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Extraction and Analysis of Auxins in Plants Using Dispersive Liquid−Liquid Microextraction Followed by High-Performance Liquid Chromatography with Fluorescence Detection

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Auxin plays an important role in cell differentiation, apical dominance, and tropism in plants. A new method based on dispersive liquid−liquid microextraction (DLLME) combined with high-performance liquid chromatography−fluorescence detection (HPLC-FLD) has been established to detect auxin. A mixture of $CHCl_3$ (extraction solvent) and acetone (disperser solvent) was injected quickly into a sample solution with desired salt concentration and pH value, and then a cloudy solution consisting of many dispersed fine droplets of $CHCl_3$ was formed. After centrifugation, the sedimented phase was withdrawn and directly analyzed by HPLC-FLD. Under optimal conditions, four auxins were baseline separated within 3.5 min, with the minimal limit of detection of 0.02 ng mL^{-1} and coefficient correlations in the range of 0.9980−0.9995. This simple method was successfully applied to real sample analysis. Experimental results showed that DLLME was a high-performance and powerful preconcentration method to extract and enrich related plant auxin.

KEYWORDS: Auxin; dispersive liquid−liquid microextraction; high-performance liquid chromatography; fluorescence detection

INTRODUCTION

Plant hormones have caused great concern due to their crucial importance in the development of plants. They are structurally diverse compounds in minor amount and regulate almost every aspect of plant life, including the growth and development of plants, response to biotic and abiotic stress, and so on. As an important type of plant hormones, auxins are known for stimulating growth processes such as cell differentiation, apical dominance, and tropism in plants (*1*). Generally, auxins consist of some derivatives such as indole, naphthalene, and chlorinated benzene compounds. As is known, indole and naphthalene derivatives usually contain rigid planar structures and a big π-conjugated system, which are key structural characteristics of fluorescent substances. Therefore, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), and 1-naphthylacetic acid (NAA) all have natural fluorescence properties. Among them, IAA and IBA are endogenous auxins in plants, whereas synthetic auxins including IPA and NAA are mainly used as plant growth regulators. Because exogenous application of IPA and NAA is popular, trace amounts of them in the environment may be expected due to agricultural use, but high concentrations of residues of these compounds in the environment are prohibited in consideration of human health. In fact, there have been some reports (*2*, *3*) involving the analysis of IPA and NAA in soil, fruit, surface water, underground water, and so on. Therefore, it is necessary to establish a sensitive and rapid method to detect IPA and NAA.

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In the past few decades, study on auxins has attracted considerable interest due to their biological and physiological significance. Several analytical techniques have been employed, including high-performance liquid chromatography (HPLC) (*4*, *5*), gas chromatography−mass spectrometry (GC-MS) (*6*, *7*), capillary electrophoresis (*8*), liquid chromatography−mass spectrometry (LC-MS) (*9*−*11*), etc. Among the above methods, HPLC has been proven to be a powerful separation tool for its inherent advantages such as wide application, short analysis time, and high separation efficiency. HPLC can provide very high selectivity and sensitivity with fluorescence detection (FLD). Especially for naturally fluorescent substances, HPLC-FLD shows outstanding superiority in simple operation, direct analysis, and no derivative reaction. As far as we know, application of HPLC-FLD for analysis of IAA or other auxins is rarely reported (*12*, *13*). For example, in the work of Sánchez and co-workers (*13*), a complex method named micellar LC−derivative fluorometry was performed to analyze seven plant growth regulators. Moreover, 23 min was required for chromatographic separation with unsatisfactory peak shapes; then a combination of liquid−liquid extraction (LLE) and solid phase extraction (SPE) was used for sample pretreatment, with limits of detection (LODs) for IAA and other auxins of about 110 ng mL^{-1}. On the basis of the disadvantages above, further improvement is still needed for simultaneous analysis of multiple auxins with HPLC-FLD.

Sample pretreatment techniques are always the bottleneck in the development of analytical chemistry (*14*). Plant hormones including auxins are difficult to analyze due to their very low amounts and many interfering substances coexisting in plants;

