ISSN 1615-9306 · JSSCCJ 38 (2) 171–356 (2015) · Vol. 38 · No. 2 · January 2015 · D 10609

JSS

JOURNAL OF SEPARATION SCIENCE

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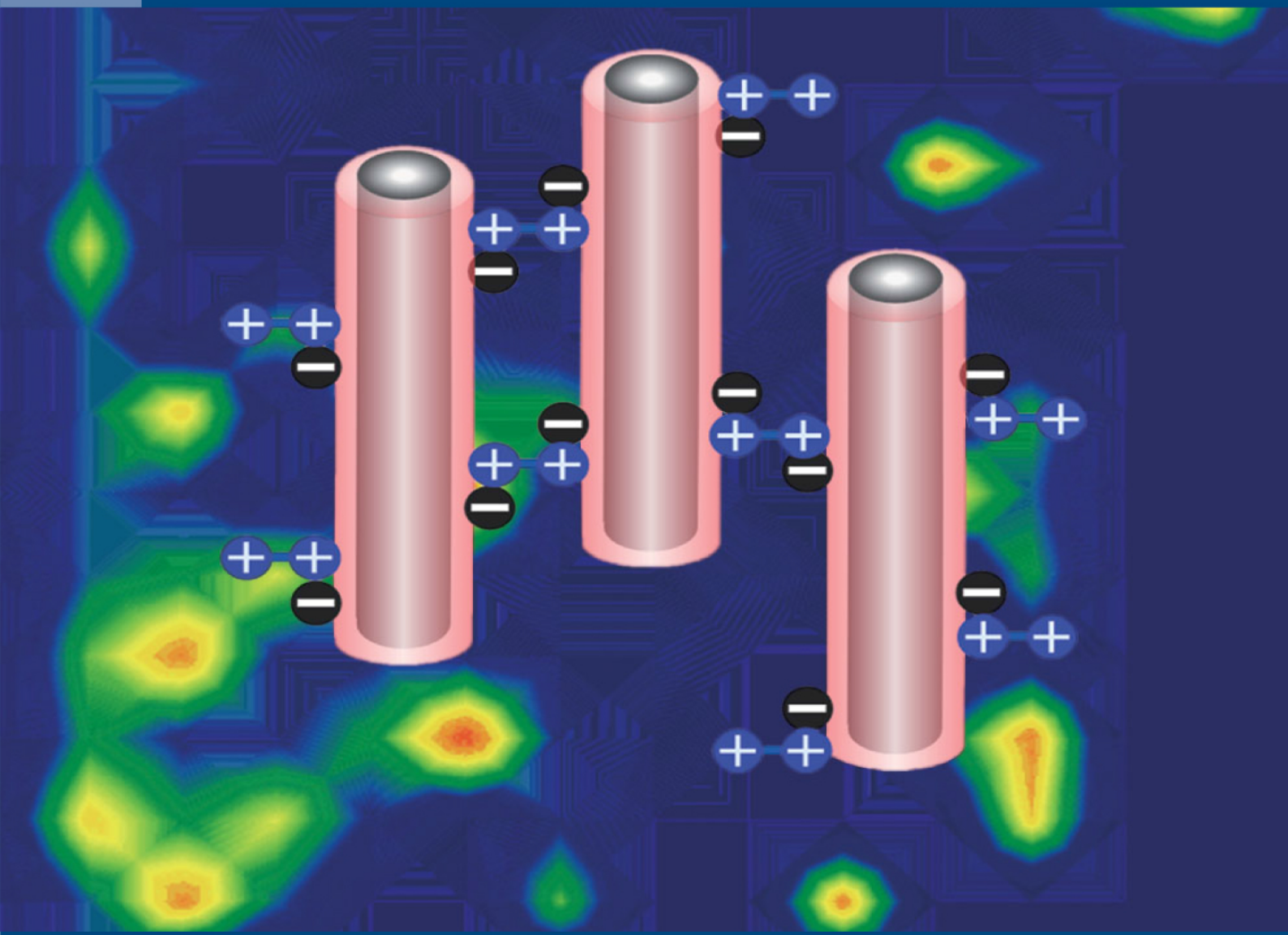

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Received October 13, 2014
Revised November 3, 2014
Accepted November 4, 2014

Research Article

Determination of multiple phytohormones in fruits by high-performance liquid chromatography with fluorescence detection using dispersive liquid–liquid microextraction followed by precolumn fluorescent labeling

Plant hormone determination in food matrices has attracted more and more attention because of their potential risks to human health. However, analytical methods for the analysis of multiple plant hormones remain poorly investigated. In the present study, a convenient, selective, and ultrasensitive high-performance liquid chromatography method for the simultaneous determination of multiple classes of plant hormones has been developed successfully using dispersive liquid–liquid microextraction followed by precolumn fluorescent labeling. Eight plant hormones in fruits including jasmonic acid, 12-oxo-phytodienoic acid, indole-3-acetic acid, 3-indolybutyric acid, 3-indolepropionic acid, gibberellin A_3, 1-naphthylacetic acid, and 2-naphthaleneacetic acid were analyzed by this method. The conditions employed for dispersive liquid–liquid microextraction were optimized systematically. The linearity for all plant hormones was found to be >0.9993 (R^2 values). This method offered low detection limits of 0.19–0.44 ng/mL (at a signal-to-noise ratio of 3), and method accuracies were in the range of 92.32–103.10%. The proposed method was applied to determine plant hormones in five kinds of food samples, and this method can achieve a short analysis time, low threshold levels of detection, and a high specificity for the analysis of targeted plant hormones present at trace level concentrations in complex matrices.

