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Highly sensitive and quantitative profiling of acidic phytohormones using derivatization approach coupled with nano-LC–ESI-Q-TOF-MS analysis

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ABSTRACT

In current study, we developed a highly sensitive method for the quantitative profiling of acidic phytohormones. Tandem solid-phase extraction (SPE) and liquid–liquid extraction (LLE) was employed to efficiently purify acidic phytohormones, which were further derived by 3-bromoactonyltrimethylammonium bromide (BTA) to increase the ionization efficiency in electrospray ionization-mass spectrometry detection. Additionally, fifteen BTA-derived acidic phytohormones, including ten gibberellins (GAs), were well separated with a salt gradient on poly(methacrylic acid-*co*-ethylene glycol dimethacrylate) (MAA-*co*-EDMA) monolithic column. By employing online trapping system, the signal intensities of the analytes were significantly improved. The limits of detection (LODs, Signal/Noise = 3) of targeted phytohormones ranged from 1.05 to 122.4 pg/mL, which allowed the highly sensitive determination of low abundant acidic phytohormones with tiny amount plant sample. Good reproducibility was obtained by evaluating the intra- and inter-day precisions with relative standard deviations (RSDs) less than 10.9 and 11.9%, respectively. Recoveries of the target analytes from spiked rice leave samples ranged from 88.3 to 104.3%. By employing the method developed here, we were able to simultaneously determine 11 endogenous acidic phytohormones from only 5 mg of rice leave sample, which dramatically decreased the required sample amount (three orders of magnitude lower) for the profiling of low abundant acidic phytohormones compared to previous reports. Taken together, the method provided a good solution for the highly sensitive and quantitative profiling of endogenous acidic phytohormones.

1. Introduction

Phytohormones have been considered to be vital regulators in plant growth and development. The subtle changes of phytohormones can influence basic developmental processes of plant [1,2]. Profiling of endogenous phytohormones is critical for in-depth understanding of how phytohormones regulate plant growth, development, and other life processes [3–6]. However, due to the distinctive distribution and trace concentration of acidic phytohormones in complex plant matrices, the determination of acidic phytohormones, particularly gibberellins (GAs) is still a highly challenging task.

With the advancement of analytical technologies, both the sensitivity and universality of mass spectrometry (MS) were significantly improved. Many reports have described the MS-based analysis of endogenous phytohormones [7–10]. However, the MS-based methods still have been restricted to achieve satisfactory ionization efficiency for negatively charged acidic phytohormones, which is mainly due to the low MS response of acidic analytes [11,12], serious matrix effect [13,14], and interference among analytes [15].

As an effective way to improve the MS signal response of negatively charged analytes, derivatization has been successfully used to sensitively quantify acidic analytes [7,9,16,17]. In our previous work, 3-bromoactonyltrimethylammonium bromide (BTA) was applied to derive acidic phytohormones by incorporating a positively charged quaternary ammonium group. BTA derivatives were then determined by capillary electrophoresis coupled with mass spectrometer (CE-MS) [7]. Using capillaries covalently coated with amino groups, the negatively charged impurities were pumped out of separation capillary by reversed electroosmotic flow (EOF), which effectively eliminated the matrix interference. Additionally, amino-coated capillaries can reduce the adsorption of BTA derivatives on the inner wall of the fused capillary. However, the detection sensitivity was restricted due to the dilution effect resulting from the sheath-liquid interface of CE-MS and 3 g rice leave sample was

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required for the detection of acidic phytohormones. Additionally, an inherent weakness of capillary zone electrophoresis (CZE) is that GA_4 and GA_{20} with the same m/z cannot be separated by electromigration mechanism. This issue can be solved by employing micellar electrokinetic chromatography (MEKC), which can separate analytes with similar m/z but exhibiting differences in hydrophobicity. However, MEKC may not be compatible with MS detection. This restriction leads to the failure in extract quantification of GA_4 and GA_{20}, respectively. Therefore, development of more sensitive and selective analytical methods for the determination of acidic phytohormones with trace concentration from plant samples is highly needed.

With nano-scale analytical system, the required sample amount can be significantly reduced. Nano-LC–ESI-Q-TOF-MS system can offer ultra-sensitive detection, but only a few studies on the profiling of phytohormones with this platform were reported [8,18]. To date, reversed-phase (RP) chromatographic separation of small acidic molecules with nano-LC platform is frequently employed with conventional particulate-packed C_{18} column [19–27]. However, particulate-packed C_{18} column seldom provides enough retention and resolution towards permanently charged analytes with quaternary ammonium group. Besides, bio-analysis performed on particulate-packed C_{18} column usually required long separation time and relative high back pressure [18]. In this respect, monolithic stationary phases show promising alternative for nano-LC due to the good permeability, ease of preparation, and broad selectivity [28,29]. These advantages suggest that hydrophobic/cation exchange monolithic capillary column may provide good separation resolution for BTA-derived acidic phytohormones.

In current work, we developed a highly sensitive method to determine the endogenous acidic phytohormones. Tandem solid-phase extraction (SPE) followed by liquid–liquid extraction (LLE) extraction was utilized for efficient purification of acidic phytohormones in rice leave samples. Afterwards, poly(methacrylic acid-*co*-ethylene glycol dimethacrylate) (MAA-*co*-EDMA) monolithic column was adopted on nano-LC–ESI-Q-TOF-MS system to achieve baseline separation, whereas online trapping system was employed to lower the limit of detection. This proposed method was successfully used for the profiling of acidic phytohormones including ten different species of GAs in 5 mg rice leave sample, which demonstrated that this method possessed excellent performance on the quantitative profiling of endogenous acidic phytohormones in complex matrices.

