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ORIGINAL

Development of an Efficient HPLC Fluorescence Detection Method for Brassinolide by Ultrasonic-Assisted Dispersive Liquid–Liquid Microextraction Coupled with Derivatization

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Abstract A novel hyphenated technique based on ultrasonic-assisted dispersive liquid–liquid microextraction (UA-DLLME) coupled with derivatization has been established for the determination of brassinolide (BL, a representative of brassinosteroids) by HPLC fluorescence detection. 9-Phenanthreneboronic acid is used as labeling reagent of BL. UA-DLLME parameters containing type and volume of extraction and disperser solvent, pH and ultrasonication time are optimized. Derivatization parameters are optimized included amount of 9-phenanthreneboronic acid, volume ratio of pyridine, derivatization time and temperature. Under optimal conditions, quantitative linear range of BL is 50–1,000 ng L^{-1} and excellent linear response is observed with correlation coefficient of 0.9996. Limit of detection and limit of quantification are calculated as 8.0 and 25.0 ng L^{-1}, respectively. RSDs of retention time and peak area are in the range of 0.68–0.97 % and 4.61–6.54 % for intra-day precision, 1.32–1.94 % and 7.28–9.75 % for inter-day precision, respectively. Accuracy is satisfactory in the range of 82.3–125.1 %. RSDs' values of repeatability are in the range of 0.82–1.79 and 3.95–8.53 % for retention time and peak area, respectively. Enrichment factor for BL is 189. The results of recovery and matrix effect are in the range of 82.0–108.6 and 90.0–115.3 %, respectively. The proposed method has been applied for the determination of BL in *Arabidopsis thaliana*, *Daucus carota* and *Brassica campestris* L. leaves with much higher sensitivity than many other methods.

Keywords Column liquid chromatography · Ultrasonic-assisted dispersive liquid–liquid microextraction derivatization · Fluorescence detection · Brassinolide · Plant matrix

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Introduction

Brassinosteroids (BRs), a class of important plant polyhydroxy steroids, were first discovered in 1970 [1, 2]. BRs have been widely recognized as the sixth plant hormone. They play an important role in plant growth and regulations [3]. BRs are widely distributed in almost every part of higher plants [4]. Brassinolide (BL) is the first member of the BRs analogs isolated from rape pollen by Grove [5], which possesses the highest activity in the growth and development of plants. BL exhibits growth-promoting effects for fruits, flowering plants and commercial plants at extremely low concentrations [6]. Therefore, the establishment of a sensitive and selective determination method for BL has great significance, and the proposed analytical strategy will be easily applied for the determination of homologous compounds of brassinosteroids.

The analysis of endogenous BL in plant is a challenge due to its extremely low concentration and complex matrix

in plant extracts. To date, many techniques have been used for the determination of BL in various plant samples. The conventional detection methods are bioassay and gas chromatography–mass spectrometry (GC–MS). Bioassay method shows satisfactory detection sensitivity for BL [7]. However, it is not only semi-quantitative and time-consuming but also demanded strictly controlled conditions. GC–MS method combined with the methaneboronic acid derivatization has been used in early research [8]. However, the experimental procedures are complicated and also time-consuming. Moreover, the analytical sensitivity is not satisfactory. Enzyme-linked immunosorbent assay (ELISA) is another available determination method for BL. Although it offers several advantages such as simplicity and rapidness, the sensitivity cannot meet the requirement for the analyses of plant hormones from plant tissues [9]. Recently, high-performance liquid chromatography (HPLC) has been frequently used for the separation and analysis of BRs. Since BRs lack suitable chromophores and ionizable functional groups, a derivatization procedure is imperative before determination to make them responsive to ultraviolet (UV), fluorometric (FL), electrochemical (EC) or mass spectrometry (MS) detection [9–12]. HPLC coupled with UV and EC detection cannot satisfy the demand of selectivity, sensitivity and matrix effect in plant samples. In addition, the ionization efficiency of BRs is generally low, which restricts the sensitivity of MS detection methods. Fortunately, organic boronic acids can effectively react with the vicinal hydroxyl groups, which can satisfy the requirements of the sensitive determination for BL [12, 13]. This kind of derivatization procedure followed by HPLC fluorescence detection (HPLC-FLD) is an appropriate choice with excellent selectivity and sensitivity. What's more, the experimental procedure is simple and rapid.