Keywords: Fluorescent labeling / Fruits / High-performance liquid chromatography / Plant hormones
DOI 10.1002/jssc.201401131

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1 Introduction

Plant hormones are structurally diverse compounds that play a crucial role in regulating numerous aspects of plant growth, development, and response to a wide range of biotic and abiotic stresses [1, 2]. Plant hormones are capable of controlling crop plant size and architecture, increasing crop grain productivity, and are widely employed to renovate crop breeding and improve agricultural production [3–5]. In most cases, multiclass phytohormones existed in plants either by endogenous secretion or exogenous treatment to achieve various enhanced agricultural characteristics during some critical growth stages. The abuse of these compounds in agriculture has led to their presence in fruits, soils, and underground waters. The potential toxicity of these hormones on humans or animals, which include carcinogenicity, impaired reproduction and development, neurotoxicity, and acute toxicity [6], has raised the need for strict control of plant hormone residues. Regarding the potential risks, many countries have set up a maximum residue limit (MRL). For example, the U.S. Environmental Protection Agency (EPA) and the European Union (EU) have set a MRL value of 100 μg/kg for naphthylacetic acid (NAA) in pome fruits and a higher value (1000 μg/kg) for NAA in apples and pears [7]. The EU has set up a MRL of

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Abbreviations: ACN, acetonitrile; **BAETS**, 2-(5-benzoacridine)ethyl-*p*-toluenesulfonate; **DLLME**, dispersive liquid–liquid microextraction; **FLD**, fluorescence detection; **GA_3**, gibberellin A_3; **IAA**, indole-3-acetic acid; **IBA**, 3-indolybutyric acid; **IPA**, 3-indolepropionic acid; **JA**, jasmonic acid; **MRL**, maximum residue limit; **NAA**, naphthylacetic acid; **OPDA**, 12-oxo-phytodienoic acid

5 mg/kg for gibberellin A_3 (GA_3) in grapes [7,8], which signifies the requirements of the sensitive and accurate quantification methods for routine analyses of phytohormone residues in a number of food matrices.

The development of a multiclass phytohormones pretreatment method is often impeded by the chemical diversity of the analytes (e.g. indole-3-acetic acid (IAA), NAA, GA_3, and jasmonic acid (JA)). Several sample pretreatment methods have been developed, which generally involved liquid extraction with different acid or alkaline solvents and further purifications by SPE with a wide variety of sorbents [9, 10], whereas the amount of elution solvent used is large, resulting in a limited enrichment factor [11]. To overcome this problem, the solvent-free sample pretreatment technique termed as SPME has been developed, and this technique has been employed for phytohormone analysis [11]. Nevertheless, it requires a specialized apparatus, like an SPME holder, and the fragile SPME fibers have a limited lifetime [12]. Recently, dispersive liquid–liquid microextraction (DLLME) has become widespread, which can overcome the disadvantages above [13]. DLLME requires simple and inexpensive devices, demands less organic solvent, and offers high enrichment factors [14,15]. In addition to the merits of other microextraction techniques, a notable advantage of DLLME is significant timesaving, which is desirable in high-throughput sample preparations. In this study, DLLME technology was employed for extraction of multiclass phytohormones, and the extraction conditions were optimized systematically by a three-level, three-variable Box–Behnken design from response surface methodology.

The development of a highly sensitive and selective detection method is also critical to plant hormone determination, but accurate determination of plant hormone has been a very challenging task. For example, plant hormones are present in very low physiological concentrations against a background of a wide range of more abundant primary and secondary metabolites. Despite the application of multistep purifications of crude plant extracts, there is still a large number of interfering substances in the samples to be analyzed. Some plant hormones (e.g. 12-oxophytodienoic acid, jasmonates, gibberellins, and abscisic acid) have little UV absorption and no fluorescence absorption, thus their determination by spectrophotometry is difficult (e.g. HPLC with UV or diode array detection). Furthermore, most of the plant hormones with carboxylic group possess strong polarity that causes weak retention in RP-LC systems and makes their separation in traditional analytical methods more difficult. An increasing number of novel techniques are being developed for determination of plant hormones. Many methods based on GC, LC, and CE coupled to MS, UV, or fluorescence detection (FLD) have been proposed for the analysis of multiphytohormones [7,8,16–23]. In general, CE offers attractive features for little sample preparation and short analytical time, but the potential reproducibility might be the obvious problem. GC–MS methods require a time-consuming and intensive purification protocol and some thermally labile phytohormones are likely to break down at the high temperature of the GC injector and column, which limits the range of plant hormones fit for GC analysis. Practically, the most frequently used method for phytohormone analysis is LC combined with different detectors. However, HPLC analysis might suffer from interference of the target HPLC–UV signals by matrix coextractives, which render the separation time longer or the sample clean-up procedure more complex [16]. HPLC with MS-based methods are the most important and effective in the analysis of toxic components at very low concentrations. But while LC–MS has been adopted for routine use for quantitative analysis, the expensive isotope internal standard is necessary and HPLC–MS methods often require high-resolution MS to ensure the high detection sensitivity, not easily available in common analytical laboratories. As mature and reliable coupled detection techniques in routine use, UV or FLD are relatively cheap and convenient. In all these methods, HPLC with FLD is more selective and sensitive [24, 25], which is much preferable to the analysis of trace phytohormones. In our previous studies, many novel fluorescent labeling reagents have been synthesized and developed for the determination of trace compounds with a carboxyl group, and 2-(5-benzoacridine)ethyl-*p*-toluenesulfonate (BAETS) is one of them, which possesses excellent fluorescence properties ensuring the high detection sensitivity [26]. In this study, a convenient, rugged, selective, and ultrasensitive HPLC method for the simultaneous determination of eight phytohormones has been developed successfully using DLLME followed by BAETS fluorescent labeling. The proposed approach was applied to determine plant hormones in five kinds of food samples, and this method can achieve a short analysis time, low-threshold levels of detection, and a high specificity.

2 Materials and methods

2.1 Reagents and materials

JA, 12-oxo-phytodienoic acid (OPDA), IAA, 3-indolybutyric acid (IBA), 3-indolepropionic acid (IPA), GA_3, 1-NAA, and 2-naphthaleneacetic acid (2-NAA) were purchased from Sigma–Aldrich (USA) and their structural formulas are shown in Supporting Information Fig. S1. High-purity water purified with a Milli-Q water purification system (Millipore, Molsheim, France) was used throughout the experiment. HPLC-grade acetonitrile (ACN) was purchased from Yucheng Chemical Reagents. Other reagents used were of analytical reagent grade (Shanghai Chemical Reagents, Shanghai, China).