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thus, sample preparation and purification are quite necessary before analysis. Usually, plant hormones are first extracted from plant tissues with aqueous organic solvent (i.e., 80% methanol), and then some purification methods are adopted before modern instrumental analysis. On the basis of sufficient literature investigation, it is concluded that the pretreatment methods employed for plant hormones are mostly focused on traditional LLE, SPE, and column chromatography. As the oldest pretreatment method for plant hormones, the LLE process is usually time-consuming, with tedious operation and considerable poisonous organic solvent consumption, which will do great harm to the environment and human health (*8*, *10*). Compared with LLE, SPE uses much less organic solvent with satisfactory recovery, so SPE has become the mainstream and replaced LLE gradually. By now, many kinds of extraction columns (Oasis MCX column, amino anion exchange column, and C_{18} SPE column) are reported to purify and enrich plant hormones; however, the commercial columns are relatively expensive and the enrichment factors are limited. With regard to column chromatography, two or three kinds of different columns are combined together for purification of plant hormones. For example, the column combination of poly(vinylpolypyrrolidone), DEAE-Sephadex G-25, and C_{18} Sep-Pak cartridge in the literature (*15*) was very complex with high cost. Therefore, the search for new and good pretreatment techniques with low cost, simplicity, and rapidity is urgent and welcome. Luckily, great progress has been made in this field recently, some novel techniques including solid phase microextraction (SPME), liquid phase microextraction, and dispersive liquid−liquid microextraction (DLLME) have been successively proposed and developed. In 2007, SPME was applied to plant hormone extraction for the first time (*16*). Although SPME is a solvent-free process, it also has some disadvantages; for instance, its fiber is expensive and fragile and has a limited lifetime. Another new technique termed DLLME was first introduced by Assadi and co-workers in 2006 (*17*). In brief, DLLME is a miniaturized LLE. In the DLLME system, water-immiscible extraction solvent dissolved in water-miscible disperser solvent is rapidly injected into a sample solution. Then, a cloudy emulsion involving extraction solvent, disperser solvent, and aqueous matrix containing the target analytes is formed. The enormous contact area between the organic droplets and sample solution is beneficial for the fast mass transfer of the target analytes, which are extracted into fine droplets and separated by centrifugation. Thus, the target compounds are sedimented in the bottom of the conical test tube for subsequent determination (*18*, *19*). As a good alternative to SPME, DLLME has become increasingly popular for its superior merits such as high enrichment ability, simple operation, low organic solvent consumption, and short time requirement. So far, DLLME has been successfully coupled to different instruments with various detectors for the extraction of numerous analytes, for example, DLLME-HPLC-ultraviolet detector (UV) for analysis of polycyclic aromatic hydrocarbons (*20*) and psychotropic drugs (*21*), DLLME-HPLC-MS for 7-aminoflunitrazepam determination (*22*), DLLME-HPLC-FLD for pesticide (*23*) and biogenic amine detection (*24*), DLLME-GC-electron-capture detection for chlorophenols analysis (*25*), DLLME-GC-flame photometric detection for extraction of organophosphorus and organosulfur pesticides (*26*, *27*), DLLME-GC-MS for qualitation of rose water constituents (*28*), and DLLME-atomic absorption spectrometry for measurement of trace lead (*29*) and gold (*30*), etc. However, most research focusing on DLLME was done in relatively simple matrices (i.e., environmental water and soil) and rarely applied to complex matrices including urine and food (*21*, *24*, *26*). To the best of our knowledge, this may be the first research to select DLLME as a pretreatment process to purify and enrich related hormones in plant tissues. Furthermore, the direct analysis of plant hormones with very simple sample treatment will be interesting and attractive.

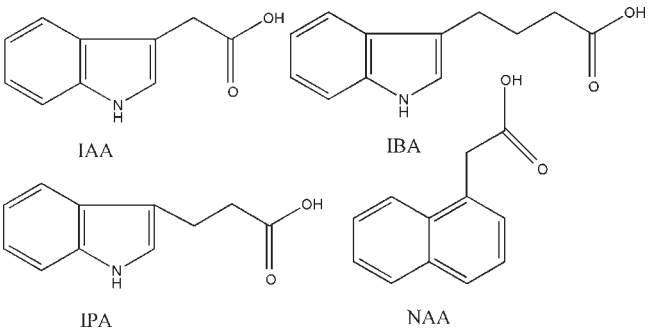

Figure 1. Chemical structures of four auxins.

The aim of this work was to propose a DLLME-HPLC-FLD method for powerful preconcentraion and sensitive detection of four common auxins (their chemical structures are shown in **Figure 1**). Several factors such as extraction solvent type and its volume, disperser solvent type and its volume, extraction time, pH of the matrix, and ion strength were optimized in order. Finally, the proposed method was successfully applied to real sample analysis.

MATERIALS AND METHODS

Reagents and Chemicals. IAA, IPA, IBA, and NAA standard substances (purity > 98%) were all purchased from J&K Chemical (Shanghai, China). The above standards were individually dissolved in acetonitrile (ACN) at a stock concentration of 1.0 mg mL^{-1} and stored at 4 °C. Working standard solutions were obtained by diluting them with ACN prior to use. HPLC-grade ACN and methanol were obtained from Merck (Darmstadt, Germany). Other reagents used were of analytical reagent grade (Shanghai Chemical Reagents Corp., Shanghai, China). Distilled water was deionized in a Milli-Q SP Reagent water system (Millipore, Bedford, MA). All of the solvents were passed through a 0.45 μm cellulose filter (Shanghai Xinya Purification Apparatus Factory, Shanghai, China) before use.

Chlorella vulgaris (*Chlorophyta*, *Chlorophyceae*, *Chlorococcales*, *Oocystaeeae*, *Chlorella*, unicellular green algae) was provided generously by Fujian Institute of Aquctic Product in Fresh Water, China. Duranta (*Duranta repens* var. 'Golden Leaves', Verbenaceae, Duranta, evergreen shrub) was collected from the garden at Fuzhou University, China.