The concentrations of BRs are about 1–100 μg kg^{-1} fresh weight in pollen and immature seeds, and 0.01–0.1 μg kg^{-1} fresh weight in shoots and leaves [14]. BL exists in plants at ultra low concentration as a kind of BRs. Moreover, the matrix of plant sample is quite complicated. Hence, a simple and rapid pretreatment procedure is necessary. Until now, lots of pretreatment techniques have been employed for the pretreatment of BRs, such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), magnetic solid-phase extraction (MSPE) and solid-phase microextraction (SPME) [9, 15–20]. However, most of these methods are tedious, solvent-consuming, and demand large amount of plant materials. Dispersive liquid–liquid microextraction (DLLME) is a novel sample pretreatment technique developed by Rezaee et al. [21]. It is mainly based on a ternary component solvent system. The mixture of disperser solvent (miscible with both extraction and aqueous solvents) and a water-immiscible extraction solvent is rapidly injected into an aqueous sample containing analytes. After being shaken for a while, a cloudy solution containing tiny droplets of the extraction solvent, which is totally dispersed in the aqueous phase, is formed in a test tube. Analytes are transferred into the extraction phase quickly due to the large contact area between the aqueous phase and the extraction solvent. The enriched analytes in the sedimented phase are analyzed by various techniques after centrifugation [22]. Owing to its simplicity of operation, low cost, rapidity, high recovery and enrichment factor, low consumption and weak interference of the matrix effect, it has been applied for the determination of various analytes in different matrices [23–25]. Recently, ultrasonic radiation has been used as an efficient method for the acceleration of mass transfer process. Ultrasonic-assisted dispersive liquid–liquid microextraction (UA-DLLME) attracts lots of attentions as an emerging DLLME technique [26, 27].

In this study, a novel hyphenated technique of UA-DLLME and derivatization for the determination of BL has been developed. The separation and detection of analytes are performed by HPLC-FLD. The method shows excellent selectivity and sensitivity with a slightly matrix effect. Experimental conditions of microextraction and derivatization are optimized by single-factor analysis method. The method has been successfully applied for the determination of BL in plant samples. To the best of our knowledge, this is the first report about the combined use of UA-DLLME and derivatization for the analysis of BL by HPLC-FLD.

Experimental

Instrumentation

Agilent 1260 series, made up of an online vacuum degasser, a quaternary pump, an autosampler, a thermostated column compartment and a fluorescence detector, was used for HPLC separation and analysis of samples. The mobile phase was filtered through a 0.2 μm membrane. A Xiangzhi TGL16M high-speed refrigerated centrifuge (Changsha, China), an automatic electronic water-bath (Jintan, China), a KQ2200E ultrasonic cleaner (Kunshan, China) and a VX-200 vortex mixer (Labnet, USA) were equipped for derivatization and UA-DLLME experiments.

Chemicals and Materials

9-Phenanthreneboronic acid was purchased from Tokyo Chemical Industry Co. LTD. (Tokyo, Japan). BL (99.0 %) was purchased from Aladdin Chemical Reagent Co. (Shanghai, China). Acetonitrile and formic acid were of HPLC grade purchased from Damao Chemical Reagent