2.2 Instrumentation

Experiments were performed using an Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, CA, USA). The HPLC system consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostatted column compartment (model G1316A), and a FLD. The mass spectrometer

(MSD Trap SL, model G2445D) from Bruker Daltonik (Bremen, Germany) was equipped with an APCI source (model G1947A). Ion source conditions: APCI in positive-ion detection mode; nebulizer pressure 60 psi; dry gas temperature, 350°C; dry gas flow, 5.0 L/min. APCI Vap temperature 350°C; corona current 4000 nA; capillary voltage 3500 V. The ultrasound-assisted dispersive liquid-liquid microextraction of phytohormones was carried out using an ultrasonic cleaner (SB-5200DTD, 40 kHz, Xinzhi Biotech, Ningbo, China).

2.3 Preparation of standard solutions

BAETS solution (1.0×10^{-2} mol/L) was prepared by dissolving 8.86 mg BAETS in 10 mL ACN. The standard mixture solution of each phytohormone (1.0×10^{-3} mol/L) was prepared in ACN/DMF (1:1, v/v), and diluted to the working solutions with different concentrations by ACN/DMF (1:1, v/v). Stock solutions (1 mg/mL of each analyte) were prepared by dissolving phytohormones in DMF, respectively. Working standard solutions were obtained by stepwise dilution of their stock standard solutions with DMF. When not in use, all reagent solutions were stored at 4°C in a refrigerator.

2.4 Ultrasound-assisted DLLME

Fruit samples were randomly collected from local markets in Jining City, China. All samples were homogenized with a high-speed homogenizer. The sample extraction was carried out according to the previous study [8]. The prepared samples (1.0 g) were further homogenized for 2 min with methanol (10 mL), and then ultrasonicated for 20 min by an ultrasonic cleaner. The mixture was centrifuged (3000 × *g*, 15°C) for 10 min. The supernatant was diluted with pure water to obtain the mixed solution with 10% of methanol, and a 5 mL portion of the prepared samples was transferred into a glass tube with a conical bottom, and then a 0.25 g of NaCl was placed in the glass tube and dissolved completely. The pH was adjusted to 2.0 with 0.1 mol/L HCl by a pH meter. Then 1.20 mL of acetone (as disperser solvent) and 100 μL $CHCl_3$ (as extraction solvent) were mixed, and rapidly injected into the sample solution by using the 2 mL glass syringe, and then the tube was immersed in an ultrasonic water bath for 1.5 min. The mixture was centrifuged at 4000 rpm for 3.0 min and the upper aqueous phase was removed, and the sediment phase was evaporated to dryness by a gentle nitrogen stream, and redissolved in 0.5 mL DMF. The solution was filtered through a 0.45 μm filter and then stored at 4°C for further analysis.

2.5 Fluorescence labeling of phytohormones

The fluorescence labeling was carried out according to several reported studies [27]. The scheme is shown in Fig. 1a and the procedure was as follows: (i) to a solution containing 20 μL of standard mixtures (or 150 μL sample solution when real sample labeling) in a vial, 100 μL BAETS reagent solution, 60 mg K_2CO_3, and 50 μL DMF was added, respectively; (ii) the vial was sealed and placed in a water bath at 90°C with shaking at 5 min intervals for 20 min; (iii) the mixture was cooled down to room temperature and diluted with ACN for HPLC analysis.

2.6 HPLC analysis

Separation of phytohormone derivatives was carried out on a Hypersil C_{18} column (200 mm × 4.6 mm, 5 μm, Agilent) combined with a linear gradient elution. Eluents A and B were ACN/H_2O (20:80; v/v) and 100% ACN, respectively. The gradient elution program was as follows: 0 min = 60% B, 13 min = 80% B, 20 min = 90% B. The flow rate was constant at 1.0 mL/min and the column temperature was set to 35°C. The injection volume was 10 μL. The fluorescence excitation and emission wavelengths were set to λex = 250 nm and λem = 405 nm, respectively.

2.7 Method validation

The developed HPLC method was validated by evaluation of the variation of retention times and peak area for analytes, building of calibration curves, LOD, LOQ, and accuracy. Linearity was measured at seven concentration levels. Calibration curves were constructed by plotting peak area (*Y*) versus concentration (*X*) in the range of 0.001–10 nmol/mL for each of the analytes. LOD and LOQ were calculated at S/N of 3 and 10, respectively. The method repeatability was investigated by six injections of 10 μL standard solution. The precision was expressed as the percentage RSD. The accuracy of the analytical method was determined by spiking with a known amount of standard into real samples.

3 Results and discussion

3.1 Selection of extraction solvent and disperser solvent

Extraction solvent can significantly affect extraction efficiency in DLLME. The optimum extraction solvent should have low solubility in water, high affinity to analytes, high density, and good chromatographic behavior. Among the solvents with density higher than water (mainly chlorinated solvents), chlorobenzene (C_6H_5Cl), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), tetrachloromethane (CCl_4), dichloroethane ($C_2H_4Cl_2$), and tetrachloroethane ($C_2H_2Cl_4$) were tried [13], and extraction efficiencies of various solvents investigated were studied, respectively. Results indicated that CH_2Cl_2 and $C_2H_4Cl_2$ were not suitable for extraction solvent, because there was no cloudy state and no sedimented droplet of organic solvent at the bottom of the tube after centrifugation. When the other four solvents were tested cloudy states and emulsion systems were formed, which is probably