HPLC Performance. Chromatographic separation was performed on an Agilent 1100 series HPLC system, which includes an autosampler, a quaternary pump, a degasser, and a fluorescence detector. A ChemStation was employed for instrument control, data acquisition, and processing. An Eclipse XDB-C_{18} reversed-phase column (5 μm, 4.6 × 150 mm, Agilent) maintained at 35 °C was used as the separation channel. The mobile phase was composed of ACN/water (50:50, v/v %) as flow rate of 1.0 mL min^{-1}. The fluorescence detector was set with the excitation wavelength (Ex) of 230 nm and the emission wavelength (Em) of 360 nm. The injection volume was 5 μL for each analysis.

Sample Pretreatment. In our study, one lower plant (*C. vulgaris*) and one higher plant (*Duranta*) were chosen as the experimental materials. Half a gram of fresh plant tissue (*C. vulgaris* and *Duranta* young leaves) was accurately weighed and ground into fine powder in the presence of liquid nitrogen. The powdered tissues were transferred to a 7 mL microcentrifuge tube, followed by 3 mL of 80% methanol (stored at 4 °C before use) containing 1 mmol/L butylated hydroxytoluence as an antioxidant, and finally the extracting mixture was maintained at 4 °C overnight. Then this mixture was centrifuged in a supercentrifuge at 4000 rpm for 5 min at 4 °C (Beckman), and the supernatant was collected. After the residues had been rinsed with 1 mL of methanol and extracted for another hour, the filtrate was combined and diluted with distilled water for the DLLME procedure described as follows (the dilution factor was 1:5).

Table 1. Optimization of HPLC Conditions in the Orthogonal Experiment Design[a]

expt	column temp (°C)	ACN proportion (%)	flow rate (mL min^{-1})	test indices[b] Rs min	Rt max
1	20	45	0.6	3.1	7.1
2	20	50	0.8	2.0	4.2
3	20	55	1.0	1.4	2.9
4	20	60	1.2	0.9	2.2
5	25	45	0.8	2.8	5.1
6	25	50	0.6	2.2	5.5
7	25	55	1.2	1.2	2.4
8	25	60	1.0	1.0	2.5
9	30	45	1.0	2.5	4.0
10	30	50	1.2	1.4	2.7
11	30	55	0.6	1.6	4.6
12	30	60	0.8	1.1	3.0
13	35	45	1.2	2.1	3.1
14	35	50	1.0	1.6	2.9
15	35	55	0.8	1.4	3.3
16	35	60	0.6	1.2	3.9

[a] The HPLC separation was performed on an Eclipse XDB-C_{18} reversed-phase column (5 μm, 4.6 × 150 mm, Agilent) with ACN/water as the mobile phase and 5.0 μL injection volume; [b] Test indices included the minimal resolution (Rs min) and the maximal retention time (Rt max).

DLLME Procedure. A 5.00 mL portion of the sample described above containing analytes of interest and 0.375 g of NaCl was exactly placed in a conical test tube, with pH 4.0 adjusted with 0.1 mol L^{-1} HCl by a PHS-3C precise pH-meter (Shanghai Dapu Instrument Limited Co.). Then 50 μL of $CHCl_3$ (as extraction solvent) and 1.0 mL of acetone (as disperser solvent) were mixed. Once the organic solvent had been injected into the sample solution with a 2.0 mL syringe, a cloudy solution consisting of many dispersed fine droplets of $CHCl_3$ was formed, and the mixture was gently shaken for 0.1 min as extraction time. After centrifugation at 4500 rpm for 3.0 min, the sedimented phase (about 25 μL) in the bottom of the conical test tube was withdrawn with a 50.0 μL LC syringe (Shanghai Gaoge Industrial and Trading Co. Ltd., Shanghai, China) and then placed into a vial insert fitted with polymeric feet (Agilent). Each of the vial inserts was placed in a 2 mL sample vial and positioned in a sample tray for HPLC analyses.

RESULTS AND DISCUSSION

Optimization of LC Conditions. The composition of mobile phase is an important parameter in adjusting retention time, selectivity, and peak shape in HPLC separation. After many kinds of organic solvents (methanol, ACN, etc.) had been tested, ACN was found to show well-shaped peaks and better chromatographic behavior. Therefore, ACN and H_2O were selected as the mobile phase in this reverse-phase LC. Then three kinds of chromatographic columns with different internal diameters and lengths (2.1 × 150 mm, 3.0 × 250 mm, 4.6 × 150 mm, C_{18}, particle size = 5 μm, Agilent) were also studied, and the results showed that four auxins were separated well using the 4.6 × 150 mm chromatographic column. The following three factors, namely, ACN percentage, pump flow rate, and column temperature, have joint influence on the separation resolution (Rs) and retention time (Rt), so orthogonal experiment design and variance analysis were adopted (**Table 1**). The test indices including the minimal Rs and the maximal Rt were evaluated, respectively. From the results of orthogonal experiments, the variation of flow rate and ACN percentage had a strong influence on Rs and Rt of the studied compounds. In other words, when 50% acetonitrile, a 1.0 mL min^{-1} pump flow rate, and 35 °C column temperature were chosen, baseline separation of four auxins was achieved within 3.5 min with Rs > 1.5.

Optimization of the DLLME System. In the present research, a series of parameters that influenced extraction efficiency were investigated systematically. The following optimization process was carried out using a single-factor method with concentration of standard mixture at 0.04 μg mL^{-1} in diluted plant matrix.

