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PAPER

Analysis of plant hormones by microemulsion electrokinetic capillary chromatography coupled with on-line large volume sample stacking

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A novel method of microemulsion electrokinetic capillary chromatography (MEEKC) coupled with on-line large volume sample stacking was developed for the analysis of six plant hormones including indole-3-acetic acid, indole-3-butyric acid, indole-3-propionic acid, 1-naphthaleneacetic acid, abscisic acid and salicylic acid. Baseline separation of six plant hormones was achieved within 10 min by using the microemulsion background electrolyte containing a 97.2% (w/w) 10 mM borate buffer at pH 9.2, 1.0% (w/w) ethyl acetate as oil droplets, 0.6% (w/w) sodium dodecyl sulphate as surfactant and 1.2% (w/w) 1-butanol as cosurfactant. In addition, an on-line concentration method based on a large volume sample stacking technique and multiple wavelength detection was adopted for improving the detection sensitivity in order to determine trace level hormones in a real sample. The optimal method provided about 50–100 fold increase in detection sensitivity compared with a single MEEKC method, and the detection limits ($S/N = 3$) were between 0.005 and 0.02 μg mL^{-1}. The proposed method was simple, rapid and sensitive and could be applied to the determination of six plant hormones in spiked water samples, tobacco leaves and 1-naphthylacetic acid in leaf fertilizer. The recoveries ranged from 76.0% to 119.1%, and good reproducibilities were obtained with relative standard deviations (RSDs) less than 6.6%.

Introduction

Plant hormones play crucial roles in mediating plant growth, such as growth, differentiation, metabolism, and morphogenesis.[1,2] These hormones also respond to environmental stimuli including photoperiod, temperature, water and nutrition supply.[3] Based on their structure and physiological function, these hormones are categorized into several major classes including auxins, cytokinins (CK), abscisic acid (ABA), gibberellins (GAs), ethylene (ET), brassinosteroids (BR), jasmonates (JA) and salicylic acid (SA).[4,5] Recently, some synthetic hormones emerged in mediating plant growth, which had similar activity of exogenous hormones.[6] For example, 1-naphthylacetic acid (NAA) is an important synthetic plant growth regulator which is widely used in promoting seed germination, stem elongation, cone production *etc*. Plant hormones have some side effects; excessive use is one of the prime factors causing water and soil pollution and long-term consumption of plant hormone residues in fruits and vegetables can cause endocrine disorders.[7] Therefore, it is necessary to develop a rapid and sensitive method for effective monitoring of the plant hormone residues in the environment.

Nowadays, several methods have been used for the analysis of plant hormones. Bioassay methods were widely used in the field of the plant hormone analysis due to their high sensitivity and specificity.[8] However, the antibody preparation required for this technique was rather laborious, and it is inevitable to encounter cross-reactivity of the antibody with other plant hormone analogues. Meanwhile, chromatographic methods including gas chromatography-mass spectrometry (GC-MS)[9,10] and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS)[11–14] have been applied in the field of plant hormone analysis, where derivatization steps are required to improve volatility and sensitivity of the hormones in the GC-MS method. Derivatization is complicated reaction between various analytes and derivatization reagents, which may further introduce complicated byproducts,[15] and the high temperature in the GC injector and column also leads to the thermal breakdown of labile compounds such as abscisic acid glucosyl ester.[16] Recently, LC-ESI-MS has been widely employed for the quantitative analysis of plant hormones such as indole-3-acetic acid (IAA),[17,18] ABA,[19] and CK[20] without derivatization. In addition, a few groups have reported the determination of IAA and ABA;[21] IAA, ABA, and JA;[18] IAA, ABA, CK, and GA;[22] and IAA, ABA, CK, GA, JA, and SA.[23] Capillary electrophoresis

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(CE) has been recognized as a powerful method for analyzing biomolecules. Several literatures have reported the determination of plant hormones by CE.[24–26] Extensions of the CE technique including capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC) and microemulsion electrokinetic capillary chromatography (MEEKC) have been developed for bioanalysis. Among them, CZE and MEKC have been used for the analysis of plant hormones.[27–29]