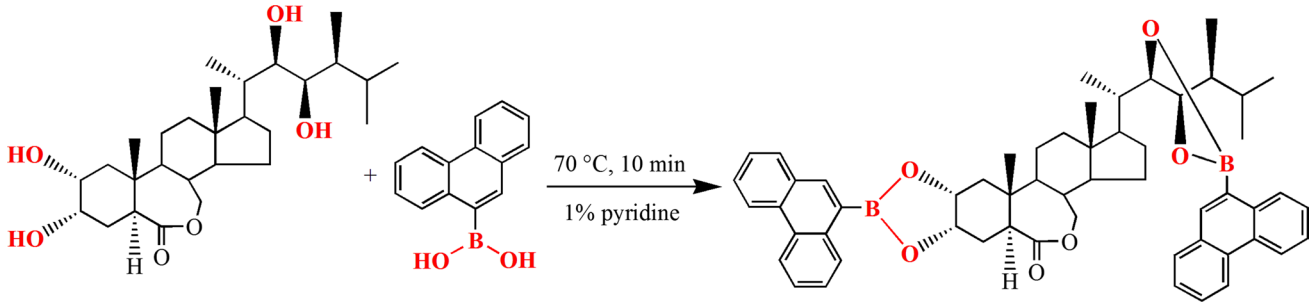

Fig. 1 Derivatization scheme of 9-phenanthreneboronic acid with brassinolide

Co. (Tianjin, China). Other reagents were of analytical grade. Water was purified using a Millipore system (Bedford, MA, USA).

Preparation of Standard Solutions and Quality Control Samples

Stock solution of BL was prepared in acetonitrile at the concentration of 0.2 mg mL^{-1}, and the solution was then diluted with acetonitrile to obtain standard working solutions. The derivatization reagent solution (0.1 mg mL^{-1}) was prepared by dissolving 2.5 mg 9-phenanthreneboronic acid in 25 mL pyridine–acetonitrile (1 %, v/v). The quality control (QC) samples were prepared at three concentration levels of 50, 200, 1,000 ng L^{-1} for BL by diluting stock solutions with acetonitrile. All the solutions were stored at 4 °C in a refrigerator when not in use.

HPLC Conditions

HPLC separation of BL derivative was carried out on a reversed-phase Agilent Zorbax SB-C18 column (4.6 mm × 150 mm, 5 μm) by a gradient elution. Eluent A was 20 % acetonitrile (v:v) and B was acetonitrile. Both of them contained 0.1 % of HPLC formic acid. The gradient elution program was as follows: 0 min = 80 % B; 5–8 min = 100 % B. Injection volume was 10 μL. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 305 nm and λ_{em} 375 nm, respectively.

UA-DLLME Combined with Derivatization Procedure

To a 5 mL aqueous solution spiked with 1 mg BL standard, a mixture of 150 μL chloroform and 300 μL methanol was rapidly injected into a centrifugal tube. The mixture was handled by ultrasonication for 90 s. Then, the extraction solvent was completely dispersed into the aqueous phase and formed a cloudy solution. After that, the mixture was centrifuged at 10,000 rpm for 2 min. The sediment chloroform phase could be collected at the bottom of the centrifuge tube and transferred to a vial using a syringe. The sediment was dried under nitrogen and redissolved in 100 μL acetonitrile.

The reaction scheme of derivatization is shown in Fig. 1. To the redissolved solution, 500 μL of 9-phenanthreneboronic acid (0.1 mg mL^{-1}) in pyridine–acetonitrile (1 %, v/v) was added. The vial was sealed and heated for 10 min in a water bath at 70 °C. After cooling, 20 μL 20 % acetic acid solution was added to adjust the pH in the range of 5.0–7.0. Finally, the solution was filtered through a 0.22 μm membrane and injected for HPLC analysis.

Method Validation

QC samples were used for the method validation. Calibration standards ranging from 50 to 1,000 ng L^{-1} were prepared. The calibration curve of BL was constructed by plotting the peak areas of BL versus their corresponding concentrations. Limit of detection (LOD) and limit of quantification (LOQ) were calculated when the signal to noise ratios were above 3:1 and 10:1, respectively. Precision and accuracy were evaluated at three concentration levels (50, 200 and 1,000 ng L^{-1}). Six replicates of each concentration level were analyzed three times over 3 days to determine the intra- and inter-day precision and accuracy using freshly prepared calibration curves. The recovery was evaluated by spiking specified amount of standard solution to blank *Arabidopsis thaliana*, *Daucus carota* and *Brassica campestris* L. leaves' samples. The method repeatability was investigated by measuring the RSDs of peak areas and retention times for BL derivative ($n = 6$) under identical conditions. The matrix effect was assessed by comparing the peak areas of *Arabidopsis thaliana*, *Daucus carota* and *Brassica campestris* L. leaves' samples spiked after UA-DLLME procedure to that of an equivalent concentration of the standard solution.

