Supporting Information

for

A dual role of boronate affinity in high-sensitivity detection of vicinal diol brassinosteroids from sub-gram plant tissues *via* UPLC-MS/MS

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Tandem MAX-MCX SPE of plant samples

The collected plant tissues were first grounded to a fine powder with a MM-400 mixer milling (Verder RETSCH Trading Co., Ltd, Shanghai, China). 0.5g of the plant material powder was extracted with 2.5 mL of 80% aqueous methanol twice. MAX and MCX Cartridges (6mL, 500mg) were purchased from WATERS Corporation (Milford, MA, USA). The MAX cartridge was activated and equilibrated with 10 mL methanol, water, 1M KOH, 10%MeOH and 80%MeOH in turn, while MCX with 10 mL methanol, water, 5%FA and 80%MeOH. After the two columns were connected with a connector, 2.5 mL 80% methanol extracts of 500mg plant materials was subjected to the tandem cartridges. For detection, the eluents was dried by N_2 chilling and then reconstructed in 200µL ACN to react with 30µg MPyBA.

Optimization of derivatization reagent

Three organic boronic acid candidates including 6-methoxy-3-pyridinylboronic acid (MPyBA), 6-bromo-3-pyridinylboronic acid (BPyBA) and 3-pyridinylboronic acid (PyBA) (Fig.S1) were used to test the derivatization efficiency. In order to obtain the best derivitization condition, several conditions were optimized with 24-epiBL and the results were depicted in Fig.S2. As is seen from this picture, when reacting with MPyBA dissolved in ACN at 75°C, the MS response of the resulting product was the highest. However, taking the stability of some molecules at high temperature into account, the derivatization of plant materials was finally carried out with MPyBA in ACN at 40°G for 1 h.



Fig.S1 Chemical structure of derivatization reagents



Fig.S2 Derivatization efficiency under different reaction conditions

Determination of recovery and matrix effect of MAX-MCX SPE procedure

To determine the recovery of BRs in MAX-MCX SPE procedure, 24-epiBL and CS standards were used as a probe. 500pg of 24-epiBL and CS were dissolved in 3mL 80% methanol to pass through the tandem MAX-MCX columns and the peak areas of them in the eluents detected via UPLC-MS/MS were marked as S_P. At the same time, 3mL 80% methanol was also loaded onto the tandem columns and the same amount of probe molecules were added to the eluents. This mass response was marked as S. The recovery was calculated to be S_P/S ratio. The results were shown in Table S1.

 D_3 -BL used to measure the matrix effect of the MAX-MCX flow-through matrix. 60pg of D_3 -BL was added to 2.5mL flow-through matrix and then detected via UPLC-MS/MS with a peak area S_M . 60pg D_3 -BL was also dissolved in 80% methanol and detected with peak area S. The matrix effect equals S_M /S ratio. The results were shown in Table S2.

Compound	S _P /S	Average	SD
	89.8%		
24-epiBL	97.5%	95.1%	4.6%
	98.0%		
	93.6%		
CS	99.6%	96.6%	3.0%
	96.6%		

Table S1. Recovery of 24-epiBL and CS in the MAX-MCX SPE procedure

Table S2. Matrix effect of the MAX-MCX flow-through matrix

Sample NO.	S _M ∕S	Average	SD
1	26.7%		
2	17.2%	25.2%	6.0%
3	31.7%		

Preparation and characterization of BA-MNPs

BA-MNPs were prepared following Scheme S1. Superparamagnetic Fe_3O_4 nanoparticles was synthesized via H_2O_2 oxidation of $Fe(OH)_2$ suspensions according to a previous published paper.¹ After the nanoparticles were modified with oleic acid (OA, Sinopharm chemical reactant co., LTD, Beijing, China) in water and 3-(trimethoxysilyl)propyl methacrylate (TMSPMA, ACROS ORGANICS, New Jersey, USA) in toluene in sequence. The modified MNPs were mixed with 4-vinyl phenylboronic acid (4-VPBA, Yingfa co., LTD, Ningbo, China), ethylene glycol dimethacrylate (EDMA, ACROS ORGANICS) , 2, 2'-Azobisisobutyronitrile (AIBN, Fine Chemical Research Institute of Tianjin, China), Polyvinylpyrrolidone (PVP, K-30, MW=40000, Sinopharm chemical reactant co., LTD) in ethanol and the surface polymerization was performed at 70 °C for 7h. Then the BA-MNPs were washed with ethanol three times and dried for use. The details of the preparation process and the optimization of the conditions will be published elsewhere. A brief characterization of BA-MNPs used was shown in Fig.S3.



Scheme S1. Process for preparation of BA-MNPs



Fig. S3 Transmission electron microscopy image (left) and magnetization curve (right) of synthesized BA-MNPs

BA-MNPs treatment of plant sample

Eluents from the MAX-MCX SPE step was first adjusted to basic at pH=9.5-10.0 with 35μ L NH₃OH and then incubated with different amounts of BA-MNPs for 60min under shaking at 500rpm with a circular oscillator. The supernatant was separated with BA-MNPs with a magnet and dried by N₂ chilling for derivatization and final detection.

UPLC-MS/MS method

BRs-MPyBA detection was performed on UPLC (Water, Milford, MA, USA) combined online with tripe quadrupole ion trapping tandem mass spectrometer (5500Q, AB Sciex, Foster City, CA). equipped with an ESI source. The inlet method was as follows: mobile phase A: 0.05% acetic acid in water, B: 0.05% acetic acid in ACN. Gradient: 0-9min, 60%B to 90%B; 9-10min, 90%B; 10-11.5min,90%B to 60%B; 11.5-14min, 60%B. BRs- MPyBA was detected in positive MRM mode. The source parameters were set as: IS voltage-5400V, TEM-550°C, GS1-45, GS2-45, curtain gas-27. The optimized compound-dependent parameters were listed in Table S3.