MEEKC is an attractive electrodriven separation technique, which offers the possibility of highly efficient separation of both charged and neutral solutes covering a wide range of water solubilities.[30] MEEKC can be regarded as an extension of MEKC with the micelles being replaced by the oil droplets wrapped by surfactant as the pseudostationary phase. In addition to the surfactant, a cosurfactant such as a short-chain alcohol is often added to further stabilize the microemulsion system as it bridges the oil and water interface and reduces the surface tension. Analytes are able to more easily penetrate the surface of oil droplets as compared with the more rigid surfaces of micelles, which enhances their solubilities in the microemulsion solution. Therefore, excellent separation of highly hydrophobic analytes can be expected. Furthermore, due to a higher electrophoretic mobility of the microdroplets compared to micelles, MEEKC offers higher sample capacity[31] and a wider migration window[32] than MEKC does. Due to the unique properties, MEEKC has gained considerable attention and widespread application in various fields.[33–38] Although MEEKC is regarded to be a good separation technique, it is well known that its detection sensitivity still restricts its application in the analysis of trace-level composition. Therefore, it is necessary to develop MEEKC coupled with an on-line sample concentration technique. On-line sample concentrations techniques include electrostacking, field-amplified sample injection (FASI), large volume sample stacking (LVSS), sweeping, anion-selective exhaustive injection (ASEI) and isotachophoresis (ITP).[29] These concentration methods can offer an enrichment factor ranging from 10^1 to 10^6.[39] However, no papers have been reported so far on MEEKC coupled to an on-line sample concentration technique for the determination of plant hormones.

The purpose of this study was to develop a novel MEEKC method for analysis of six plant hormones: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), 1-naphthaleneacetic acid (NAA), abscisic acid (ABA) and salicylic acid (SA) (their molecular structures are shown in Fig. 1). The feasibility of an on-line sample concentration procedure coupled with MEEKC was evaluated in this paper. The different aspects affecting MEEKC separation were investigated in detail, and a large volume sample stacking was designed to increase the detection sensitivity. Furthermore, the proposed study provided a simple, effective and sensitive method for determination of plant hormones in water samples, tobacco leaves and 1-naphthylacetic acid in leaf fertilizer.

Experimental

Chemicals

IAA, IBA, IPA, NAA, ABA and SA were obtained from J&K Chemical (Shanghai, China). Sodium dodecylsulfate (SDS) was purchased from Acros Organics (Geel, Belgium). Salicylic acid (SA), tetraborate, 1-butanol, and ethyl acetate (analytical grade) were purchased from Kermel Chemical Reagents Development Centre (Tianjin, China). Acetonitrile (HPLC-grade) were provided by Sinopharm Chemical Reagents (Shanghai, China). An uncoated fused-silica capillary was a product of Yongnian Optic Fiber Factory, (Hebei, China). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

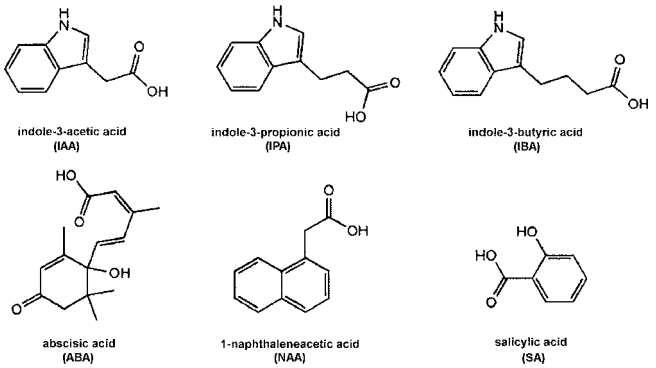

Fig. 1 Chemical structures of the six plant hormones.

Instrumentation

An Agilent CE^{3D} system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) was employed for the separation and determination of the target plant hormones. Data acquisition and processing were performed with Agilent ChemStation software. All pH values of running buffer was measured by a PHS-3C meter (Shanghai Dapu Instument Company, Shanghai, China). Prior to use, all mobile phases for MEEKC were degassed with a KQ3200E ultrasonic bath (Kuisan, China).