2. Materials and methods

2.1. Chemicals and reagents

Stable isotope-labeled compounds and standards, [$^2H_5$] IAA, [$^2H_6$] ABA, [$^2H_2$] SA, [$^2H_2$] GA_1, [$^2H_2$] GA_3, [$^2H_2$] GA_4, [$^2H_2$] GA_{12}, [$^2H_2$] GA_{24}, [$^2H_2$] GA_{53}, IAA, ABA, JA, SA, IBA, GA_1, GA_3, GA_4, GA_7, GA_9, GA_{12}, GA_{19}, GA_{20}, GA_{24}, and GA_{53} were purchased from Olchemim Ltd. (Olomouc, Czech Republic). Ammonium formate ($HCOONH_4$), formic acid (FA), and triethylamine (TEA) (33 wt%, solution in ethanol) were purchased from Shanghai General Chemical Factory. HPLC-grade acetonitrile (ACN) and methanol were obtained from TEDIA Company Inc. (OH, USA). Milli-Q water (Millipore, Bradford, USA) was used in all experiments. C_{18} SPE cartridges (3 mL, 50 mg) were obtained from Micromole Separation and Testing Technology (Beijing, China). SAX SPE cartridges (3 mL, 200 mg) were purchased from Weltech Co., Ltd. (Wuhan, China). BTA was synthesized according to previous work [7].

2.2. Preparation of plant samples

Rice was grown in growth chambers in a 16 h light/8 h dark photoperiod with 80% humidity under 28 °C. Light intensity was fixed to 120 lx/m^2/s. After approximate 7 days growing, 5–500 mg (fresh weight) rosette leaves were harvested.

Rice leaves were collected, weighted, immediately frozen in liquid nitrogen, and then stored at −80 °C. As shown in Fig. 1, to extract phytohormones, plant samples (5 mg) were frozen in liquid nitrogen and finely ground followed by extraction with 500 μL modified *Bieleski* solvent (methanol/H_2O, 80/20, v/v) at 4 °C for 12 h. [$^2H_5$] IAA (15.0 ng/g), [$^2H_6$] ABA (50 ng/g), [$^2H_2$] SA (500 ng/g), [$^2H_2$] GA_1 (1.00 ng/g), [$^2H_2$] GA_3 (1.00 ng/g), [$^2H_2$] GA_4 (1.00 ng/g), [$^2H_2$] GA_{12} (1.00 ng/g), [$^2H_2$] GA_{24} (2.00 ng/g), and [$^2H_2$] GA_{53} (2.00 ng/g) were added to plant samples as internal standards (I.S.) prior to grinding.

Then, the supernatants were sequentially passed through the tandem SPE cartridges containing C_{18} adsorbent (50 mg) and SAX adsorbent (200 mg). Before SPE extraction, the tandem cartridges were pre-conditioned with 8 mL H_2O, 8 mL methanol, and 8 mL modified *Bieleski* solvent. After sample loading, the C_{18} cartridge was removed and the SAX cartridge was rinsed with 2 mL methanol/H_2O (20/80, v/v). After that, 3 mL ACN with 1% FA (v/v) was applied to elute the targeted acidic phytohormones and the eluent was evaporated under mild nitrogen stream at 35 °C followed by re-dissolving in 100 μL H_2O.

The resulting solution (100 μL) was then acidified with 10 μL FA, and extracted with ether (2 × 1 mL). The ether phase was combined, dried under nitrogen gas and reconstituted in 100 μL ACN. To the resulting solution, 10 μL triethylamine (TEA) (20 μmol/mL) and 10 μL BTA (20 μmol/mL) were added. The reaction solution was vortexed for 30 min at 35 °C and evaporated under nitrogen gas followed by re-dissolving in 200 μL H_2O/ACN (90/10, v/v) for further analysis.

2.3. Nano-LC–ESI-Q-TOF-MS

All nano-LC experiments were carried out on a Shimadzu Prominence nano-flow liquid chromatography system (Kyoto, Japan) with two LC-20AD nano pumps, two vacuum degassers, a LC-20AB HPLC pump, a SIL-20AC HT autosampler and a FCV nano valve (Fig. 2).

The analytical column of poly(MAA-*co*-EDMA) monolithic column (100 μm *i.d.*, 360 μm *o.d.*, 30-cm long, purchased from Weltech Co., Ltd., Wuhan, China) was connected to nano-LC system and conditioned with the mobile phase (ACN/H_2O, 50/50, v/v) at a flow rate of 600 nL/min for 30 min. The poly(MAA-*co*-EDMA) monolithic column was coupled with a ESI emitter (7 cm × 25 μm, with a 8 ± 1 μm tip) (PicoTip company, USA) by a stainless steel union.