		Collision	Declustering	Entrance	Collision Cell
Compounds	Q1/Q3	Energy	Potential	Potential	Exit Potential
		/eV	/v	/v	/v
BL-MPyBA	598.35/178.1	57	20	10	12
24-epiBL-MPyBA	598.30/178.1	60	20	10	12
CS-MPyBA	582.40/178.1	60	20	10	12
TE-MPyBA	566.30/178.1	60	20	10	12
TY-MPyBA	566.35/154.1	68	20	10	12
Glucose-MPyBA	298.20/178.1	37	150	10	12

Table S3. Optimized MRM parameters of investigated compounds

Relationship between MS responses of 24-epiBL-MPyBA and Glucose-MPyBA

The transition 298.20/178.1 was used to monitor the response of Glucose-MPyBA in the eluents after the tandem MAX-MCX SPE step. Then the peak area of 24-epiBL-MPyBA and the response of the ion channel at the retention time window of 24-epiBL-MPyBA were plotted against

the amounts of BA-MNPs used. The results were shown in Fig.S4.



Fig. S4 UPLC-MS/MS chromatograms of BRs and glucose in rice panicles Inset graph: plot of MS response of 24-epiBL-MPyBA and glucose-MPyBA against the amount of BA-MNPs applied

UPLC-QTOF method for investigation of matrix effect

The origin of ionization and the elimination mechanism of BA-MNPs were studied on an Acquity UPLC (Waters, Milford, MA, USA) combined with QTOF-MS (Synapt G2 HDMS, Waters, Manchester, UK) system also equipped with an ESI source. UPLC method was set same as described above. All experiments were carried out in positive resolution mode to give a resolution at 20000 with a scan range from 100 to 600. To ensure the accurate mass analysis, leucine - enkephalin was used as lock mass in MS/MS mode (m/z 278.1141 and 556.2771 for positive ion mode) at a concentration of 200pg/mL at 10mL/min. The source parameters were set as: Capillary voltage-3000V, sampling cone-40V, extraction cone-4V, source temperature-100°C, desolvation temperature -350°C, cone gas -50L/h, desolvation gas -800L/h. To obtain the MS/MS scan spectrum of the precursor ions m/z 280.2636, 436.2654 and m/z 438.2810, argon was used as collision gas and collision energy was varied to monitor the product ions of interest. The results were shown in Fig.S5.



Fig.S5A MS/MS spectrum of m/z 436.2654



The three ions m/z 245.2268, 263.2371, 280.2636 were one compound

To further confirm these 3 ions were one compound, enhanced product ion (EPI) mass spectra of m/z 263.2, 245.2 and MS/MS/MS spectra of m/z 280.3>263.2>, 280.3>245.2> were performed on UPLC combined online with tripe quadrupole ion trapping tandem mass spectrometer (5500Q, AB Sciex, Foster City, CA). UPLC conditions and source parameters were set as described above. For EPI scan of m/z 263.2 and 245.2, CE was set as 28V and 25V respectively. For MS/MS/MS scan of m/z 280.3>263.2> and m/z 280.3>245.2>, excitation energy (AF2) was set as 0.05V and 0.06V respectively. The results were shown in Fig.S6.



Fig.S6A MS³ spectrum of m/z 280.3>245.2>





Fig.S6C MS³ spectrum of m/z 280.3>263.2>



Elution of interferences from BA-MNPs

To wash the interferences out from used BA-MNPs, 2.5mL of 5%FA in 80%methanol was utilized. The mixture was also shaken at a speed of 500rpm by a circular oscillator. At last, the supernatant was also collected through magnetic separation for detection on UPLC-QTOF-MS. The extracted ion chromatograms (EIC) of specific ions were shown in Fig.S7.



Validation of the proposed method

In order to validate the overall proposed method, including MAX-MCX SPE, BA-MNPs treatment and UPLC-MS/MS analysis, a series of different amounts of D₃-BL and D₃-CS were added to 250 mg of grounded plant tissues. The mean areas of detected D₃-BRs (n=4) were plotted against D₃-BRs concentrations to generate a calibration curve. Good linearities were obtained with coefficients 0.9997, 0.9996 for D₃-BL and D₃-CS respectively. Limits of quantification (LOQs) of the proposed method were determined as D₃-BRs concentration corresponding to S/N of 10. From this point, the LOQs of BL and CS are 1.12 pg and 1.45 pg respectively. The precision of the method was investigated with the determined endogenous 24-epiBL and CS levels from 8 replicates and the RSD (n = 8) was 10.7% and 2.58%. The recovery of the whole method was determined at two levels of 50 pg and 200 pg/250 mg fresh weight (FW) similarly to the MAX-MCX SPE procedure described before. The difference is using real plant extracts instead of 80% methanol. All the results were summarized in Table S4.

Table S4. Validation of the whole method

Compound	Equation of linear regression	r²	Linear range (pg)	LOQ (pg)	Added (pg/250mg FW)	Recovery (%)
24-epiBL	Y=0.0021X-1.20	0.9997	1.12-500	1.12	50	77.2
					200	82.6
CS	Y=0.0020X-3.89	0.9996	1.45-500	1.45	50	77.2
					200	73.3

References

1. M. Tada, S. Hatanaka, H. Sanbonsugi, N. Matsushita and M. Abe, J Appl Phys, 2003, 93, 7566-7568.