Preparation of running buffer for MEEKC

Borate buffer was prepared from tetraborate and pH-adjusted with sodium hydroxide or hydrogen chloride. The optimum microemulsion was prepared as follows: 0.6% (w/w) SDS, 1.2% (w/w) 1-butanol, 1.0% (w/w) ethyl acetate and 97.2% (w/w) 10 mM borate buffer (pH 9.2) were mixed. The mixture was then sonicated for 30 min to obtain the stable and optically transparent microemulsion system. The solutions were filtered through a 0.22 μm microfilter prior to vialing.

Preparation of standard solutions and samples

A stock standard solution of 1.0 mg mL^{-1} of each analyte was prepared in acetonitrile and then stored in the refrigerator at 4 °C. They were mixed and subsequently diluted with deionized water as needed. Water samples were collected from drinking water, which were filtered with 0.22 μm microfilter before use. Approximately 0.5 g of fresh tobacco leaves were frozen in liquid nitrogen and ground into a powder. A 3 mL aliquot of methanol–H_2O (3 : 1, v/v) was added and shaken for 30 min and deposited for 12 h at 4 °C. Methanol–H_2O (3 : 1, v/v) (1 mL) was added, re-shaken for another 30 min and then centrifuged at 5000 rpm for 10 min. The combined extract was filtered through

a 0.45 μm filter and distilled at 45 °C under reduced pressure, then the 1 mL methanol was added to dissolve the obtained extraction for analysis. The leaf fertilizer powder (approximately 8.0 mg) was dissolved in 250 mL of deionized water and then stored at 4 °C. Prior to analysis, the sample was diluted to the desired concentration and injected directly into the capillary. All the standard solutions and sample solution were filtered through a syringe cellulose acetate filter (0.22 μm) and sonicated before use.

MEEKC and LVSS procedures

Fresh capillaries were pretreated by flushing with 0.1 M NaOH for 30 min, water for 30 min and then running buffer for 30 min. This procedure was also used at the beginning of each sequence of runs for the repeatability of migration times. Electrophoretic separation was carried out in a 63 cm fused-silica capillary (54.4 cm effective length) × 50 μm i.d. × 375 μm o.d with separation voltage of 20 kV. Before the introduction of a sample, the capillary was flushed with buffer for 2 min. Normal sample injection mode with a pressure of 30 mbar for 6 s was adopted and the cassette was thermostated at 25 °C. DAD was performed by multiple wavelength scanning at 204 nm (for SA), 214 nm (for IAA, IPA, IBA and NAA) and 246 nm (for ABA).

After the capillary had been conditioned with the microemulsion separation solution, the LVSS injection mode was performed at 50 mbar for 240 s. Firstly, the injection end of the capillary was placed on a microemulsion separation solution and a voltage of −5 kV was applied. Then, when the current was 95% and the capillary was only filled with background solution, the voltage was turned off and the voltage separation began.

Results and discussion

Effect of pH and concentration of borate buffer

As we know, the pH of the running buffer plays an important role in the separation selectivity, which can affect both the ionization of analytes and the magnitude of EOF.[40] Also, it has a great effect on the degree of dissociation of solutes and their solubility in the microemulsion. A series of borate buffers with different pH values in the range of 7.0–10.0 were investigated. The experimental results showed that the influence of pH on separation was significant for IPA and NAA, but slight for IAA, IBA, ABA and SA. The peaks of IPA and NAA happened to partly overlap and peak shapes of analytes were not symmetrical below pH 9.2 of running buffer, the reasonable explanation was possibly the affinity between hydrophobic analytes and the oil droplet. When pH values varied from 9.2 to 10.0, six plant hormones were well separated and peak shapes were improved due to the pK_a values less than 7.0 (IAA, pK_a 4.75; IPA, 6.15; IBA, 4.80; ABA, 6.8; NAA, 4.23; SA, 2.98). The result further indicated that more analytes were ionized with the increase of pH, which led to a stronger repulsive force between the increasing negative charge of the analytes and the negative microemulsion droplets. However, pH values exceeding 10 in CE caused damage to the silica surface of the capillary. Considering separation efficiency and the analysis time, the pH of 9.2 was selected for the running microemulsion conditions.

The effect of borate concentration was also investigated at the pH of 9.2 in MEEKC separation. It was found that the migration time of analytes increased with varying the borate concentration from 5 to 50 mM, whereas the resolution showed no difference. Considering with analysis time and Joule heat generated in the capillary, the concentration of 10 mM was chosen in the separation.