Results and Discussion

Optimization of HPLC Separation

BL derivative (1,000 ng L^{-1}) was used for the optimization of chromatographic separation. Different mobile phases (methanol and acetonitrile) and additives (formic acid and acetic acid at various concentrations) were evaluated. Compared with methanol/water, acetonitrile/water offered a more rapidly elution program of BL derivative. Since the complex matrix of plant samples, isocratic elution could not obtain an ideal chromatographic separation. Gradient elution could achieve very satisfactory chromatographic separation and the peak shape was improved. Therefore, gradient elution was used in this study. A series of experiments showed that the best mobile phase composition was eluent A acetonitrile/H_2O (20:80; v/v) and eluent B acetonitrile. The best signal response was obtained when 0.1 % formic acid was added. In view of the resolution and analysis speed, a reversed-phase Agilent Zorbax SB-C18 column (4.6 mm × 150 mm, 5 μm) was chosen to perform the separation of BL derivative with gradient elution. The optimum flow rate and column temperature were 1 mL min^{-1} and 30 °C respectively. The chromatogram of BL standard derivative is shown in Fig. 2a, and the representative chromatogram of an *Arabidopsis thaliana* sample and its spiked one is shown in Fig. 2b.

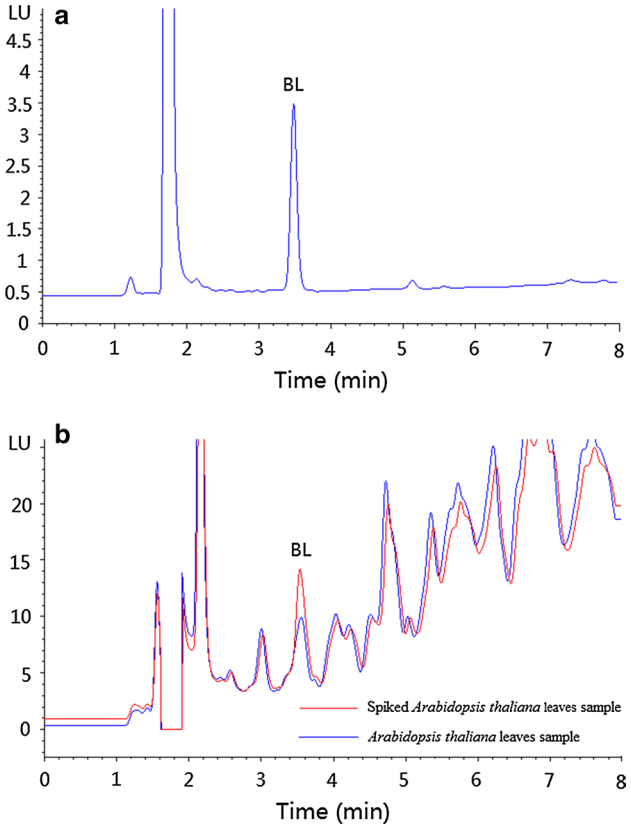

Fig. 2 Chromatograms of brassinolide derivative from **a** standard derivative, **b** *Arabidopsis thaliana* sample and its spiked one

Optimization of UA-DLLME Extraction Procedure

To get reliable extraction efficiency for BL, numerous factors that might influence the UA-DLLME procedure were investigated. A series of experiments were designed to optimize these parameters using 5 mL of ultrapure water sample spiked with 200 ng L^{-1} of BL standard.

Effect of Extraction Solvent and its Volume

The selection of extraction solvent was the most important factor for the UA-DLLME procedure. In traditional DLLME, the extraction solvent should have a high-density and low solubility in water. Thus, the droplets of extraction solvent could be collected at the bottom of the tube after centrifugation. Five kinds of high density organic solvents were optimized as extraction solvents in this study, namely dichloromethane, chloroform, chlorobenzene, trichloroethylene and tetrachloroethylene. Five milliliters of ultrapure water sample spiked with 200 ng L^{-1} of BL standard was transferred to a centrifuge tube, a mixture of 100 μL extraction solvent and 400 μL methanol used as disperser solvent was rapidly injected into the sample solution. As shown in Fig. 3a, chloroform showed the best extraction efficiency. Further, the volume of chloroform was also optimized by changing its consumption from 50 to 250 μL, while the volume of methanol was constant at 400 μL. As can be seen from Fig. 3b, the peak areas of BL derivative increased with the increase in the volume of chloroform in the range of 50–150 μL and tended to decrease when the volume was above 150 μL. Therefore, 150 μL of chloroform was eventually selected as the optimized type and volume of extraction solvent.