Extraction Solvent and Its Volume. Extraction solvent can significantly affect extraction efficiency in DLLME. The extraction solvent selected herein ought to have low solubility in water, high affinity to analytes, high density, and good chromatographic behavior. Considering the requirements above, five kinds of organic solvents such as chlorobenzene (C_6H_5Cl), chloroform ($CHCl_3$), tetrachloromethane (CCl_4), dichloromethane (CH_2Cl_2), and tetrachloroethylene (C_2Cl_4) were tried as the possible extraction solvents, and their extraction efficiencies were studied, respectively. Other DLLME conditions were as follows: 60 μL of extraction solvent, 1.0 mL of acetone as disperser solvent, extraction time of 0.1 min, centrifugation at 4500 rpm for 3.0 min. When CH_2Cl_2 was adopted as extraction solvent, there was no cloudy state and also no sedimented droplet of organic solvent at the bottom of the test tube after centrifugation. Additionally, cloudy states and emulsion systems were formed when the other four solvents were tested. Therefore, CH_2Cl_2 was not suitable for extraction solvent; meanwhile, the extraction efficiencies of the other four solvents were investigated and compared. As can be seen from **Figure 2A**, $CHCl_3$ had the highest signal response and enrichment factors. The extraction efficiency was then investigated for each of the four auxins by using 40−70 μL of $CHCl_3$ to extract the standard solutions. In this optimization process, $CHCl_3$ as extraction solvent, 1.0 mL of acetone as disperser solvent, extraction time of 0.1 min, and centrifugation at 4500 rpm for 3.0 min were respectively selected as other DLLME conditions. By increasing the $CHCl_3$ volume in the range of 40−70 μL at 10 μL intervals, the volume of the sedimented phase was gradually increased from 10 to 65 μL. **Figure 2B** depicts the change trend of signal intensity versus the $CHCl_3$ volume. It was clear that $CHCl_3$ could not extract the analytes of interest absolutely at its low volume (40 μL). As $CHCl_3$ increased to 50 μL, higher extraction efficiency was achieved; however, extraction efficiency decreased when $CHCl_3$ exceeded 60 μL due to a dilution effect (with the increase of the sedimented organic phase volume, the concentration of target analytes reduced, which is referred to as a "dilution effect"). Therefore, 50 μL was selected as optimal extraction volume of $CHCl_3$ in the following work.

Disperser Solvent and Its Volume. In DLLME, the selection principle of disperser solvent is that it should be miscible with both extraction solvent and aqueous sample. To seek the most appropriate disperser solvent, several solvents (ACN, methanol, acetone, and ethanol) were evaluated. Other DLLME conditions used were as follows: 50 μL of $CHCl_3$ as extraction solvent, 1.0 mL of disperser solvent, extraction time of 0.1 min, and centrifugation at 4500 rpm for 3.0 min. As can be observed in **Figure 3**, the maximum signal responses of four auxins were accomplished when acetone was selected as disperser solvent in this research. Selecting a suitable volume of disperser solvent is another crucial factor influencing the extraction efficiency. Experiments were performed with different volumes of acetone (400−1200 μL) as the disperser solvent. It could be concluded that the peak areas of four auxins were enhanced greatly by increasing the volume of acetone to 1000 μL, whereas the extraction efficiency degraded with 1200 μL of acetone. The reason was that a low amount of acetone could not disperse $CHCl_3$ completely and a cloudy state was not formed well. On the other hand, too high a volume of acetone increased the solubility of analytes and $CHCl_3$ in aqueous sample, thereby, the extraction abilities decreased. Subsequently, 1000 μL of acetone was adopted as optimal disperser solvent, with other DLLME conditions including 50 μL of $CHCl_3$ as extraction solvent, 0.1 min as extraction time, and centrifugation at 4500 rpm for 3.0 min.