Effect of surfactant

In the MEEKC system, the surfactant has a remarkable effect on the separation selectivity, which can affect the charge and size of the microemulsion droplet, the direction and magnitude of the EOF and the level of ion-pairing with charged analytes.[40] SDS, which is a commonly employed surfactant in the MEEKC system, was used in this study, and the influence of SDS concentration on separation was then evaluated over the 0.3%–1.8% (w/w) concentration range using 1.2% (w/w) 1-butanol, 1.0% (w/w) of ethyl acetate and 97.2% (w/w) 10 mM borate buffer (pH 9.2). As shown in Fig. 2, when the SDS concentration was 0.3% (w/w) or 0.6% (w/w), baseline separation of plant hormones was obtained and the migration time of analytes changed slightly. However, with the increase of SDS concentration, the peaks of IPA and NAA happened to overlap and when the SDS concentration started to exceed 1.2% (w/w), the migration time of all analytes increased gradually. A higher concentration of the surfactant increases the capacity factor of neutral solutes as it increases the charge density on the oil droplet, and the higher surfactant concentration also increases the ionic strength of buffer, reducing the EOF and increasing migration time.[41] Due to the stability of the microemulsion requiring enough surfactant, a SDS concentration of 0.6% (w/w) was employed for further investigation in this study.

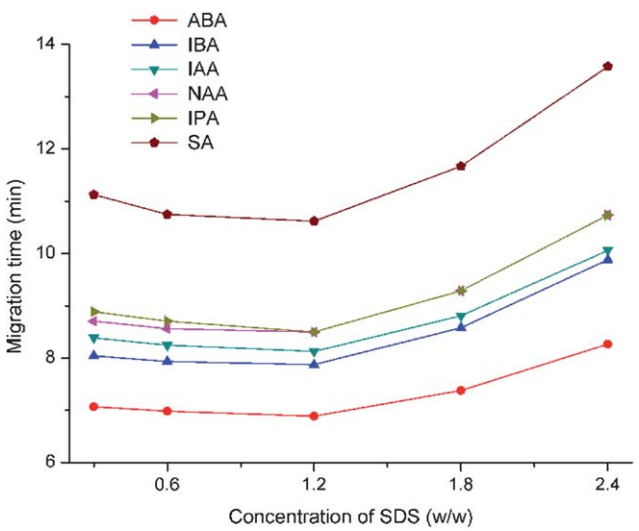

Fig. 2 Effect of SDS percentage (w/w) on migration time. Conditions: 10 mM borate buffer (pH 9.2) containing various SDS percentages. 1.2% (w/w) 1-butanol,1.0% (w/w) ethyl acetate and 97.2% (w/w) 10 mM borate buffer with various pH; capillary, 63 cm (54.4 cm effective length) × 50 μm i.d. × 375 μm o.d.; applied voltage, 20 kV; hydrodynamic injection, 30 mbar for 6 s; detection wavelength, 214 nm; temperature, 25 °C.

Effect of cosurfactant

In MEEKC, the cosurfactant is the most influential constituent of the microemulsion on separation selectivity, which can be solubilized into the microemulsion layer and allows the formation of liquid film in the microemulsion droplets and increases the mechanical strength of the composite membrane and stability of the microemulsions.[42] In our experiment, 1-butanol was used in the MEEKC system as a cosurfactant. The effect of 1-butanol concentration on the stability of the microemulsion and separation of six plant hormones was investigated. The results indicated that the selectivity of separation did not change with variation of 1-butanol concentration at a wide range of 0.3–2.4% (w/w). However, when the 1-butanol concentration was above 1.2% (w/w), the migration time of analytes was slightly longer, and we also found that the microemulsion was damaged with a 1-butanol concentration above 2.4%(w/w) in the study. To shorten analysis time and obtain a more stable microemulsion, a concentration of 1.2% (w/w) was finally chosen as the optimum parameter.