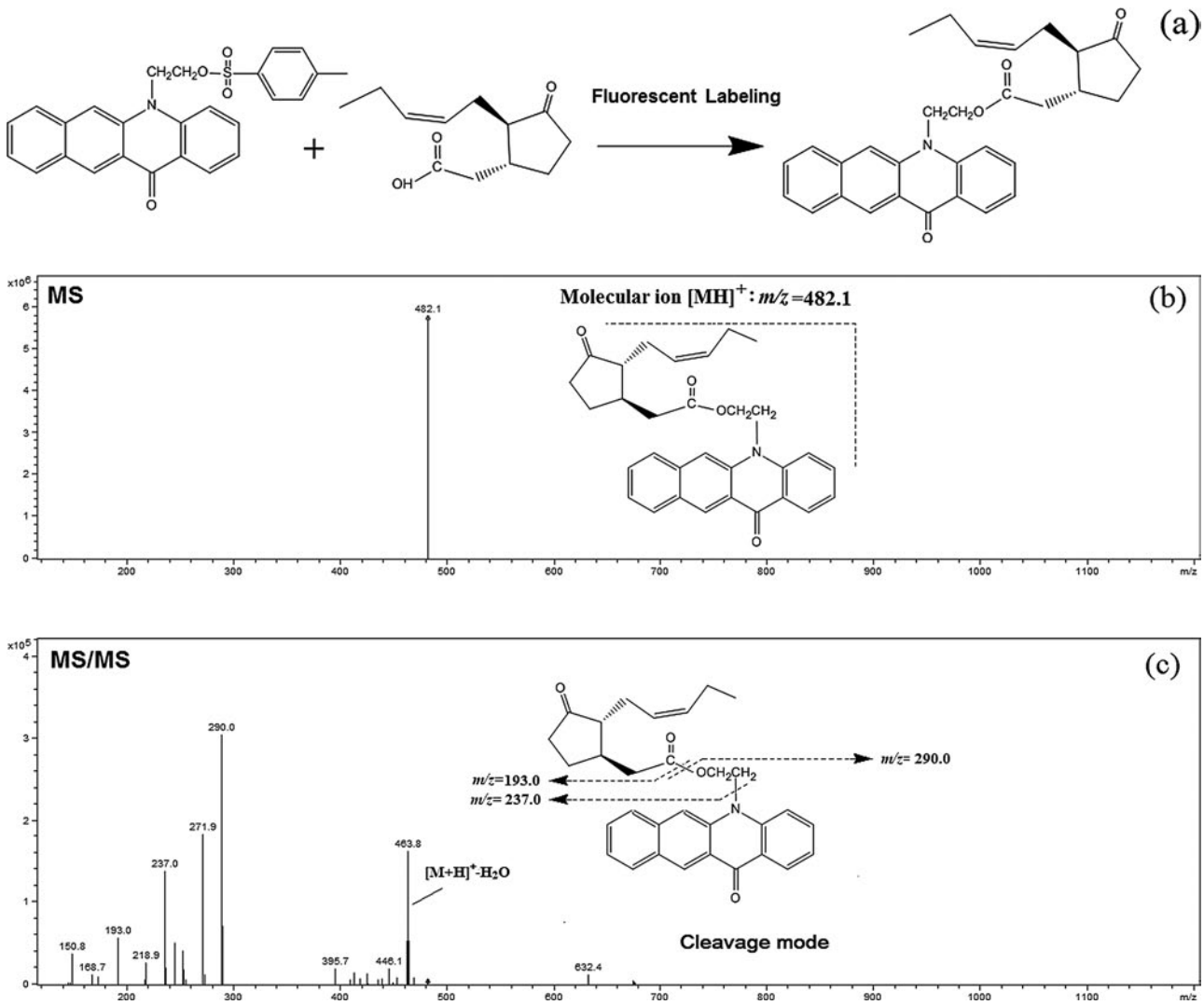

Figure 1. The representative labeling scheme for BAETS with jasmonic acid (a), and the representative MS data (MS and MS/MS) and cleavage mode for BAETS-JA derivative (b and c).

because the densities of C_6H_5Cl, $CHCl_3$, CCl_4, and $C_2H_2Cl_4$ are higher than those of CH_2Cl_2 and $C_2H_4Cl_2$, and the miscibilities of C_6H_5Cl, $CHCl_3$, CCl_4, and $C_2H_2Cl_4$ in the organic solvents are lower. $CHCl_3$ as extraction solvent gave the highest signal response for eight targeted compounds comparing to other solvents. Meanwhile, in the case of $CHCl_3$ as extraction solvent, a stable two-phase system was formed and its sedimented phase can be easily removed by microsyringe. So $CHCl_3$ was selected as the extraction solvent.

The miscibility of the disperser solvent in the extraction solvent and aqueous phase is the most important factor affecting the selection of disperser solvent in DLLME. The selection of a dispersive solvent is limited to solvents such as methanol, ACN, and acetone, which are miscible with both water and extraction solvents. The experiments were performed by using 1.0 mL of each dispersive solvent containing 50 μL $CHCl_3$ and three replicate tests were performed for each type of dispersive solvent. The results are illustrated in Fig. 2, which indicated that acetone exhibited the highest extraction efficiency. Furthermore, acetone can give more stable and uniform cloudy solutions, so it was selected as the disperser.

3.2 Selection of sample solution pH and ionic strength

Plant hormones possess carboxylic groups, thus the solution pH of the sample can significantly affect the extraction recoveries. When the pH changes, the acid–base equilibrium for plant hormones shifts significantly toward the neutral forms or ionic forms. In acidic conditions, the plant hormone becomes uncharged and is extracted more efficiently into the fine droplets of the organic phase [23]. The effect of the sample solution pH on the extraction efficiency from water samples was studied within the range of 2–6 using HCl (Fig. 3). The extraction efficiencies of the targeted phytohormones were highest when the pH was set to 2.

