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# Sensitive determination of brassinosteroids by solid phase boronate affinity labeling coupled with liquid chromatography-tandem mass spectrometry

Xiao-Tong Luo[a], Bao-Dong Cai[b], Lei Yu[a], Jun Ding[a], Yu-Qi Feng[a,*]

[a] Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Department of Chemistry, Wuhan University, Wuhan 430072, China
[b] Wuhan Greensword Creation Technology Co., Ltd, Wuhan 430075, China

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ABSTRACT

Brassinosteroids (BRs) are regarded as the sixth plant hormone that is widely distributed in the plant kingdom. Sensitive quantification of BRs will be greatly benefit to illuminate the detail mechanisms about how BRs play crucial role in plant developmental processes such as cell division, cell expansion, cytodifferentiation, seed germination, vegetative growth and resisting biological or abiotic stress. In the current study, we developed a method for rapid and sensitive determination of endogenous BRs in plant tissues by combining LC–MS and a novel sample preparation strategy, in which the plant tissue extract was supplied to solid phase boronate affinity labeling and extraction, followed by desorption and salt-induced phase transition extraction for further purification. Under the optimized conditions, good linearity was obtained for 6 BR with correlation coefficients ($r$) ranging from 0.9988 to 0.9999. The limits of detection (LODs, S/N = 3) ranged from 1.4 to 2.8 pg $mL^{-1}$. The recoveries were between 93.4% and 116.2% with the relative standard deviations (RSDs) ranging from 2.8% to 15.8%. Finally, the developed method was successfully applied to the analysis of 6 endogenous BR in various plant tissues including 20 mg FW *Oryza sativa* shoot, 10 mg FW *Oryza sativa* root, 20 mg FW *Arabidopsis thaliana* shoot, 4 *Arabidopsis thaliana* flowers (2.8 mg) and one *Brassica napus* stamen (3.0 mg) with concentration ranging from 0.26 to 157.28 ng $g^{-1}$ FW.


## 1. Introduction

Brassinosteroids (BRs), a category of natural steroidal lactones/ketones that are widely distributed in the plant kingdom, are confirmed as the sixth plant hormone [1]. The discovery of this hormone is greatly attributed to the study by Grove *etc.* in 1979 when the bioactive compound, brassinolide (BL) was isolated [2]. From then on, increasing steroidal compounds have been discovered in various plant species including 53 angiosperms, 24 algae, one bryophyte and one pteridophyte [3]. In spite of the trace amount (down to 0.01–0.1 ng $g^{-1}$ FW level) detected, BRs play a crucial role in many developmental processes in plants such as cell division, cell expansion, cytodifferentiation, seed germination, vegetative growth and help plants resist biological or abiotic stress [4,5]. Although great advances have been achieved in our knowledge of BRs [6–10], some detail mechanisms are still vague. Many studies suggested that these mechanisms may be closely related to BR concentration and action location [11]. Therefore, the distribution information of BRs will help biologists explore the mechanisms. Thus, a simple, sensitive and accurate analytical method is needed. Also, the method should be high-throughput and applicable to various plant matrix because of large amount and kinds of the biological sample.

Nowadays, LC–MS has been widely used for BRs analysis because of its selectivity and sensitivity [12–34]. However, some difficulties still present and restrict the development of analytical method of BRs to be simpler, more selective and more sensitive simultaneously. First, the complex sample matrix would disturb BR enrichment. Therefore, many pretreatment methods were proposed. In early stage, the purification of BR was performed with a multi-step procedure in which large amount of plant sample was required [35–37]. During the past few years, more kinds of proficient methods were used, such as dispersive liquid-liquid micro-extraction (DLLME) [38], matrix solid-phase dispersion (MSPD) [28], magnetic solid-phase extraction (MSPE) [22,26,33,39], solid-phase micro-extraction (SPME) [34], solid-

* Corresponding author.
*E-mail addresses:* yqfeng@whu.edu.cn, 864544745@qq.com (Y.-Q. Feng).

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phase extraction (SPE) [21,23,29] and pipette-tip solid-phase extraction [24,31]. However, most of these methods were based on hydrophilic or hydrophobic interaction between material and BRs, which were not selective enough. For improving the selectivity of sample pretreatment procedure, methods based on boronate affinity interaction were developed in which *cis*-hydroxyl containing BRs could be selectively captured [26,27,33,34,40]. Although good selectivity had been approved, material synthesis was complex and the enrichment procedure was tedious. Also, the strong interaction made it difficult to elute BRs from the sorbent that made the sample pretreatment procedure difficult to be controlled [26,40].