Effect of oil type and concentration

Octane, ethyl acetate, heptane and hexane are the most commonly used oils in MEEKC. In our experiment, oil types (ethyl acetate, heptane and hexane) were investigated. The results indicated that the six plant hormones had better separation with ethyl acetate than heptane and hexane, and our results agree with the literature.[43] A reasonable explanation is that heptane and hexane lead to microemulsions with a high surface tension, which means that for the microemulsion to be stabilized, the level of the surfactant must be high. When the microemulsion includes heptane (or hexane), 1-butanol, SDS and borate buffer, the separation was slow because a high concentration of SDS was required to form droplets. With the ratio of the surfactant increasing, the EOF decreases which slows down the migration of droplets and analytes to the detector. However, ethyl acetate has a lower surface tension and has been widely used in MEEKC.[43–45] Ethyl acetate could be used to form microemulsions with less surfactant and reduce the analysis time. Therefore, ethyl acetate was adopted as the oil phase and its concentration was optimized. The experimental results indicated that the six plant hormones were separated in the range of 0.5–2.5% (w/w) ethyl acetate. The migration time of analytes became longer with the increase of the concentration of ethyl acetate. Considering analysis time and the stability of microemulsion, a concentration of 1.0% (w/w) was selected.

Enhancing sensitivity through LVSS procedures

As previously reported, CE is not a very sensitive technique and some applications need highly sensitive methods if some analytes are to be determined at trace levels. To improve the detection sensitivity, several on-line concentration techniques have been developed.[29] In our experiment, FASI and LVSS were applied to enhance the detection limits. However, when the FASI technique was used in this study, the results showed that the FASI technique could not match with these plant hormones and induced poor separation efficiency. So, LVSS was selected as an on-line concentration in this study. LVSS is a model of sample concentration in CE, which could overcome the drawbacks of too high injection volume in FASI concentration techniques.

In LVSS, firstly, a large sample zone is introduced into the capillary, and then the sample matrix must be removed by EOF or external pressure to facilitate subsequent separation. Due to analytes stacking in the inlet of the capillary, detection sensitivity is improved without the loss of separation efficiency, resulting in an enrichment factor up to 100-fold. However, its apparent limitation is that only negative or positive species can be effectively concentrated in a single run. So, the pH of borate buffer was selected at 9.2 to ensure the six plant hormones were all anionic species in our experiment. When the current reached 95% of its initial value, LVSS with polarity switching was performed for on-line sample concentration.

To avoid possible leakage of the sample zone caused by the injection of a large volume sample, a short plug of running buffer was introduced by hydrodynamic injection, and then the negative stacking voltage was applied. The effect of injection time at 50 mbar was investigated for the highest absorbance of reasonable peak width. The peak area of analytes gradually increased with the change of injection time. When the injection time exceeded 240 s, the peak area kept a platform, peak shape became poor and the stability of baseline deteriorated. Therefore, 240 s was selected as the optimum injection time. As shown in Fig. 3, compared with normal hydrodynamic injection, the six plant hormones were well separated and the obtained enrichment factor was in the range of 50–100 using the LVSS technique.

Method validation

The performances of analysis of several plant hormones with LVSS-MEEKC systems were compared the other reported methods. It was known that analytical time was very long and vast amounts of organic solvent were required as the mobile phase in the LC-MS method,[17–19] which would not meet the needs of throughput analysis. A previous study on the analyses of the plant hormones with a CZE method was reported by A. Segura Carretero,[46] with detection limits of around 0.45–1.04 mg L^{-1}. The developed LVSS-MEEKC method enhances the detection

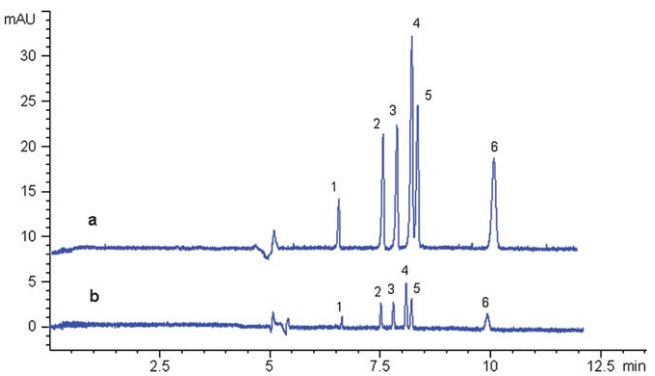

Fig. 3 Comparison of electropherograms between normal hydrodynamic injection and LVSS. Conditions: (a) LVSS: sample, 50 mbar for 240 s; buffer, 50 mbar for 5 s; stacking voltage, −10 kV for 45 s; sample concentrations, 1 μg mL^{-1} (b) Normal hydrodynamic injection: 30 mbar for 6 s; sample concentrations, 10 μg mL^{-1}. Others were same as Fig. 2. Peaks: 1. ABA, 2. IPA, 3. IAA, 4. NAA, 5. IBA, 6. SA.