For direct injection, a 50-nL sample loop was used. Optimization of separation conditions was performed by direct injection. To enhance the capacity of the analytical system, online trapping system was employed. Four types of trapping columns, including poly(MAA-*co*-EDMA), poly(2-acrylamido-2-methyl-1-propanesulfonic acid-*co*-ethylene glycol dimethacrylate) (AMPS-*co*-EDMA), hypercrosslinked poly(methacrylic acid-*co*-vinylbenzene chloride-*co*-divinylbenzene) (MAA-*co*-VBC-*co*-DVB), poly(2-acrylamido-2-methyl-1-propanesulfonic acid-*co*-divinylbenzene-*co*-ethylene glycol dimethacrylate) (AMPS-*co*-DVB-*co*-EDMA) (7-cm long, 100 μm *i.d.*, 360 μm *o.d.*), were purchased from Weltech Co., Ltd., Wuhan, China. For online trapping, the sample loop was replaced by the trapping column. And 20 μL of sample was automatically injected into the trapping column at a flow rate of 10 μL/min for 9 min with carrier solution, as the loading tube volume was ~85 μL. The trapped BTA-derived

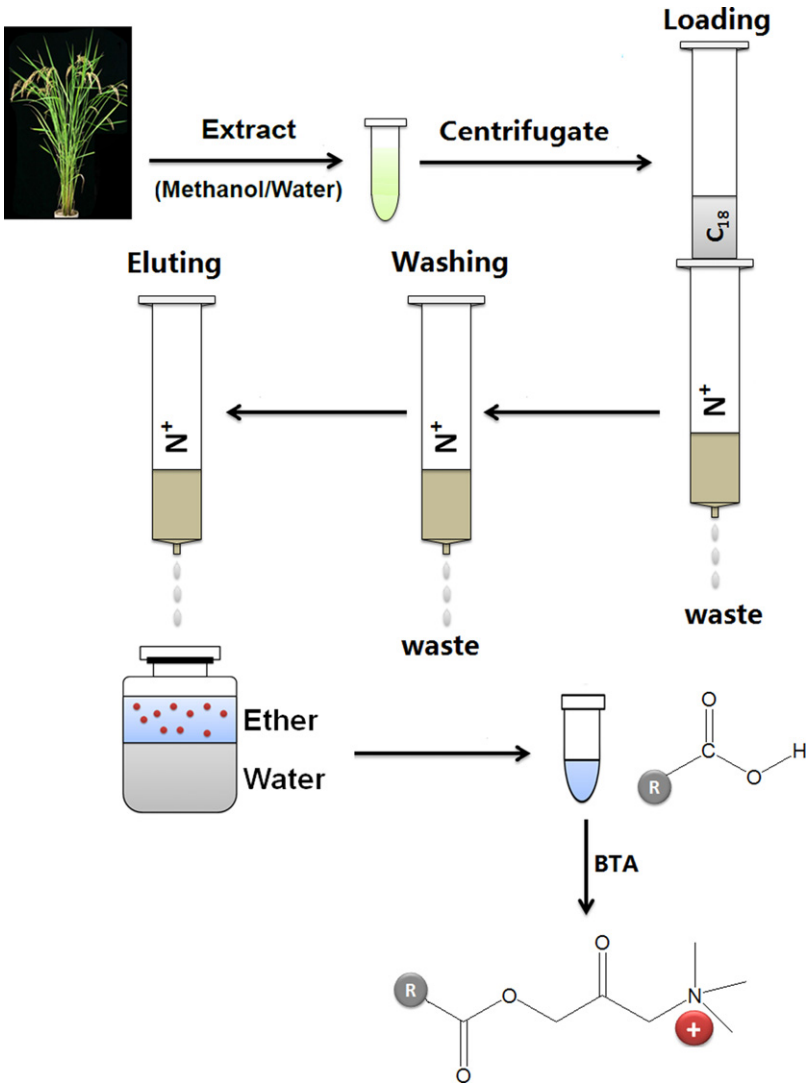

Fig. 1. The scheme for the sample preparation by tandem SPE followed by LLE.

phytohormones were then separated at a flow rate of 600 nL/min on the poly(MAA-*co*-EDMA) monolithic column.

MS analysis was performed using a MicroTOFq orthogonal-accelerated TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) controlled by Bruker Daltonics Control 3.2, and Bruker Daltonics Data analysis 3.4 software was employed for the data analysis. Transfer parameters were optimized by direct infusion of an ESI tuning mix (Agilent Technologies, Waldbronn, Germany). Spectra were collected with a time resolution of 1 s. Postrun internal mass calibration was performed using sodium formate cluster ions $Na^+(HCOONa)_{1-9}$ ranging from 90.9766 to 430.9137 m/z, which were detected in the last part of the chromatography after sample injection.

The BTA-derived acidic phytohormones were detected under positive ion mode condition. The optimized online nano-ESI parameters were as follows: capillary voltage −1.5 kV; dry gas 3.5 L/min; dry temperature 130 °C; funnel 1 RF 200.0 Vpp; funnel 2 RF 200.0 Vpp; ISCID energy 10.0 eV; hexapole RF 200.0 Vpp; pre pulse storage 5.0 μs. Ultra-pure nitrogen gas was used as drying gas and nebulizer gas. Spectra were acquired by summarizing 5000 single spectra. Full scan mode was used. Extraction of centroid spectra peaks with a width of 0.01 Da was used to pick up the extracted ion chromatograms (EICs) from the total ion chromatogram (TIC).

3. Results and discussion

3.1. Establishment of nano-LC with online trapping process

Considering that the BTA-derived phytohormones contained positive charge, the strong cation exchange mechanism (SCX) will lead to long retention of analytes and therefore high concentration of salt in mobile phase will be needed for the elution. On the contrary, anion exchange (AX) mechanism can cause electrostatic repulsion between analytes and stationary phase, which will result in low separation resolution of analytes. In this respect, weak cation exchange (WCX) retention with the assistance of hydrophobic or hydrophilic mode could be the appropriate option for the separation of BTA-derived acidic phytohormones. Since poly(MAA-*co*-EDMA) monolithic column could provide weak cation exchange sites (carboxyl groups of monolith) and hydrophobic interaction sites (backbone of monolith) [30], it was employed as the separation column in our experiment.