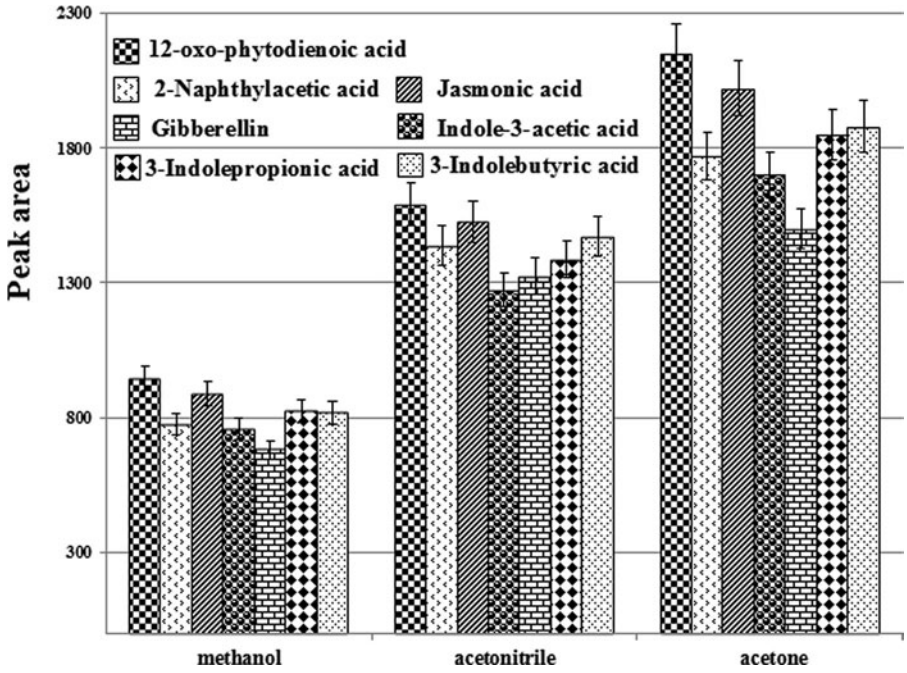

Figure 2. The effect of dispersive solvents on extraction efficiency (peak area) in DLLME.

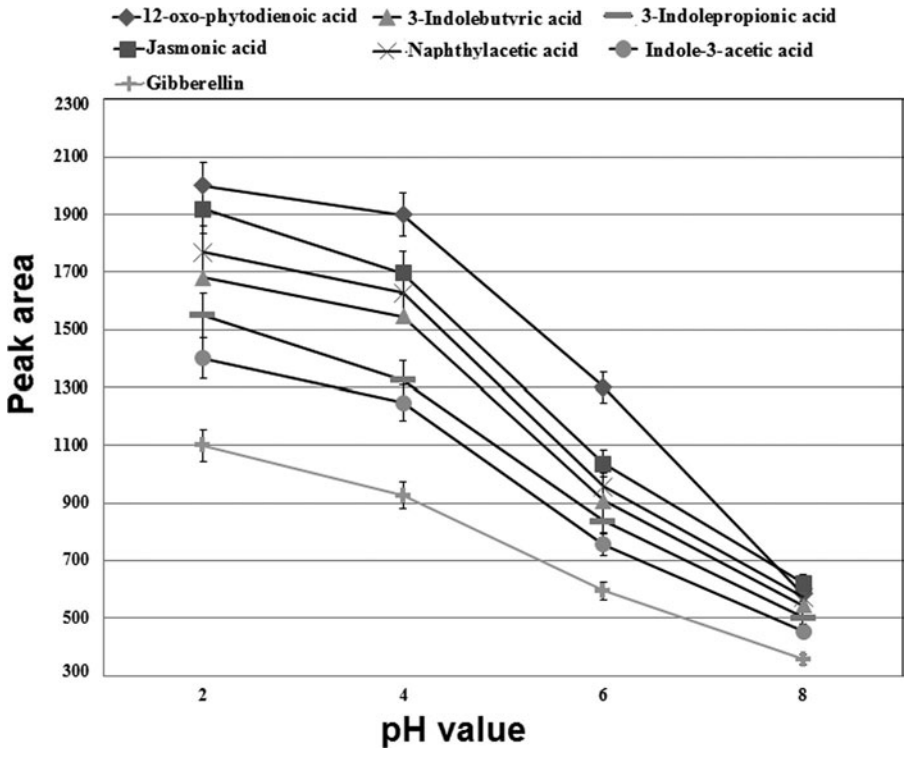

Figure 3. The effect of pH on extraction efficiency (peak area) in DLLME.

The increase in the ionic strength can lead to a decrease in the solubility of the analytes in sample solution, thus, extraction efficiency may be enhanced. For investigating the influence of ionic strength on the extraction efficiency of DLLME, various experiments were performed by adding different amount of NaCl (0–15%, w/v) with other experimental conditions keeping constant. Results indicated that the peak area increased with the ionic strength growth (from 0 to 5%), while a slight decrease was observed at the higher ionic strength of >5%. Furthermore, the higher percentage of NaCl could cause no sedimentation at the bottom after centrifugation; this could be due to the density of the aqueous solution, which increased with high amounts of NaCl. Based on these results, 5% w/v NaCl was chosen as the optimal ionic strength in the DLLME procedure.

Table 1. The conditions used and experimental data for the peak area of eight phytohormones obtained from Box–Behnken design ($n = 3$)

No.	Parameters			IPA	NAA	IAA	IBA	JA	OPDA	GA
	EV	DV	Time							
1	50	1500	2.5	350	330	170	451	407	420	124
2	150	1500	2.5	893	748	536	1097	1030	1154	332
3	100	500	4	799	745	559	966	980	966	270
4	150	500	2.5	711	676	304	1071	1142	1326	592
5	100	1500	4	777	640	393	686	613	864	260
6	50	1000	1	682	504	296	572	528	668	240
7	100	1000	2.5	785	763	509	988	965	1198	633
8	100	1000	2.5	869	850	590	1053	1101	1241	432
9	100	1000	2.5	798	844	610	880	1065	1343	354
10	100	500	1	747	661	423	540	620	1047	268
11	100	1000	2.5	843	847	628	1022	1088	1253	447
12	150	1000	4	770	734	495	944	965	904	223
13	150	1000	1	998	692	330	1078	988	972	302
14	100	1000	2.5	973	935	630	1190	1203	1193	349
15	50	500	2.5	376	433	336	447	600	872	231
16	100	1500	1	620	657	391	533	637	1068	266
17	50	1000	4	612	572	251	693	677	754	214
18-Opt[a)]	110	1200	1.5	782	709	472	705	784	1136	365
19-Verif[b)]	110	1200	1.5	790	740	460	727	795	1240	357

a) The optimized conditions by the model and the predicted peak area.

b) The results for verified experiments under the optimized conditions ($n = 3$).