Table 1 Regression equations, linearity, detection limits and repeatability for the method[a]

Compound	Regression equation[b]	R^2	Linear range (μg mL^{-1})	Detection limit (μg mL^{-1})	RSD (%) (n = 5) Migration time	RSD (%) (n = 5) Peak area
ABA	$y = 25.254x + 5.3351$	0.9941	0.10–5.00	0.02	2.9	9.1
IBA	$y = 43.848x + 2.4807$	0.9982	0.10–5.00	0.01	3.5	3.6
IAA	$y = 39.784x + 1.7345$	0.9941	0.10–5.00	0.008	3.4	4.2
NAA	$y = 72.953x + 2.0352$	0.9998	0.10–5.00	0.005	3.5	6.2
IPA	$y = 39.918x + 0.6837$	0.9983	0.10–5.00	0.007	3.3	5.7
SA	$y = 136.2x + 6.2546$	0.9990	0.10–5.00	0.012	4.2	1.8

[a] The conditions were the same as in Fig. 3a. [b] y: peak area, (mAU × s); x: mass concentration, μg mL^{-1}.

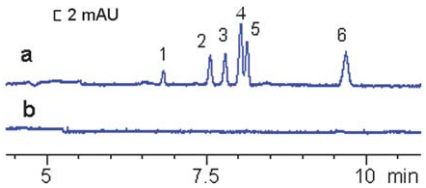

Fig. 4 Electropherogram of six plant hormones in a spiked water sample: (a) sample spiked with 0.5 μg mL^{-1} standard mixture; (b) blank water sample. Conditions were same as in Fig. 3a.

limits about 50–100 fold. The method was validated under the optimum microemulsion background electrolyte containing 1.0% (w/w) ethyl acetate, 0.6% (w/w) SDS, 1.2% (w/w) 1-butanol and 97.2% (w/w) 10 mM borate buffer at pH 9.2, and the analytical time of six plant hormones was less than 10 min. Because the UV spectra of the six plant hormones are different, it is difficult to find a wavelength at which all the analytes have the largest absorption if a single detection wavelength is employed. For quantitative application and detection sensitivity, multiple wavelength detection was carried out with a 2-fold improvement for ABA (246 nm) and SA (204 nm).

After the method had been established, a calibration step was carried out. A series of mixed standard solutions of the six plant hormones with different concentrations were prepared for the determination of the linearity and detection limits of this method. The results were presented in Table 1. The calibration curves exhibit excellent linearity and the limit of detections (based on a signal-to-noise ratio of 3) for analytes were from 0.005 to 0.02 μg mL^{-1}. Repeatability was determined by a mixed standard solution of six plant hormones. The results indicated the RSDs of migration time within 4.2% and peak areas less than

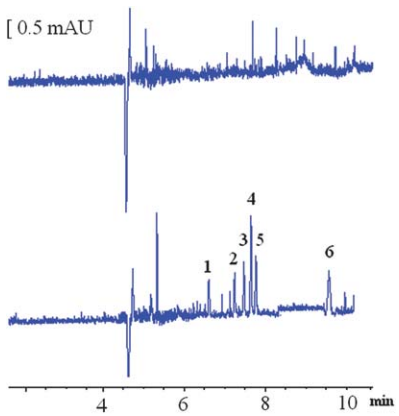

Fig. 5 Electropherogram of six plant hormones in tobacco leaves sample spiked with 2.0 μg mL^{-1}. Conditions were same as in Fig. 3a.

9.1% (n = 5) were also obtained. The above results demonstrated that the proposed method of MEEKC coupled with on-line large volume sample stacking have a great potential in the analysis of plant hormones.