Firstly, the influence of ACN content from 20% to 98% (v/v) in mobile phase on the separation of BTA-derived acidic phytohormones was investigated using GA_1, GA_3, GA_4, GA_{24}, abscisic acid (ABA) as probes. As shown in Supplementary Fig. S1, the separation resolution of these BTA-derived acidic phytohormones decreased with a decrease of ACN content from 98 to 80% (v/v), which may

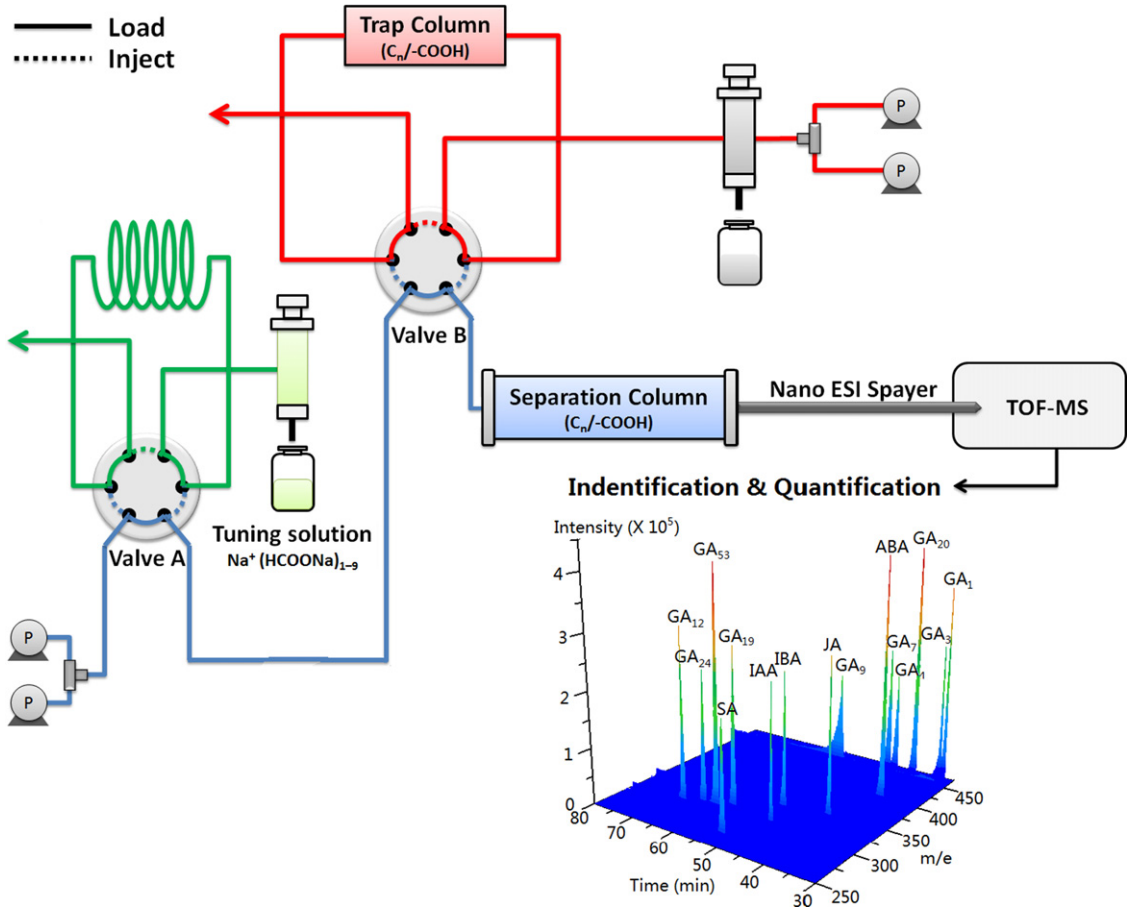

Fig. 2. The scheme for the analysis of phytohormone by nano-LC–ESI-Q-TOF-MS system.

be attributed to that the hydrophilic interaction between analytes and monolithic stationary phase was weakened. In contrast, the decrease of ACN content from 80 to 20% (v/v) caused the separation resolution increased. We reasoned that the hydrophobic interaction between BTA-derived acidic phytohormones and monolithic stationary phase was strengthened. Since 50% (v/v) ACN content provided good separation resolution, a stable spray and high MS response, 50% (v/v) ACN was chosen for further experiment.

Subsequently, the separation conditions of 15 BTA-derived acidic phytohormones were optimized. Due to the electrostatic interaction between quaternary ammonium groups of BTA-derived phytohormone and carboxylic acid groups of poly(MAA-*co*-EDMA) monolith, all peaks of these phytohormones were tailed. To improve the symmetry of peaks, $HCOONH_4$ was added in the mobile phase from 0 to 200 mM. The results showed that the retention time of GA_{24} with two quaternary ammonium groups was extremely long (>90 min) at 30 mM $HCOONH_4$, though GA_1, GA_3, GA_4, ABA, can be well separated. When the concentration of $HCOONH_4$ increased to 80 mM, the retention time of GA_{24} was 55 min with a symmetrical peak, but the separation resolution (R) of other phytohormones was less than 1.5. Hence, a gradient of $HCOONH_4$ concentration from 30 to 50 mM was applied (Supplementary Table S1). As shown in Fig. 3, all 15 acidic phytohormones were well separated, particularly GA_4 and GA_{20} which have the same m/z value. Thus, these phytohormones can be individually identified and quantified without any interfere from each other. Due to the relatively strong electrostatic interaction between SA and online trapping column as well as monolithic stationary phase, the elution capability of mobile phase towards SA was relatively weak; therefore, the peak of SA was tailed. In addition, we found that the increased salt concentration in mobile phase had little influence on the MS response of analytes. This phenomenon may be attributed to the permanently positive charge of the quaternary ammonium group, which ensured a constant ionization efficiency of the BTA-derived acidic phytohormones [31,32].