3.3 Optimization of DLLME conditions

An ultrasonic process was applied to accelerate the formation of a fine cloudy dispersive mixture in DLLME. The conditions including ultrasonic time, the volume of extraction solvent, and disperser solvent can significantly affect the extraction efficiency. In this study, these conditions were further optimized by a three-level, three-variable Box–Behnken design from response surface methodology. The peak area of each analyte was selected as the response variable. This design involved a total of 17 randomized runs (Table 1). The software Design Expert (Version 7.1.3, Stat-Ease, Minneapolis, MN, USA) was employed for experimental design, data analysis, and model building. Statistical analysis of the model was performed to evaluate the ANOVA. Results of the analysis indicated that all the linear parameters and quadratic parameters were significant at the level of $p < 0.01$. The *F*-value for the lack of fit was insignificant ($p > 0.05$), meaning that this model was sufficiently accurate for predicting the relevant responses.

The response surface curves were plotted to investigate the interactions of the variables and determine the optimal level of each variable for the maximum response. The typical 3D response surface plots for NAA and IBA are shown in Fig. 4a and b, which reflect the effect of DLLME conditions (ultrasound time (*T*), volume extraction solvent volume (EV), and disperser solvent volume (DV) on the extraction efficiency. For example, Fig. 4a-1 shows interaction between EV and DV on the peak area of NAA. As shown in Fig. 4a-1,

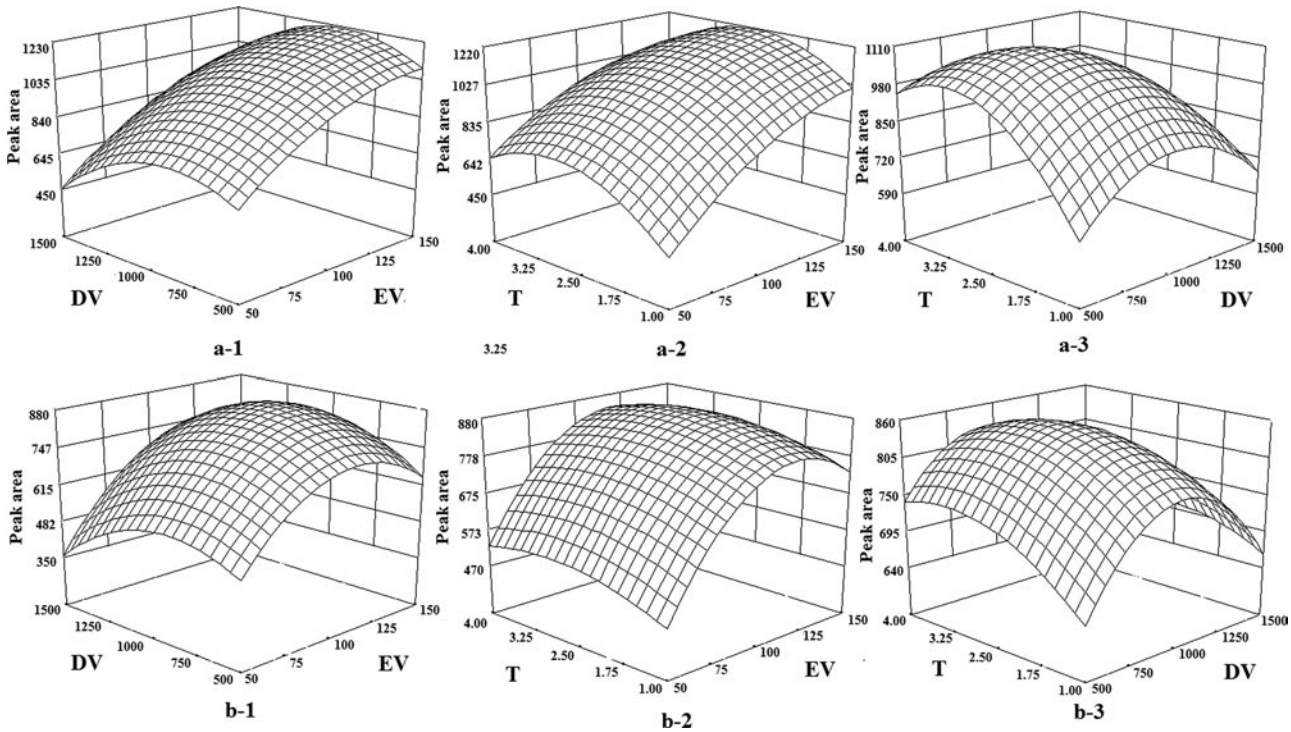

Figure 4. The 3D response surface plots of extraction efficiency for the representative NAA (a) and IBA (b) affected by ultrasound time (*T*), the volume of extraction solvent (ES), and disperser solvent (DV) in dispersive liquid–liquid microextraction.