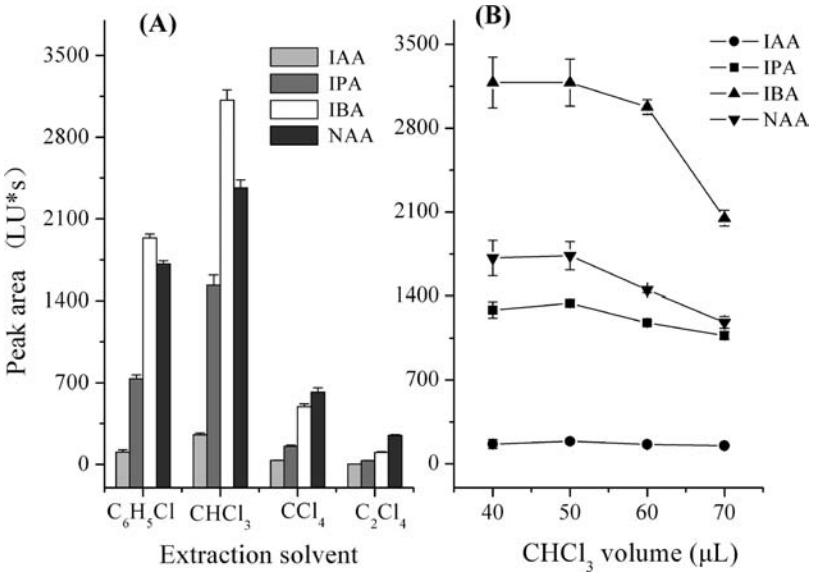

Figure 2. Effects of different extraction solvents (**A**) and volumes of $CHCl_3$ (**B**) on peak areas of analytes. The unit LU*s in the peak area measurements refers to adsorption peak width. LC and DLLME conditions in (**A**): Agilent Eclipse XDB-C_{18} column (5 μm, 4.6 $\times$ 150 mm) maintained at 1.0 mL $min^{-1}$and 35 °C, ACN/water = 50:50 (%, v/v), FLD Ex/Em 230/360 nm; 60 μL extraction solvent, 1.0 mL acetone as disperser solvent, extraction time 0.1 min, centrifugation at 4500 rpm for 3.0 min. The standard mixture was 0.04 μg mL^{-1}. HPLC and DLLME conditions in (**B**): different volumes of $CHCl_3$ as extraction solvent; other conditions as in (**A**).

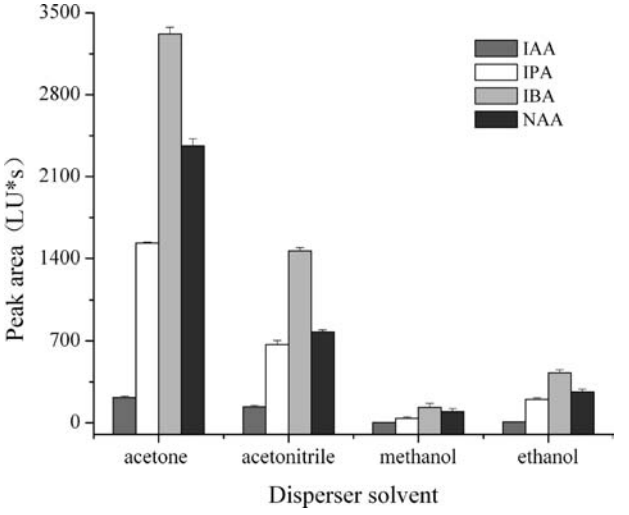

Figure 3. Effects of different disperser solvents on peak areas of analytes. Conditions: 50 μL of $CHCl_3$ as extraction solvent; 1.0 mL of different types of disperser solvents; other DLLME and HPLC conditions as in **Figure 2A**.

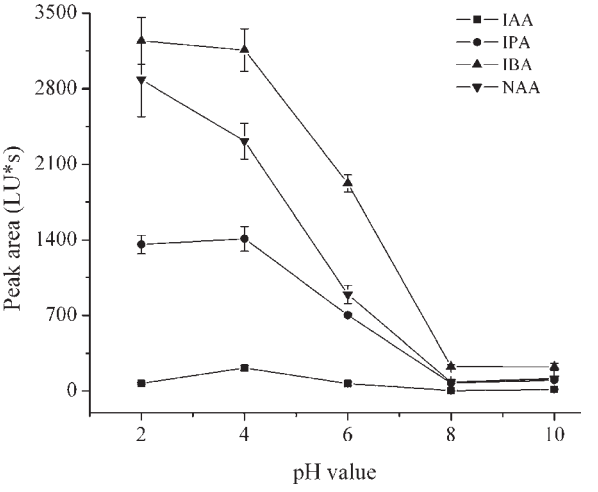

Figure 4. Effects of different pH values on peak areas of analytes. Conditions: 50 μL of $CHCl_3$ as extraction solvent; 1.0 mL of acetone as disperser solvent; other DLLME and HPLC conditions as in **Figure 2A**.

Extraction Time. Extraction time in DLLME refers to the interval time from the injection of a mixture of extraction solvent and disperser solvent to the moment of centrifugation. The effects of extraction time on the extraction efficiency were examined over the range of 0.1−60 min (0.1, 2, 5, 8, 10, 20, 30, and 60 min, respectively). The results exhibited that there was no significant difference with the change of various extraction times. It seemed that the contact area between the extraction solvent and the aqueous sample was infinitely large after the formation of the cloudy state; thus, mass transfer of the analytes from aqueous phase to extraction phase was quickly realized. Therefore, 0.1 min was enough for effective extraction. In comparison with conventional LLE or SPE, it was notable that DLLME exhibited prominent merits such as much time-saving and simple operation. In short, DLLME is a time-independent method.

pH Value of Sample Solution. In general, analytes are expected to be in a nonionic state in DLLME for higher extraction efficiency. Because analytes in neutral forms are much easier to extract by extraction solvent than those in ionic forms, accordingly, partition coefficient and extraction ability in this system are enhanced. As is known, pH can affect the existing forms of compounds in solution. To investigate the effect of the solution pH on extraction efficiency, different samples with pH 2−10 were prepared by adding 0.1 mol/L HCl or NaOH to adjust. Other DLLME conditions were 50 μL of $CHCl_3$, 1.0 mL of acetone, 0.1 min extraction time, and 3.0 min of centrifugation at 4500 rpm, respectively. It can be seen from **Figure 4** that the peak areas of four auxins decreased obviously with the pH values in range of 4−10. This can be explained by the pK_a values of IAA, IPA, IBA, and NAA of 4.75, 6.15, 4.80, and 4.23, respectively. Four auxins were all in neutral form at pH 4.0, so the signal