For the online trapping processing, we selected four different types of monoliths (Supplementary Table S2) and investigated their performance (Table 1). The hydrophilic poly(MAA-*co*-EDMA) monolith was the same as the separation column; poly

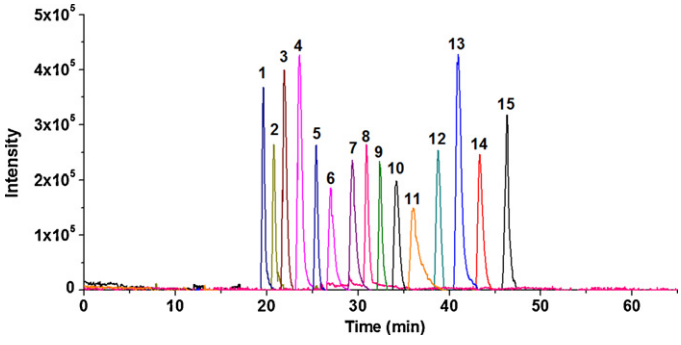

Fig. 3. The extracted-ion chromatography of BTA-derived acidic phytohormone standards obtained with nano-LC–ESI-Q-TOF-MS system. Experimental conditions: separation column, poly(MAA-*co*-EDMA) monolithic column (100 μm × 30 cm); flow rate, 600 nL/min; sample loading amount, 5 pg for each BTA-derived acidic phytohormone standard; mobile phase A, H_2O/ACN (50/50, v/v); mobile phase B, 80 mM $HCOONH_4$/ACN (50/50, v/v); separation gradient was provided in Supplementary table* S1. Order of peaks: 1, GA_1; 2, GA_3; 3, ABA; 4, GA_{20}; 5, JA; 6, GA_4; 7, GA_7; 8, IAA; 9, IBA; 10, GA_9; 11, SA; 12, GA_{19}; 13, GA_{53}; 14, GA_{24}; 15, GA_{12}.

Table 1
The performance of four monoliths as trapping column. The performance of the four monoliths for 15 BTA-derived acidic phytohormones in free plant matrix (50 mg rice) was indicated by color. The concentrations of the indicated phytohormones in rice were from literature [9]. Red color represents the analytes can be captured by the monolith (efficiency > 80%); blue color represents the analytes cannot be captured by the monolith (efficiency < 20%); white color represents the analytes can be captured by the monolith but with low efficiency (20–80%).[a]

Analytes	Charge state	BTA-derived phytohormones *m/z*	Monolith				Concentration of hormones in rice (ng/g)
			A	B	C	D	
GA_1	+1	462.249					0.89
GA_3	+1	460.233					0.92
GA_4	+1	446.254					1.66[b]
GA_7	+1	444.238					0.67
GA_9	+1	430.259					5.56
GA_{12}	+2	280.191					2.1
GA_{19}	+2	295.178					16.8
GA_{20}	+1	446.254					1.66[b]
GA_{24}	+2	287.181					5.56
GA_{53}	+2	288.188					3.93
ABA	+1	378.228					78.4
JA	+1	324.217					14.3
IAA	+1	289.155					42.7
IBA	+1	317.186					67.1
SA	+1	252.123					2130

Monolith A	Poly(AMPS-*co*-EDMA)
Monolith B	Poly(AMPS-*co*-DVB-*co*-EDMA)
Monolith C	Poly(MAA-*co*-EDMA)
Monolith D	Hypercrosslinked poly(MAA-*co*-VBC-*co*-DVB)

[a] Efficiency is calculated by ratio of $Area_{online\ trapping}$ to $Area_{direct\ injection}$ with the same loading amount.
[b] The value represents the concentration of GA_4 and GA_{20}.

(AMPS-*co*-EDMA) monolith with sulfonic groups can provide SAX interaction; poly(MAA-*co*-VBC-*co*-DVB) monolith and poly (AMPS-*co*-DVB-*co*-EDMA) monolith possessed a large surface area over 300 m^2/g. Our results showed that the poly(MAA-*co*-EDMA) monolithic column had the best performance on the capturing of BTA-derived acidic phytohormones. Remarkably, the salt in the mobile phase also contributed to the compression of the sample zone in the trapping column and therefore further improved the column efficiency.