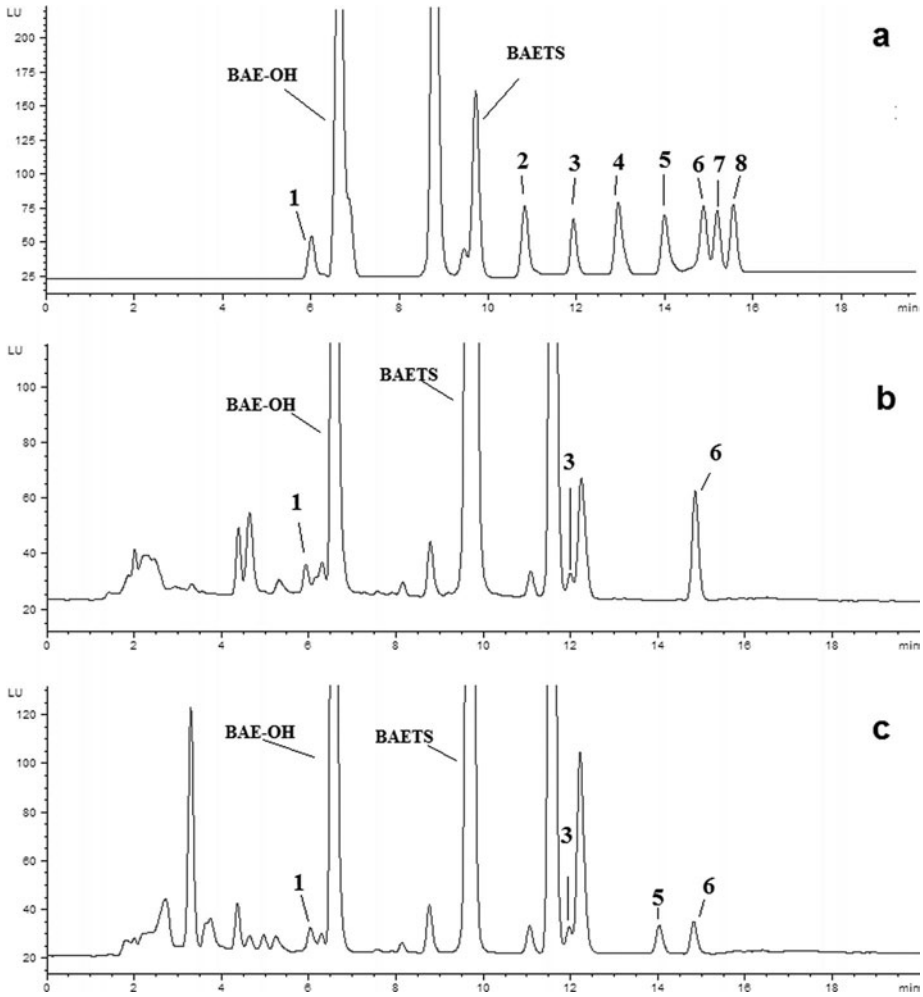

Figure 5. The representative chromatograms for eight standards (a), litchi (b), and grape (c). Peak labels: (1) gibberellin A_3; (2) indole-3-acetic acid; (3) 3-indolepropionic acid; (4) 3-indolybutyric acid; (5) jasmonic acid; (6) 12-oxo-phytodienoic acid; (7) 1-naphthylacetic acid; and (8) 2-naphthaleneacetic acid.

with a definite time, the peak area increased with the increasing amount of DV and reached a maximum value, followed by a decline with its further increase. It shown in Fig. 4a-2, T and EV had a remarkable interaction. With a given DV, the peak area increased with the increase of T and reached the highest value around 2 min, and then a little decline was observed with its further increase. Similarly, Fig. 4a-3 describes the effect of T and DV on extraction efficiency. The optimum parameters for DLLME given by RSD were 100 μL of EV, 1200 μL of DV, and ultrasound emulsification time of 1.5 min, and the predicted values given by the model is shown in Table 1 (No. 18). Under the above optimal conditions, DLLME were carried out for verification of the optimization ($n = 3$) and the result is shown in Table 1 (No.19), which was close to the theoretical predicted value. The excellent correlation between predicted and measured values verifies the model validation and existence of an optimal point.

3.4 HPLC separation and MS identification

LC columns with different stationary phases were trialed (i.e. Hypersil C_{18}, Hypersil BDS C_8, Hypersil BDS C_{18}, and Spherisorb C_{18}). Hypersil C_{18} gave better separation and peak shape compared to other columns, which was chosen for the next HPLC condition optimization. Due to the hydrophobic character of the labeled analytes, organic solvent-rich mobile phases are typically used for their rapid elution under RP conditions. In a preliminary series of experiments, mixtures of water with two common HPLC organic modifiers (ACN, methanol) were examined as mobile phases. The flow rate was set at 1 mL/min and the sample injection volume at 20 μL. Results indicated that the usage of ACN offered better peak symmetry and was therefore selected for subsequent studies. Furthermore, optimal chromatographic conditions were obtained in gradient elution mode (Section 2.6), allowing better and rapid separation of eight analytes. The typical chromatogram for eight standards is shown in Fig. 5a.

The ionization and fragmentation of the plant hormone derivatives are studied by LC–APCI-MS in positive-ion detection mode. All hormone derivatives produced intense molecular ion peaks at m/z $[M + H]^+$. The MS and MS/MS spectra of the JA derivative are shown in Fig. 1b. The collision-induced dissociation spectra (MS/MS) of molecular ions (MS, $[M + H]^+$ ion) produced the fragment ions at m/z 193.0, 237.0, 290.0, and 463.8 (Fig. 1b). In most cases, the collision-induced

Table 2. Linear regression equation, correlation coefficients, LOD, LOQ, reproducibility, accuracy, and intra- and interday precision ($n = 6$)

Analytes	Linearity		LOD (ng/mL)	LOQ (ng/mL)	Repeatability RSD (%) ($n = 6$)			
	$y = Ax + B$[a)]	R			Intraday		Interday	
					Retention time	Peak area	Retention time	Peak area
Gibberellin	$y = 21.32x + 9.74$	0.9993	0.38	1.32	0.03	2.2	0.97	3.4
Indole-3-acetic acid	$y = 36.92x + 9.50$	0.9999	0.19	0.77	0.02	2.4	0.91	4.1
3-Indolepropionic acid	$y = 35.19x + 8.93$	0.9997	0.21	0.81	0.02	3.8	0.87	3.1
3-Indolebutyric acid	$y = 40.55x + 10.35$	0.9995	0.26	0.83	0.03	1.4	0.83	3.8
Jasmonic acid	$y = 32.01x + 8.84$	0.9995	0.33	0.92	0.02	2.7	0.81	4.1
12-Oxo-phytodienoic acid	$y = 34.36x + 6.50$	0.9996	0.44	1.01	0.03	2.6	0.79	3.2
1-Naphthylacetic acid	$y = 30.82x + 14.90$	0.9993	0.38	0.81	0.04	3.5	0.78	5.4
2-Naphthylacetic acid	$y = 35.1x + 4.22$	0.9993	0.41	0.82	0.02	3.3	0.79	3.4

a) y, peak area; x, injected amount of each phytohormone (ng).