Effect of Disperser Solvent and its Volume

The selection of disperser solvent was also one of the most important factors in the DLLME process. It mainly depended on its miscibility with extraction solvent and aqueous phase. Four solvents viz., acetone, acetonitrile, methanol and ethanol were selected as disperser solvents for the optimization of UA-DLLME. The extraction solvent (chloroform 150 μL) was mixed with 400 μL of various disperser solvents. Other operations were identical with previous result. As can be seen from Fig. 3c, the maximum extraction efficiency was obtained when methanol was selected as disperser solvent. Thus, methanol was selected as disperser solvent in this study. The volume of methanol was also optimized. Other

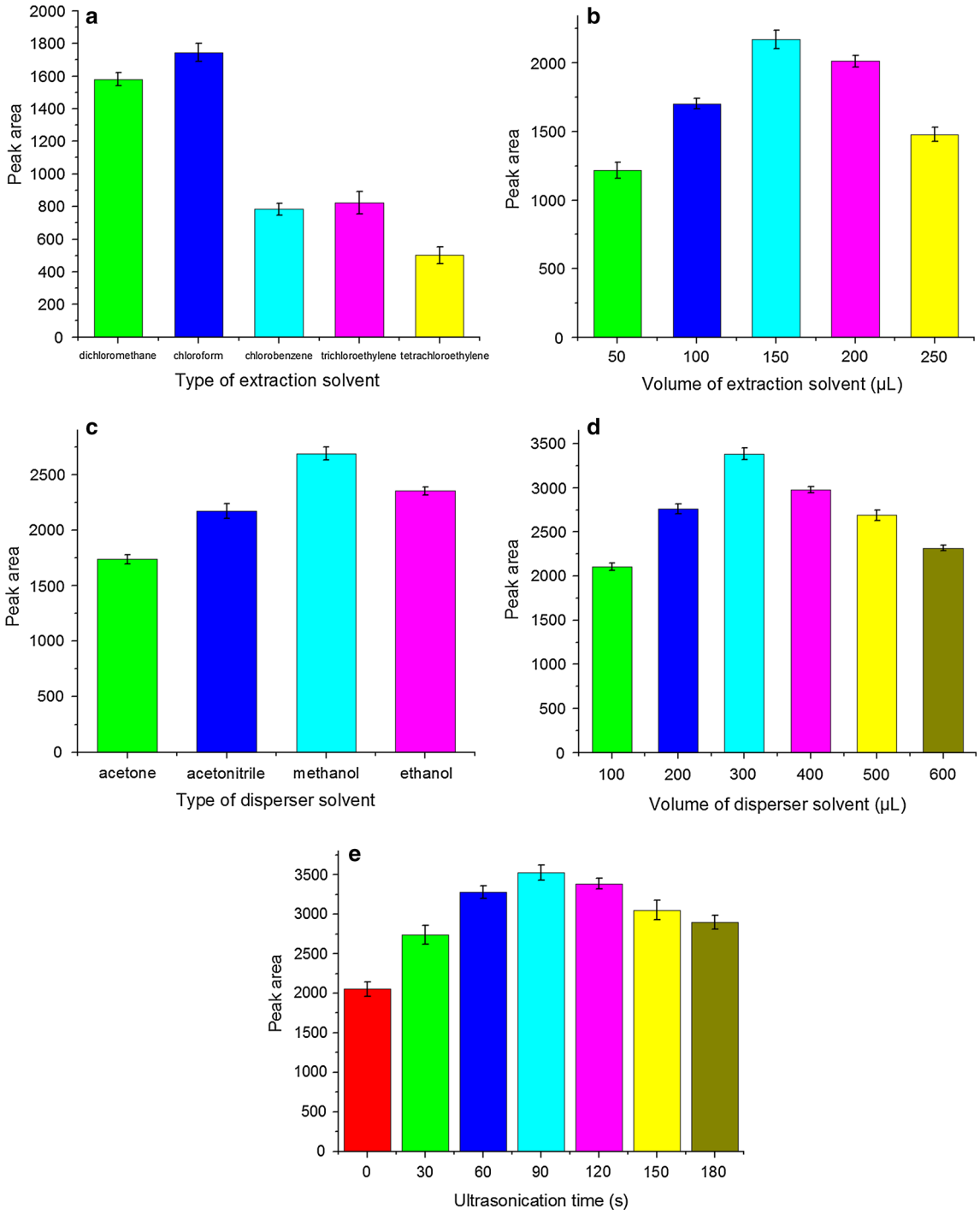

Fig. 3 Optimization of different parameters of UA-DLLME at 200 ng L^{-1} of brassinolide. **a** Type of extraction solvent; **b** volume of extraction solvent; **c** type of disperser solvent; **d** volume of disperser solvent; **e** ultrasonication time

parameters which had been optimized were kept constant and the volume of methanol was changed from 100 to 600 μL. The maximum peak area of BL derivative was obtained when the volume of methanol was at 300 μL (Fig. 3d). In the end, methanol at a volume of 300 μL was selected as disperser solvent for further research.

Effect of pH and Ultrasonication Time