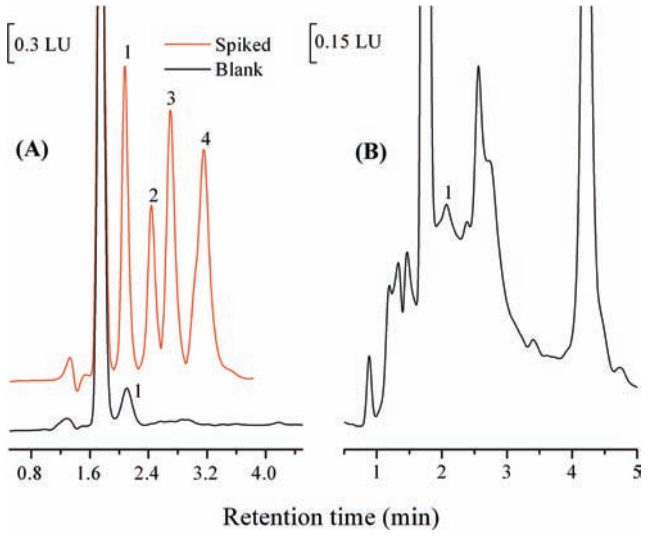

Figure 5. Analysis of IAA in *Chlorella vulgaris* (**A**) and *Duranta* (**B**). The unit LU in the peak height measurements refers to adsorption. Peaks: (1) IAA; (2) IPA; (3) IBA; (4) NAA. The red line in (**A**) refers to the recovery experiment by adding known amounts of analytes (10.0 ng mL^{-1} for IAA and 1.0 ng mL^{-1} for IPA, IBA, and NAA) to *C. vulgaris* matrix. Optimal DLLME conditions: 50 μL of $CHCl_3$, 1.0 mL of acetone, 0.1 min for extraction, centrifugation at 4500 rpm for 3.0 min, pH 4.0, 7.5% (w/v) NaCl. The HPLC conditions were the same as in **Figure 2A**.

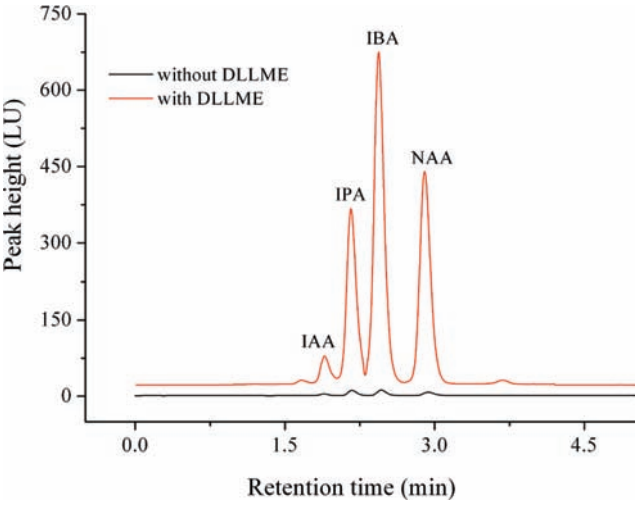

Figure 6. Chromatogram contrast of signal response before DLLME and after DLLME extraction. The optimal conditions were the same as **Figure 5**. The standard mixture was 0.4 μg mL^{-1}.

intensities of analytes reached maximum. On the other hand, the extraction efficiency was reduced at higher pH values, because four auxins were supposed to be in ionic forms, which were not easy to extract by $CHCl_3$. Meanwhile, IAA was hardly detected at pH 2 because its stability was poor at low pH value (i.e., pH $\leq$2.0). Therefore, the sample solution was adjusted to pH 4.0, with the highest sensitivity of all these compounds at this pH.

Influence of Ion Strength. Usually, an increase of the ionic strength can lead to a decrease in the solubility of the analytes in sample solution; thus, extraction efficiency may be enhanced. To examine the effect of ion strength on extraction efficiency, various concentrations of NaCl over the range of 0−15.0% (w/v) were added into the solution. Other experimental conditions were kept constant with pH 4.0 of sample solution. As NaCl increased from 0 to 7.5%, better extraction was obtained on the basis of experimental results, whereas a slight decrease in signal response was observed at higher percentages of NaCl. That was because a salting-out effect increased the sedimented organic phase volume and dilution effect of $CHCl_3$ occurred. In addition, when 15.0% NaCl was added, the extraction solvent was at the upper layer of the test tube, not sedimented at the bottom after centrifugation (this phenomenon was in agreement with ref *19*); perhaps the density of the aqueous solution varied at high amounts of NaCl. Consequently, adding 7.5% (w/v) of NaCl into the sample solution was suitable in subsequent experiments.