Table 3. Determination of eight phytohormones in real samples under the optimized experimental conditions ($n = 3$)

Plant sample	(ng/g)	GA_3	IAA	IPA	IBA	JA	OPDA	1-NAA	2-NAA
Nectarine	Added	0	0	0	0	0	0	0	0
	Found	4.47	0	0.78	0	2.37	3.46	0	0
	Added	60	15	15	15	15	60	15	15
	Found	59.90	14.11	15.28	14	17.48	64.99	14.07	14.45
	Recovery (%)	92.91	94.08	96.83	93.30	100.65	102.41	93.79	96.30
Litchi	Added	0	0	0	0	0	0	0	0
	Found	21.71	0	0.83	0	0	26.11	0	0
	Added	60	15	15	15	15	60	15	15
	Found	78.24	14.42	14.99	15.47	14.08	83.72	14.14	14.57
	Recovery (%)	95.75	96.14	94.67	103.10	93.89	97.22	94.28	97.10
Cherry	Added	0	0	0	0	0	0	0	0
	Found	1.34	0	0.70	0	0	0.46	0	0
	Added	60	15	15	15	15	60	15	15
	Found	59.21	14.14	14.86	14.22	15.32	57.06	14.39	14.34
	Recovery (%)	96.53	94.28	94.67	94.77	102.12	94.38	95.95	95.60
Apple	Added	0	0	0	0	0	0	0	0
	Found	3.80	0	0.65	0	0	10.47	0	0
	Added	60	15	15	15	15	60	15	15
	Found	61.46	14.54	14.45	14.85	14.80	66.44	13.88	15.17
	Recovery (%)	96.34	96.93	92.32	98.98	99.28	94.28	92.52	101.10
Grapes	Added	0	0	0	0	0	0	0	0
	Found	2.57	0	1.23	0	1.70	2.20	0	0
	Added	60	15	15	15	15	60	15	15
	Found	63.65	14	15.62	14.89	16.20	60.59	15.07	14.58
	Recovery (%)	101.73	93.30	96.24	99.28	97.02	97.42	100.45	97.20

dissociation spectra of m/z $[M + H]^+$ for plant hormone derivatives produced the specific fragment ion by losing H_2O molecule, producing the ion at m/z $[MH - H_2O]^+$, which was a specific fragment ion for the identification of plant hormone derivatives (Fig. 1b).

3.5 HPLC method validation and application

As shown in Table 1, all plant hormone were found to give linear responses with correlation coefficients of >0.9993. With the injections of the highly diluted standard sample, the calculated LOD (S/N = 3:1) was from 0.19 to 0.44 ng/mL, and the value of LOQ was in range of 0.77–1.32 ng/mL, which demonstrated the high sensitivity of this method. The instrument precision was examined by the continuous six injections of standard solution. The intraday RSD of retention time and peak area for ($n = 6$) was <0.04 and <3.8%, respectively. The interday RSD of retention time and peak area was <0.97 and <5.4%, respectively. Meanwhile, the method precision was determined by adding 20 pmol standard solution to the original sample, then through the whole procedure including

Table 4. The overall comparison of the developed method with the reported methods

Analyte	Method	Analytical time	LOD (nM)	Reference
GA_3, IAA, JA, IBA, NAA, 2,4-D	HPLC–FLD	~80 min	3.6–6.7 nM	[28]
IBA, NAA, 2,4-D	HPLC–FLD	~80 min	4.43–14.8 nM	[29]
JA	HPLC–ECD	~12 h	50 nM	[30]
IAA, ABA, GA_3	LC–MS/MS	~14 h	11 nM to 12.6 μM	[31]
IAA, IBA, JA, SA, ABA, GA_3	DART-MS	~30 min	3.0–208 nM	[32]
GA_3, IAA, IPA, IBA, JA, 1-NAA, 2-NAA, OPDA	HPLC–FLD	~50 min	1.03–2.30 nM	This study

DART, direct analysis in real-time.

extraction, derivatization, separation, and quantitation to ensure the final result. The method accuracies (Table 2) were in the range of 92.32–103.1%. These results demonstrated the suitability of the proposed method for determination of trace plant hormones in plant extract.

The proposed method of DLLME combined with precolumn fluorescent labeling HPLC analysis was applied to the determination of phytohormones in five kinds of fruits. The typical chromatograms for litchi and grape are presented in Fig. 5b and c, respectively. The analytical results are shown in Table 3. Different phytohormones were detected in nectarine, litchi, cherry, apple, and grapes. As shown in Table 3, the concentrations of the phytohormones varied greatly in the various fruits. For example, the content of GA_3 in litchi was as high as 21.71 ng/g, while GA_3 in cherry was 1.34 ng/g. IAA, IBA, 1-NAA, and 2-NAA were not found in all tested fruits.

A comparison of the proposed method with the recently reported methods is provided in Table 4. As a result, our method showed many advantages. For example, the developed method in this study offered the LOD of 1.03–2.30 nM, which was lower than the methods in Table 4. The completed analytical time was much shorter than the methods presented in Table 4. The HPLC separation conditions of our method including mobile phase and elution program were simpler. In addition, HPLC–FLD can be easily available in common analytical laboratories compared to direct analysis in real-time MS and HPLC–MS/MS.

4 Conclusion

In this study, a new HPLC method for the simultaneous determination of eight phytohormones has been developed successfully. The strategy of DLLME followed by precolumn fluorescent labeling allows the convenient sample preparation, high sensitivity, and selectivity. Satisfactory results were obtained in terms of linearity, sensitivity, method accuracies, and repeatability. When applied to real sample analysis, the proposed method showed excellent applicability. Overall, this method can achieve a short analysis time, low-threshold levels of detection, and a high specificity for analysis of targeted plant hormones present at trace-level concentrations in complex matrices.

This work was supported by The National Natural Science Foundation of China (Nos. 31301595, 21475074, and 21275089), Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety (GDPKLAPPS1401), the Natural Science Foundation of Shandong Province, China (ZR2013BQ019), PhD research start-up funds of Qufu Normal University (bsqd 2012017), and the Soft Science Project of Shandong Province (2013RKA08020).

The authors have declared no conflict of interest.

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