Effect of pH value of the aqueous sample was also investigated. A series of experiments were carried out to test the effect of the variation of sample pH by altering the pH of aqueous sample from 2 to 11. The peak area of BL

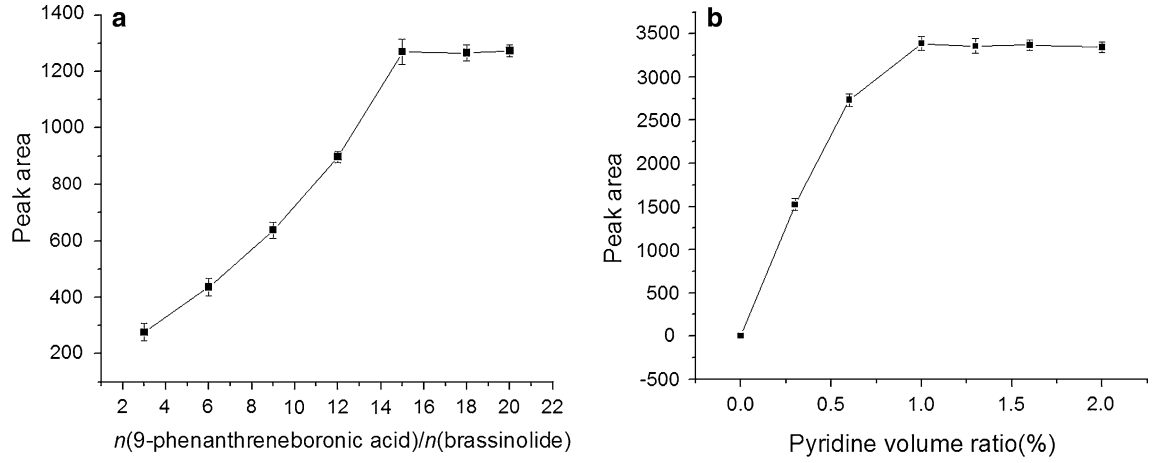

Fig. 4 Effect of **a** mole ratio of 9-phenanthreneboronic acid and brassinolide and **b** pyridine volume ratio

derivative was higher in acidic aqueous solution than in alkaline aqueous solution. In addition, the peak area had not much difference in the pH range of 2–7. This should be due to the fact that BL was less soluble in the acidic water phase than in the alkaline water phase. Therefore, all aqueous samples were prepared between pH 5–7 in this study. Ultrasonication could observably accelerate the mass transfer process of BL from aqueous phase to the organic extraction phase. The effect of ultrasonication was investigated by changing ultrasonication time from 0 to 180 s (Fig. 3e). Results indicated that, the extension of ultrasonication time would reduce the extraction efficiency and the most suitable ultrasonication time was 90 s. This could be explained as follows. As the ultrasonication time increasing, a lot of small vacuum bubbles or void was formed. When these small vacuum bubbles or void violently collapsed, the local temperature rose rapidly which might influence the extraction efficiency of UA-DLLME [28].