Effect of Plant Matrix. As mentioned above, 80% aqueous methanol was used to extract endogenous auxins. The presence of too much methanol would increase the solubility of $CHCl_3$ and aqueous solution, so no extraction solvent was sedimented at the bottom of the test tube. That is, too much methanol was an unfavorable factor for the DLLME system. In consideration of the above problem, diluting the matrix to some extent was a convenient method. Therefore, the plant tissues involved in our work were all diluted to 5-fold volumes after crude extraction. The sample in **Figure 5A** was *C. vulgaris*, whereas the sample in **Figure 5B** was *Duranta*. Notably, these two plants were processed according to the same sample pretreatment with the same dilution. Because of the severe background interference of *Duranta* in **Figure 5B**, this matrix was only suitable for qualitative analysis of IAA without further quantification. On the basis of our experimental results, *C. vulgaris* matrix was directly selected for the following optimization process and method validation.

As discussed in the previous sections, the detailed conditions of DLLME-HPLC-FLD system were summarized as follows. Four auxins were separated with an Eclipse XDB-C_{18} column (5 μm, 4.6 × 150 mm) maintained at 35 °C, ACN/water (50:50, v/v %) as the mobile phase with a flow rate of 1.0 mL min^{-1} and FLD Ex/Em of 230/360 nm. In the DLLME process, 50 μL of $CHCl_3$ and 1.0 mL of acetone were injected quickly into a sample solution (5.0 mL, pH 4.0) containing 7.5% (w/v) of NaCl. After extraction for 0.1 min, the mixture was centrifuged at 4500 rpm for 3.0 min, and the sedimented $CHCl_3$ was withdrawn and injected for HPLC analyses. Under the optimal conditions, signal responses in DLLME-HPLC-FLD system were compared with those with ordinary HPLC-FLD determination (**Figure 6**). As can be seen, the Rt values were 1.89, 2.17, 2.47, and 2.94 min for IAA, IPA, IBA, and NAA, respectively. There was no obvious difference in retention time of the four analytes using pure HPLC-FLD and DLLME-HPLC-FLD. In the literature (*17*), the enrichment factor (EF) was defined as C_{sed}/C_0, where C_{sed} and C_0 represent the concentration of analytes in the sedimented phase and in initial aqueous solution, respectively. **Figure 6** also shows that the enrichment effects were obvious after DLLME procedure, with the EFs approximately 10-fold for IAA but 40−60-fold for the other three auxins.

Analytical Performance. The quantitative analysis of the proposed system was performed on the basis of the external standard method. A series of standard mixtures with different concentrations were prepared in the plant matrix (*C. vulgaris*). Under the optimized conditions, two kinds of calibration curves were plotted by simple linear regression of the fluorescence intensity (peak area) versus the concentration of auxins. One calibration curve was constructed without DLLME enrichment, whereas the other was performed after the DLLME procedure. The linear range, correlation coefficients (R), and LODs for all target compounds are summarized and compared in **Table 2**. As can be seen, all calibration curves exhibited good linearity, with R > 0.9980.

Table 2. Analytical Performance Data for Four Auxins in Plant Matrix with Two Methods[a]

	without DLLME procedure			with DLLME enrichment		
analyte	linear range (ng mL^{-1})	*R*	LOD (ng mL^{-1})	linear range (ng mL^{-1})	*R*	LOD (ng mL^{-1})
IAA	8−800	0.9996	2.0	0.5−80	0.9993	0.1
IPA	8−800	0.9997	1.0	0.05−8.0	0.9980	0.02
IBA	8−800	0.9997	1.0	0.08−8.0	0.9995	0.02
NAA	8−800	0.9997	1.0	0.05−5.0	0.9990	0.02

[a] The DLLME and HPLC conditions were the same as in **Figure 5**.

Table 3. Comparison of the Present Technique with Reported HPLC-Related Methods[a]

detector	MS	FL	UV	FL
sample pretreatment method	LLE	combination of SPE and LLE	SPME	DLLME
LOD (ng mL^{-1})	10.0 (IAA), 8.0 (IPA), 9.0 (IBA), 30.0 (NAA)	110 (IAA), 110 (IBA)	0.15 (IAA), 0.12 (IBA), 0.06 (NAA)	0.1 (IAA), 0.02 (IPA, IBA, and NAA)
literature	*10*	*13*	*16*	this work

[a] The LOD of the DLLME-HPLC method was compared with those of other works by HPLC with various detectors based on different sample preparation techniques.

Table 4. Recoveries of Four Auxins in Spiked *Chlorella vulgaris* Samples (n = 3)[a]

	spiked at 0.5 ng mL^{-1}[b]		spiked at 1.0 ng mL^{-1}		spiked at 5.0 ng mL^{-1}	
analyte	found, mean ± SD[c] (ng mL^{-1})	recovery (%)	found, mean ± SD[c] (ng mL^{-1})	recovery (%)	found, mean ± SD[c] (ng mL^{-1})	recovery (%)
IAA	5.38 ± 0.25	107.6	11.40 ± 0.15	114.0	48.30 ± 1.01	96.6
IPA	0.58 ± 0.20	116.0	1.15 ± 0.06	115.0	4.74 ± 0.10	94.7
IBA	0.53 ± 0.06	106.5	1.14 ± 0.15	114.0	4.80 ± 0.70	96.0
NAA	0.46 ± 0.50	92.5	1.09 ± 0.35	108.8	4.85 ± 1.20	97.1

[a] The HPLC and DLLME conditions were the same as in **Figure 5**. [b] The concentration of IAA was 10-fold compared with those of the other three auxins in the standard mixture. [c] SD refers to standard deviation (n = 3).