We also optimized the trapping conditions, including ACN content in the carrier solution, the loading flow rate, and the length of trapping column. It showed that the BTA-derived acidic phytohormones can retain on trapping column with HILIC or RP/SCX mode. However, under HILIC mode, the salt in mobile phase may precipitate and then block the nano-valve and tubes with high ACN content. Therefore, we used low ACN content and further investigated the influence of ACN content in sample and carrier solution from 5% to 20% (v/v) (Supplementary Fig. S2). The results showed that with the increase of ACN content from 5% to 10% (v/v), the signal intensity of most of the analytes increased, while further increase of ACN content to 15% (v/v) would result in decrease of the signal intensity of GA_1, GA_3, GA_4, GA_7, GA_9, GA_{12}, GA_{53}, and IBA. Moreover, the 20% (v/v) ACN content resulted in a dramatic decrease in the MS signal intensity of all the analytes. Thus, the ACN content was set at 10% (v/v). The length of trapping column was also optimized from 3 to 10 cm and we found that 7 cm-long trapping column gave the best performance. Additionally, we investigated the effect of different loading flow rate on the trapping efficiency. The results showed that with the increase of flow rate from 1 to 20 μL/min, the MS signal response of BTA-derived acidic phytohormones was slightly affected (Supplementary Fig. S3), while the back pressure sharply increased from 1.7 to 18.3 MPa. Therefore, we chose a flow rate of 10 μL/min (back pressure ~9 MPa) for further experiments. Additionally, we investigated the capacity of trapping column by increasing the sample loading amount from 3 to 10 ng. The results showed that with the increase of sample loading amount, the signal intensity of analytes increased, indicating the large capacity of trapping column (Supplementary Fig. S4).

Taken together, the optimized conditions for online trapping process and separation were as follows: the trapping column, poly(MAA-*co*-EDMA) monolith (7-cm long, 100 μm *i.d.*, 360 μm *o.d.*); carrier solution, H_2O/ACN (90/10, v/v); loading flow rate, 10 μL/min; injection volume, 20 μL; the separation column, poly(MAA-*co*-EDMA) monolith (30-cm long, 100 μm *i.d.*, 360 μm *o.d.*); flow rate, 600 nL/min; mobile phase A, H_2O/ACN (50/50, v/v);

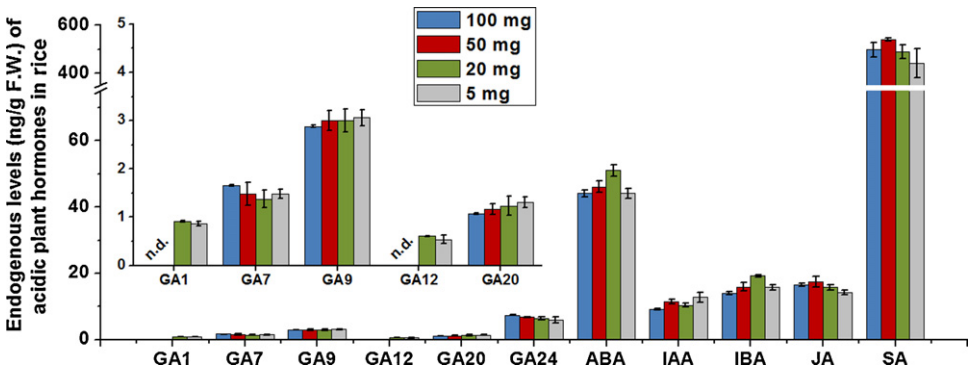

Fig. 4. The investigation of the influence of sample amount on the detection of endogenous phytohormones in rice leave sample.

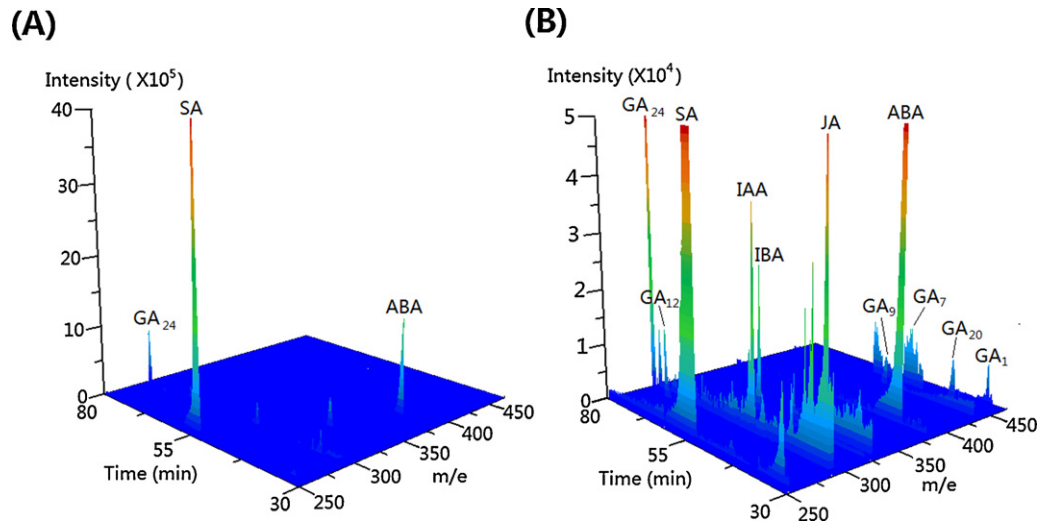

Fig. 5. The extracted-ion chromatography of endogenous acidic phytohormones from 5 mg plant sample obtained with nano-LC–ESI-Q-TOF-MS. (A and B) The 3D chromatography of detected acidic phytohormones with different *Z* axis (MS intensity) obtained from 5 mg of rice leaves.

mobile phase B, 80 mM $HCOONH_4$/ACN (50/50, v/v). The separation gradient was listed in Supplementary Table S1.