Optimization of Derivatization Conditions

The main factors affecting derivatization yields were amount of 9-phenanthreneboronic acid, the volume ratio of pyridine, the reaction time and temperature. Amount of 9-phenanthreneboronic acid described as mole ratio to BL was optimized in the range of 3–20. The results showed that the optimal mole ratio of 9-phenanthreneboronic to BL was 15 (Fig. 4a). Pyridine was used as catalyst for the derivatization reaction [12]. The effect of pyridine volume ratio was investigated in the range of 0–2 %. Figure 4b shows that derivatization reaction did not occur when the pyridine was not used. The peak area of BL derivative obviously increased along with the increasing pyridine volume ratio in the range of 0–1 %. At higher volume ratio (>1 %), the peak area of BL derivative basically kept stable. Thus, the optimized pyridine volume ratio was at 1 %. The effect of reaction temperature was evaluated ranging from 40 to 90 °C. Results indicated that the derivatization could be completely achieved at 70 °C. When the reaction temperature was less than 70 °C, a long derivatization time was needed to obtain a constant response. Complete derivatization procedure was carried out at 70 °C for 10 min. If more than 15 min, the peak area of derivative would decrease. In conclusion, the vicinal hydroxyl groups of BL could be sufficiently labeled under the above optimum conditions.

Method Validation

The calibration curve of BL derivative was plotted at six points in the range of 50–1,000 ng L^{-1}. The linear regression equation was $Y = 0.3297X - 3.302$, good linearity was obtained between concentration and peak area with correlation coefficient (r^2) of 0.9996, and the LOD and LOQ were found to be at 8.0 and 25.0 ng L^{-1}, respectively.

As can be seen from Table 1, accuracies were in the range of 82.3–125.1 %. RSDs' values of precision for retention time and peak area were in the range of 0.68–1.94 and 4.61–9.75 %, respectively. Repeatability expressed as RSDs' values were in the range of 0.82–1.79 and 3.95–8.53 % for retention time and peak area, respectively. In conclusion, good accuracy, precision and repeatability were obtained for the proposed method.

The mean recoveries of BL for *Arabidopsis thaliana*, *Daucus carota* and *Brassica campestris* L. leaves' samples were in the range of 82.0–114.6 % (Table 1). The complex plant matrix could seriously interfere with the determination of BL. Thus, the matrix effect of the proposed method was assessed. After the UA-DLLME procedure, a proper amount of standard solution of BL was spiked into the *Arabidopsis thaliana*, *Daucus carota* and *Brassica*

Table 1 The results of matrix effect, recovery, accuracy, repeatability and precision of brassinolide in plant leaves ($n = 6$)

Spiked level (ng L^{-1})	Matrix effect (%)			Recovery (%)			Accuracy		Repeatability		Intra-day precision		Inter-day precision	
	Arabidopsis thaliana	*Daucus carota*	*Brassica campestris* L.	*Arabidopsis thaliana*	*Daucus carota*	*Brassica campestris* L.	Intra-day (%)	Inter-day (%)	Peak area RSD (%)	Retention time RSD (%)	Peak area RSD (%)	Retention time RSD (%)	Peak area RSD (%)	Retention time RSD (%)
50	95.4 ± 6.5	85.4 ± 3.2	114.6 ± 5.9	82.0 ± 7.3	114.6 ± 5.0	84.8 ± 4.5	115.6	97.4	6.38	1.79	5.27	0.68	7.28	1.32
200	90.0 ± 4.9	91.3 ± 6.8	107.4 ± 4.7	96.3 ± 5.1	89.3 ± 5.3	105.6 ± 6.7	82.3	107.6	3.95	0.82	4.61	0.97	8.61	1.94
1,000	115.3 ± 8.4	89.5 ± 7.1	93.6 ± 6.3	108.6 ± 7.5	90.7 ± 7.8	112.4 ± 8.1	88.6	125.1	8.53	1.46	6.54	0.73	9.75	1.45

