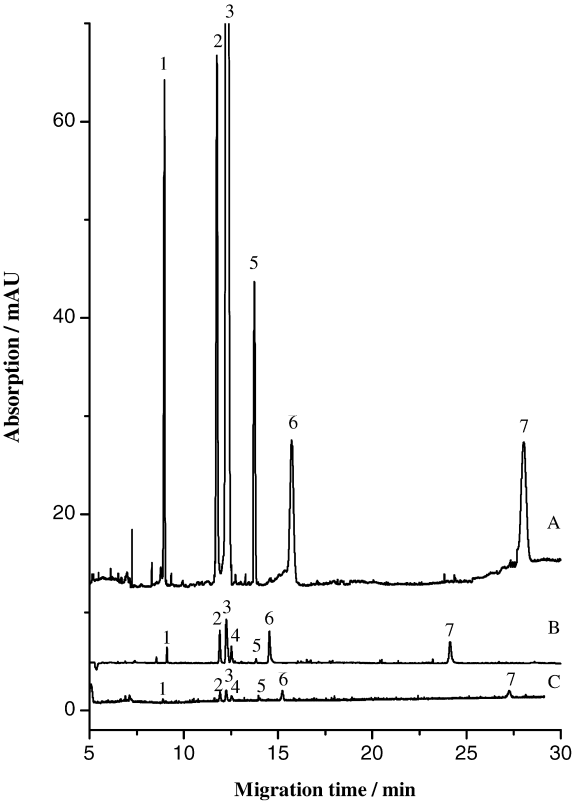

Fig. 5. Comparison of electropherograms of LVSS (A), BCC (B) and normal capillary cell (C). Conditions: (A) buffer: 50 m*M* borate containing 70 m*M* SDS (pH 9.3); bubble cell capillary: 64.5 cm (56 cm effective length)×360 μm O.D.×50 μm I.D., bubble factor: 3; detection wavelength: 210 nm; stacking voltage: −10 kV, separation voltage: +25 kV; temperature: 25 °C; sample injection: hydrodynamically, 36 s at 950 mbar; (B) buffer: 50 m*M* borate containing 70 m*M* SDS (pH 9.3); bubble cell capillary: 64.5 cm (56 cm effective length)×360 μm O.D.×50 μm I.D., bubble factor: 3; detection wavelength: 210 nm; separation voltage: −25 kV; temperature: 25 °C; sample injection: hydrodynamically, 5 s at 50 mbar; (C) conditions as in Fig. 3. Samples for A, B and C are the same. Peak identity: 1, EOF; 2, ABA; 3, IAA; 4, NAA; 5, KT; 6, GA; 7, BA.

3.9. Application to real-world samples

The developed methodology was applied to monitor the changes in ABA in flowers of transgenic tobacco under drought stress, where the availability of water is reduced. As shown in Fig. 6, ABA was successfully identified by migration time (within the RSD of this method) and UV spectrum from miscellaneous components. Because of the interfaces from other impurities such as pigments, pretreatment procedures with C_{18} column are required if detecting other hormones. It is our intention to measure the changes with varying environmental factors in the other main hormones in various tissues of transgenic plants such as root, stem, leaf, flower and seed.

Table 2
Quantification data

Species	Calibration curve ($y=a+bx$)		γ	N ($\cdot 10^5$)	RSD (%)		Linear range (μg ml^{-1})
					PA	t_m	
	a	b					
ABA	3.10	264.2	0.9998	1.71	0.12	0.45	2–0.004
IAA	10.84	322.7	0.9998	1.21	0.15	0.48	6–0.01
NAA	27.03	1497	0.9991	1.11	0.14	0.54	2–0.004
KT	0.39	487.4	0.9998	1.55	0.15	0.11	1–0.002
GA	14.42	70.05	0.9998	1.20	0.27	0.98	40–0.08
BA	18.48	135.1	0.9995	1.49	0.28	1.04	20–0.04

N, theoretical plate number; PA, peak area; t_m: migration time; x, concentration of analyte (μg ml^{-1}); y, integrated peak area (mAU s).

4. Conclusion

It was demonstrated that the main hormones in plant were successfully separated and simultaneously determined by MECC. Multi-wavelength detection and bubble detection cell were employed to improve separation sensitivity, but proved to be unable to detect low amounts of hormones in plant tissues. LVSS was developed to further enhance the sensitivity of the separation with an enrichment factor of ~10–600, and was able to detect hormones down to ng/ml level. The MECC method coupling with LVSS was successfully applied to identifying trace amounts of ABA in tobacco flowers.

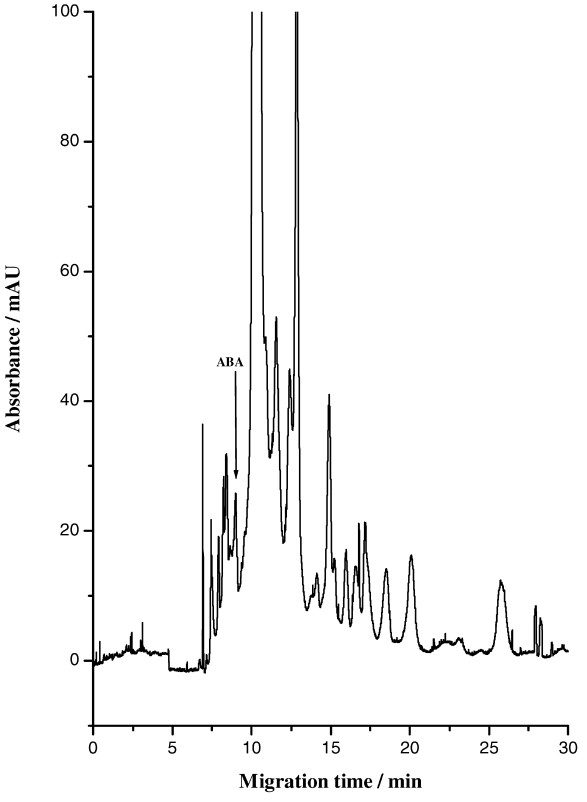

Fig. 6. Electropherogram of extraction of tobacco flowers. Conditions are the same as in Fig. 5A.

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