3.2. Purification of phytohormones

Previous studies showed that the pretreatment of plant tissues was indispensable due to the large amount of lipids and pigments interference [10,13]. The lipids and pigments can be removed using C_{18} cartridges and strong anion exchange (SAX) cartridges. However, it normally required re-dissolving of crude extract to meet the sampling condition for subsequent extraction step after removing lipids and pigments [33–35]. In current study, with tandem SPE, solvent evaporation can be avoided between C_{18} and SAX-SPE steps, which can greatly shorten the sample processing time.

Table 2
Linearity, LOD, and LOQ of 15 acidic phytohormones obtained by nano-LC–ESI-Q-TOF-MS platform.

Analytes	Regression line		R^2	Linear range (ng/mL)	LOD (pg/mL)	LOQ (pg/mL)
	Slope	Intercept				
GA_1	0.0285	0.5072	0.9995	0.004–1	1.05	3.50
GA_3	0.0459	0.3101	0.9985	0.02–10	6.52	21.8
GA_4	0.0219	0.6417	0.9965	0.01–10	2.49	8.30
GA_7	0.0559	0.4059	0.9912	0.015–10	4.44	14.8
GA_9	0.1621	0.1266	0.9965	0.05–10	14.6	48.8
GA_{12}	0.2126	0.1389	0.9947	0.005–1	1.56	5.20
GA_{19}	0.0652	0.1145	0.9920	0.03–10	15.3	51.0
GA_{20}	0.1229	5.0805	0.9968	0.05–10	12.7	42.3
GA_{24}	0.2823	0.7382	0.9944	0.05–10	10.2	34.0
GA_{53}	0.0119	0.0456	0.9923	0.05–10	17.6	58.8
ABA	0.0035	1.5736	0.9990	0.005–1	1.10	3.66
IAA	0.0012	0.0014	0.9957	0.02–10	4.38	14.6
JA	0.0123	0.9272	0.9941	0.04–10	10.9	36.4
IBA	0.0016	1.1545	0.9935	0.02–10	7.69	25.6
SA	0.0417	0.0360	0.9980	0.5–100	122.4	408

Table 3
Determination of endogenous acidic phytohormones from 5 mg rice sample.

Analytes	Found (ng/g)	Spiked concentration (ng/g)	Recovery (%)	RSD (%, $N=4$)	Found[a] (ng/g)
GA_1	0.87 ± 0.05	1.00	99.3	6.82	0.99 ± 0.10
GA_3	n.d.	0.50	98.8	3.46	n.d.
GA_4	n.d.	0.50	102.7	2.51	1.32 ± 0.22[b]
GA_7	1.48 ± 0.23	1.50	97.9	1.15	n.d.
GA_9	3.00 ± 0.21	3.00	96.8	7.98	3.25 ± 0.99
GA_{12}	1.54 ± 0.09	0.50	103.5	2.76	1.46 ± 0.23
GA_{19}	n.d.	0.50	88.8	3.91	n.d.
GA_{20}	1.16 ± 0.11	1.00	92.1	4.19	0.94 ± 0.09[b]
GA_{24}	6.75 ± 0.18	6.50	88.3	5.10	5.62 ± 0.34
GA_{53}	n.q.	0.50	92.5	6.73	n.d.
ABA	46.08 ± 1.71	50.0	96.9	4.38	50.62 ± 4.51
IAA	11.51 ± 0.71	10.0	104.8	5.11	12.06 ± 2.95
IBA	15.94 ± 1.23	15.0	102.0	6.71	14.81 ± 2.21
JA	17.38 ± 1.58	20.0	97.6	8.92	17.10 ± 1.19
SA	540.51 ± 5.57	500	89.4	9.90	604.99 ± 40.05

n.d., not detected; n.q., not quantified.
[a] The results were obtained using previous CE-MS method with 3 g rice sample.
[b] The result was the total concentration of GA_4 and GA_{20}.

Table 4
Precisions (intra- and inter-day) for the determination of 15 acidic phytohormones in rice samples.

Analytes	Intra-day precision (RSD %, $N=5$)			Inter-day precision (RSD %, $N=4$)		
	Low (0.1 ng/g)	Medium (0.5 ng/g)	High (5 ng/g)	Low (0.1 ng/g)	Medium (0.5 ng/g)	High (5 ng/g)
GA_1	4.45	5.40	5.70	7.02	8.50	8.98
GA_3	4.71	5.91	9.20	4.35	5.45	8.49
GA_4	5.81	7.29	8.02	1.73	1.06	2.87
GA_7	3.36	3.26	10.90	8.79	7.26	11.90
GA_9	3.83	5.56	5.87	7.27	10.56	10.45
GA_{12}	4.09	4.95	5.23	8.16	9.89	9.79
GA_{19}	9.67	9.72	10.38	1.66	2.01	4.14
GA_{20}	5.50	6.66	7.04	2.92	3.54	7.27
GA_{24}	6.09	7.38	7.80	5.13	6.21	6.56
GA_{53}	5.87	7.11	7.51	2.96	3.59	6.81
ABA	3.20	3.88	4.10	5.76	7.82	8.49
IAA	5.63	6.82	7.20	5.56	6.73	7.11
JA	2.66	3.22	3.40	5.76	6.25	7.06
IBA	4.30	3.54	4.30	6.33	8.45	9.38
SA	3.81	9.95	10.51	3.81	9.95	10.51

Moreover, taking advantage of the analyte solubility differences in different solvents, LLE is an effective way to purify the plant phytohormones [36,37]. Furthermore, LLE can improve the derivatization efficiency by decreasing the influence of impurities [7]. Therefore, tandem SPE followed by LLE was employed for the purification of phytohormones.