campestris L. leaves' samples. The mean values of matrix effect were in the range of 85.4–119.6 % (Table 1). Results indicated that the matrix effect of the proposed hyphenated technique of UA-DLLME and derivatization was greatly improved. This might be because UA-DLLME was a selective extraction procedure for BL in complex plant matrix, and 9-phenanthreneboronic acid derivatization was also a selective labeling procedure for the vicinal hydroxyl groups in BL under the established experimental conditions.

Enrichment Factors

The enrichment factor (EF) was defined as the ratio of the analyte concentration in the extracted phase (C_{exp}) to the initial concentration of analytes (C_0) in the sample: $EF = C_{exp}/C_0$. Under the optimum extraction and derivatization conditions above, the result of EF was 189 for BL.

Comparison with Reported Methods

In Table 2, LOD of the proposed method was compared with that of the reported methods for the determination BL in real samples [9, 12, 19, 29]. LOD of the method with a derivatization procedure by HPLC-FLD [12] was relatively higher than HPLC–MS/MS [9, 19, 29] without derivatization. This means that semplice derivatization could not greatly improve the determination sensitivity of BL. In addition, compared with SPE [9, 29] and LLE [12], double-layered solid-phase extraction (DL/SPE) combined with boronate affinity polymer monolith microextraction, this method showed relatively lower LOD [19], which indicated that the selection of a highly efficient pretreatment procedure could increase sensitivity to some extent. UA-DLLME was such kind of pretreatment procedure definitely. However, LOD of this study for BL was about 6–80 times lower than the reported methods listed in Table 2. This should be benefited from the use of hyphenated technique of UA-DLLME and derivatization by HPLC fluorescence detection, which was not reported for the determination of BL. In conclusion, the proposed strategy of UA-DLLME combined with derivatization could greatly increase the sensitivity and the selectivity, and matrix effect was also effectively improved.

Application to the Analysis of Plant Samples

The established method was applied for the determination of BL in *Arabidopsis thaliana*, *Daucus carota* and *Brassica campestris* L. leaves. Two gram leaves of 2–3 weeks old plants were used for BL extraction. Plant leaves were ground to a fine powder and extracted in ice-cold 10 % (v/v) methanol for 2 h [9, 15]. After centrifugation, 5 mL supernatant was transferred to another centrifuge tube and

Table 2 Comparison of the proposed method with reported methods for determination of brassinolide in plant

Extraction method	Detection method	LOD (ng L^{-1})	LOQ (ng L^{-1})	References
SPE	LC–ESI–MS	98	–	[9]
LLE	HPLC-FLD	625	–	[12]
DL/SPE-BA/PMME	HPLC–ESI–MS/MS	50	270	[19]
SPE	HPLC–ESI–MS/MS	230	780	[29]
UA-DLLME	HPLC-FLD	8.0	25.0	This work

"–" not included in the method

SPE solid-phase extraction, *LLE* liquid–liquid extraction, *UA-DLLME* ultrasonic-assisted dispersive liquid–liquid microextraction, *DL/SPE-BA/PMME* double layered solid-phase extraction combined with boronate affinity polymer monolith microextraction

150 μL of chloroform was added for the UA-DLLME procedure. The contents of BL in the *Arabidopsis thaliana*, *Daucus carota* and *Brassica campestris* L. leaves were 1.25, 0.63 and 137.45 ng g^{-1} ($n = 3$).

Conclusions

In this study, a highly sensitive and selective hyphenated technique of UA-DLLME and derivatization was developed for the determination of BL in plant matrix. The combination of UA-DLLME and derivatization greatly enhanced the sensitivity and effectively improved the matrix effect of *Arabidopsis thaliana*, *Daucus carota* and *Brassica campestris* L. leaves' samples. This hyphenated technique was first used for the analysis of BL and it could be further extended for the analysis of other brassinosteroids.

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