The LODs, based on a signal-to-noise ratio (S/N) of 3, were much lower in DLLME-HPLC-FLD (0.02−0.1 ng mL^{-1}) than those of ordinary HPLC-FLD (1.0−2.0 ng mL^{-1}), indicating the high sensitivity of this new DLLME-HPLC method. Additionally, a comparison was conducted between this method and other works based on different sample preparation techniques combined with HPLC. Comparison of the results is shown in **Table 3**. As can be seen, the LODs obtained in this work were at least 1 order lower than those in the literature (*10*, *13*, *16*). Besides, the proposed DLLME has advantages such as higher enrichment factor, lower consumption of organic solvent, and shorter operation time.

The repeatability of the proposed method was determined by the intraday and interday precisions, which were expressed as relative standard deviation (RSD) for all analytes. Under the optimal DLLME-HPLC conditions, seven sequential runs of 0.04 μg mL^{-1} auxins in plant matrix (*C. vulgaris*) within 1 day were conducted, and then the same concentration of standard solution was also analyzed on 5 consecutive days. RSDs of the intraday precision (n = 7) varied from 0.17 to 0.94% (Rt) and from 2.52 to 5.42% (peak areas), whereas RSDs of interday precision (n = 5) were less than 0.86 and 5.63% for Rt and peak areas, respectively. The satisfactory precision suggested that this DLLME method possessed high repeatability even in a complex matrix.

Analysis of Real Sample. The analysis of endogenous IAA or IBA is not easy for the following reasons. First, although IAA exists in most plants, it is easily oxidized or deactivated in the presence of light and high temperature during pretreatment process. Second, IAA and IBA occur in very low amounts in plants with abundant interfering substances and similar metabolites. IAA is reported to be 1−100 ng/g of fresh weight (FW) in plants, whereas IBA occurs only in some kinds of higher plants (i.e., *Zea*), and its content is far less (*1*). Therefore, a new and good sample pretreatment technique, DLLME, was developed for its attractive advantages such as high efficiency and enrichment factor.

In the present study, *C. vulgaris* and *Duranta* were selected as the real samples to validate the DLLME-HPLC method with FL detection. It is revealed that IAA exists in lower and higher plants, whereas the presence of IBA in *C. vulgaris* and *Duranta* is not identified yet. According to the sample treatment process, the plant tissues were extracted by aqueous methanol first and then purified and concentrated by DLLME before HPLC analysis. Results demonstrated that nearly no interference was observed in the matrix of *C. vulgaris* after DLLME treatment. Moreover, DLLME combined with HPLC-FLD was successfully applied to detect low concentrations of IAA in this sample. The content of IAA in the *C. vulgaris* sample was calculated on the basis of the calibration curve mentioned above. According to our experiment, IAA was 7.4 ng mL^{-1} (about 37.0 ng/g of FW) in *C. vulgaris*, whereas IBA was not detected, although it perhaps existed in ultralow amount, even though no IBA existed in the mentioned two plants. The successful real sample analysis suggested that dilution of matrices to some extent was suitable due to high sensitivity and low LODs of this DLLME method.

To investigate recoveries of the developed method, *C. vulgaris* matrix after dilution was spiked at three different concentrations of standards solution (5.0, 10.0, and 50.0 ng mL^{-1} for IAA and 0.5, 1.0, and 5.0 ng mL^{-1} for IPA, IBA, and NAA, respectively). Each treatment was performed in triplicate under optimal conditions (n = 3), and the results are summarized in **Figure 5A** and **Table 4**. As can be seen from **Table 4**, recoveries ranged from 94.7 to 116.0% with standard deviations (SDs) of < 1.2 in *C. vulgaris*

sample. The satisfactory recoveries indicated that this DLLME method was feasible for the determination of the four auxins.

In conclusion, a rapid HPLC-FLD method for the determination of auxins in plants has been established with DLLME as the sample pretreatment technique for the first time. With regard to HPLC-FLD, tedious derivatization was avoided because auxins are naturally fluorescent substances. As to DLLME, this new pretreatment technique showed many excellent merits including simplicity, low cost, time-savings, and high enrichment efficiency in comparison with traditional LLE and SPE. To sum up, the combination of DLLME-HPLC-FLD had several advantages such as simple operation, quick analysis, good repeatability and high recoveries. Furthermore, the successful application of this assay to analyze trace IAA in plants showed that the developed method was precise, selective, and sensitive. Most importantly, this research provided a new pretreatment in the analysis of trace plant hormones in complex matrix, which would be beneficial for more study on plant hormones in future work.

ABBREVIATIONS USED

DLLME, dispersive liquid−liquid microextraction; HPLC-FLD, high-performance liquid chromatography−fluorescence detection; LLE, liquid−liquid extraction; SPE, solid phase extraction; SPME, solid phase microextraction; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; IPA, indole-3-propionic acid; NAA, 1-naphthylacetic acid.

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