Sample analysis and recovery

In this study, the established method was applied to determine three kinds of real samples, including spiked water samples, tobacco leaves and leaf fertilizer. As shown in Fig. 4 and Fig. 5, the results indicated that none of the plant hormones were detected in water samples and tobacco leaves. The recoveries were determined by addition of mixed standard solutions of six plant hormones into the water sample and tobacco leaves under the same conditions. The results showed that the recoveries of

Table 2 Recovery of six plant hormones in water sample (n = 5)[a]

Compound	Added amount (μg mL^{-1})	Found amount (μg mL^{-1})	Recovery (%)	RSD (%)
ABA	0.50	0.40	80.2	6.6
IBA	0.50	0.48	96.1	2.0
IAA	0.50	0.47	94.7	1.1
NAA	0.50	0.55	110.4	0.9
IPA	0.50	0.59	119.1	4.0
SA	0.50	0.54	109.1	5.4

[a] The conditions were the same as in Fig. 3a.

Table 3 Recovery of six plant hormones in tobacco leaf sample ($n = 5$)[a]

Compound	Added amount (μg mL^{-1})	Found amount (μg mL^{-1})	Recovery (%)	RSD (%)
ABA	0.50	0.38	76.0	5.8
	2.0	1.59	79.5	5.3
IBA	0.50	0.44	88.1	4.2
	2.0	1.78	88.9	5.1
IAA	0.50	0.45	90.1	3.1
	2.0	1.74	87.3	3.7
NAA	0.50	0.51	102.2	2.5
	2.0	2.08	104.3	2.6
IPA	0.50	0.57	114.6	4.3
	2.0	2.14	107.5	4.7
SA	0.50	0.53	106.2	5.6
	2.0	2.02	101.4	6.3

[a] The conditions were the same as in Fig. 3a.

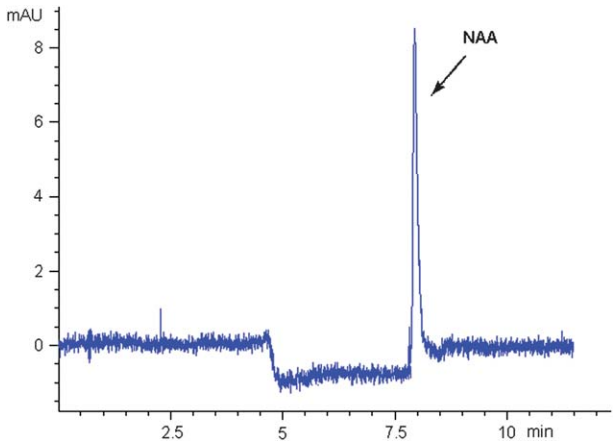

Fig. 6 Electropherogram of NAA in leaf fertilizer. Conditions were same as in Fig. 3a.

these analytes were in the range of 80.2–119.1% in Table 2 and 76.0–114.6% in Table 3, respectively. Due to the difference of nature and structure, the six plant appeared different recoveries by the same pretreatment for samples. Nevertheless, it could meet the quantitative performances.

In addition, in order to further confirm the feasibility of the method, the leaf fertilizer sample was further analyzed by the optimized LVSS-MEEKC method according to the mention in above section. As can be seen from Fig. 6, it was calculated that the content of NAA in leaf fertilizer was about 78.9 μg mg^{-1}, which was consistent with the marked content of NAA in leaf fertilizer. Then 10 μg mg^{-1} NAA was spiked in the leaf fertilizer for recovery testing, and an excellent result was obtained. These results support that the proposed method is suitable for its application to real samples.

Conclusions

Six plant hormones (ABA, IAA, IPA, IBA, NAA and SA) were well separated by MEEKC using a borate buffer solution consisting of 97.2% (w/w) 10 mM borate buffer at pH 9.2, 1.0% (w/w) ethyl acetate, 0.6% (w/w) SDS and 1.2% (w/w) 1-butanol within 10 min. In order to develop a more sensitive method for analyzing these compounds, the LSVV technique was adopted in the experiment. The results demonstrated a 50–100 fold increase in detection sensitivity compared with a single MEEKC method and the LODs of each analyte were between 0.005–0.02 μg mL^{-1}. The proposed method was successfully applied to the determination of plant hormones in real samples (water and tobacco leaves) and identifying trace amounts of NAA in leaf fertilizer. It can be expected to be an excellent alternative method for the quantification of plant hormones.

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