The parameters of SAX-SPE process were optimized, including the washing solution, FA content, and elution volume. Firstly, the methanol content of the sample solution can influence the ionization degree of carboxyl groups, thus the ion-exchange interaction between negatively charged analytes and positively charged SPE materials. It showed that all the 15 acidic phytohormones in the sample solution containing 20% (v/v) methanol can be effectively captured on this SCX-SPE cartridge. Secondly, the eluent containing 1% (v/v) FA was sufficient for the elution and further increase of FA content in the eluent decreased the derivatization efficiency (Supplementary Fig. S5). Using a buffered solution in this SPE step may enhance the elution efficiency of acidic phytohormones. However, existence of salt can influence the subsequent derivatization reaction and the efficiency of online trapping. Thus, a buffered solution was not used in SAX-SPE eluent. Thirdly, the effect of elution volume was assessed from 1 to 5 mL and the results revealed that the MS signal response of phytohormones significantly increased with the increase of elution volume from 1 to 3 mL and no obvious increase was observed when the elution volume increased from 3 to 5 mL (Supplementary Fig. S6). Fourthly, the washing solution was also optimized by changing the methanol content. The results demonstrated that 2 mL washing solution containing 20% (v/v) methanol can elute impurities from SAX-SPE cartridges but not analytes, and an increase of methanol content from 30 to 80% (v/v) resulted in the elution of analytes from SAX-SPE cartridges.

After tandem SPE purification, the LLE was adopted to eliminate the polar impurities and improve the derivatization efficiency [7]. The LLE was optimized by changing the volume of ether. The results showed that with the increase of ether volume from 500 μL to 1 mL, the signal intensity of targeted analytes increased, while the intensity of targeted analytes increased very slightly when the ether volume was over 1 mL (Supplementary Fig. S7). Thus, the ether volume was fixed at 1 mL for further experiments.

3.3. System performance

Limits of detection (LODs) and limits of quantification (LOQs) for the acidic phytohormones were calculated as the concentration of the analytes at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The results showed the LODs and LOQs for 15 acidic phytohormones were in the range of 1.05–122 pg/mL and 3.5–408 pg/mL, respectively (Table 2). Moreover, the calibration curves were constructed by comparing peak area counts to concentrations. The result showed good linearities were obtained for the determinations of all 15 acidic phytohormones with linear coefficient R^2 values greater than 0.991.

We next assessed whether the analytical system was capable for the detection of low abundance target compounds in

complex matrix by analyzing endogenous acidic phytohormones in rice leaves with different fresh weights from 2 to 100 mg (Fig. 4). With 2 mg rice leaves, the RSD of 5-times analysis was over 50%, which was not suitable for accurately quantitative analysis. As shown in Fig. 4, with the decreased amount of rice leave sample from 100 to 5 mg, the number of targeted analytes quantified was not apparently affected. Remarkably, GA_1 and GA_{12} were detected from 5 mg and 20 mg rice leaves but not from 50 mg and 100 mg rice leaves, which may be attributed to the interference of abundant analogous of acidic phytohormones and ion suppression in the more concentrated plant matrix. In this respect, the decrease of sample amount can reduce the analogous interference on the determination of GAs.

3.4. Profiling of endogenous acidic phytohormones

Fig. 5 shows the extracted ion chromatogram of 11 endogenous acidic phytohormones including 6 GAs (concentration range: 0.54–6.75 ng/g, Table 3) in 5 mg rice leaves (The 2D chromatogram was provided in Supplementary Fig. S8). In addition, the effect of rice leave matrices on the quantification of these acidic phytohormones was evaluated by spiking phytohormone standards into the rice matrices. The results showed that 15 acidic phytohormones were successfully determined in 5 mg rice leaves with 88.3–104.3% recovery (RSDs, 1.15–9.90%, $N = 4$) (Table 3). Furthermore, the reproducibility of proposed method was evaluated by investigating the intra and inter-day precisions. Our results showed that the intra- and inter-day RSDs were less than 10.9% and 11.9%, respectively (Table 4).

We also compared the results obtained by our previous CE-MS method [7] and the current nano-LC–ESI-Q-TOF-MS method (Table 3). The number and concentrations (ng/g) of GAs detected by the current method were similar to that obtained by previous CE-MS method. Notably, GA_4 and GA_{20} can be respectively quantified by the present method, indicating the improved separation capability using monolithic capillary. GA_7 was detected and quantified by current method but not previous method, which suggested that this method gave higher detection sensitivity even with significantly decreased sample amount (5 mg for current method and 3 g for previous CE-MS method).

4. Conclusion

In this study, we developed a highly sensitive method for the quantitative determination of BTA-derived acidic phytohormones by nano-LC–ESI-Q-TOF-MS platform. The extraction and purification of acidic phytohormones from plant matrix were performed by tandem solid-phase extraction (SPE) followed by liquid–liquid extraction (LLE). Poly(MAA-*co*-EDMA) monolithic column was adopted to achieve good separation of the BTA-derived acidic phytohormones with a quaternary ammonium group. Moreover, online trapping process significantly improved the detection limits. By employing the method developed here, we successfully quantify 11 endogenous acidic phytohormones from only 5 mg rice leaves. These results demonstrated the excellent performance of our method on the sensitive profiling of endogenous acidic phytohormones in complex matrix.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.08.005.

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