Second, the sensitivity of BRs on MS signal intensity was unsatisfactory because of lacking effective ionizable groups and bad fragmentation behavior in collision-induced dissociation (CID) [16,23,26,30,32]. Therefore, chemical derivatization based on the specific boronate affinity reaction between derivatization reagent and *cis*-hydroxyl group of BR molecule was introduced to improve the sensitivity. Many derivatization reagents were reported [14,22,24,27,29,31,34]. With the help of 3(4)-(N,N-dimethylamino)-phenylboronic acid (DMAPBA), the signal response of BRs on MS was enhanced by 25-fold [21,22]; as for 4-phenylaminomethyl-benzeneboric acid (4-PAMBA), 2000-fold was enhanced [31]. Later, Deng et al. proposed a pipette-tip solid-phase extraction and BRs were then derived with 4-borono-N,N,N-trimethylbenzenaminium iodide (BTBA), the sensitivities of BRs were enhanced by 1190-448785 fold [24]. The amount of plant sample could decrease to sub-milligram. Usually, the derivatization reaction is carried out after sample pretreatment procedure and this additional step is time-consuming and tedious. Therefore, *in-situ* derivatization was introduced. Based on hydrophilic interaction, Ding et al. used a $TiO_2$-coated magnetic sorbent to enrich BRs [22]. Then, the BR-adsorbed $TiO_2$ was served as "micro-reactor" for BR derivatization, which enabled extraction and derivatization simultaneously. Later, Yu et al. revealed a selective method which combined modified tip extraction and *in-situ* derivatization [31]. This method was highly sensitive and selective with only 50 mg FW of plant tissue required. Compared with the conventional derivatization, *in-situ* derivatization assuredly improves the time efficiency of sample pretreatment procedure but introduces another problem: excessive derivatization reagent. 500000-fold excessive derivatization reagent became the new man-made introduced interference which should not be neglected [22].

Herein, we developed a method for rapid and sensitive determination of endogenous BRs in plant tissues by combining LC–MS and a novel sample preparation strategy, in which plant tissues extract was supplied to solid phase boronate affinity labeling (SPBAL) & extraction, followed by desorption & salt-induced phase transition extraction (SPTE) for further purification. MCX sorbent (containing functional groups of —C8 and —$SO_3H$) was first modified with a quaternary ammonium boronate affinity labeling reagent for selectively labeling and capturing of BRs through boronate affinity interaction. Afterwards, desorption & SPTE was carried out. In brief, 90% acetone aqueous solution was served as elution solvent. As soon as 20–50 mg $CH_3COONH_4$ added, auto phase separation occurred because of salting-out effect. After being vigorously stirred and centrifuged, labelled BRs were desorbed from MCX and enriched in organic phase. Meanwhile, excessive labeling reagent was stayed in aqueous phase. This process ensured further purification together with desorption step. Also, it was time-saving for collecting organic phase to dry out and re-dissolve. Benefited from these advantages, the sensitivity of BRs was improved with 923–15000 fold. Finally, 6 endogenous BR were detected in various plant tissues including 20 mg FW *Oryza sativa* shoot, 10 mg FW *Oryza sativa* root, 20 mg FW *Arabidopsis thaliana* shoot, 4 *Arabidopsis thaliana* flowers (2.8 mg) and 1 *Brassica napus* stamen (3.0 mg).

## 2. Experimental

### 2.1. Chemicals and reagents

Phytohormone standards: brassinolide (BL), castasterone (CS), 28-homobrassinolide (28-homoBL), 28-homocastasterone (28-homoCS), typhasterol (TY), 6-deoxocastasterone (6-deoxoCS) and stable isotope-labelled standards: [$^2H_3$]BL, [$^2H_3$]CS, [$^2H_3$]TY were all purchased from Olchemim Ltd. (Olomouc, Czech Republic).

HiCapt MCX SPE sorbent (containing functional groups of −C8 and −$SO_3H$) was obtained from WELTECH (Wuhan, China). Formic acid (FA, 88%), ammonium acetate ($CH_3COONH_4$), diethyl ether anhydrous ($C_4H_{10}O$) were all purchased from Sinopharm Chemical Reagent (Shanghai, China). 4-(bromomethyl)phenylboronic acid and isoquinoline were purchased from J&K Chemicals (Beijing, China). Acetonitrile (ACN, HPLC grade) and acetone (HPLC grade) were obtained from Merck KGaA (Darmstadt, Germany). Ultra-pure water used throughout the study was purified by Milli-Q system (Milford, MA, USA).

### 2.2. Synthesis of 2-(4-Boronobenzyl) isoquinolin-2-Ium

2-(4-boronobenzyl) isoquinolin-2-ium (BBII) was synthetized according to the method reported previously [41]. Briefly, the mixture of 4-(bromomethyl)phenylboronic acid (0.1 g, 0.5 mmol) and isoquinoline (0.25 g, 1.9 mmol) (10 mL) was stirred in diethyl ether anhydrous at room temperature for 8 h. Afterwards, the precipitate was washed by 10 mL of diethyl ether anhydrous thrice and dried in vacuum oven for 12 h at 60 °C (yield, 45.1%, HPLC purity 96.5%, and $^1$H NMR purity >97%). $^1$H NMR ($D_2O$, 400 MHz): 9.70 (s, 1H), 8.43 (dd, $J$ = 6.8, 1.2 Hz, 1H), 8.28–8.31 (m, 2H), 8.10–8.15 (m, 2H), 7.92–7.96 (m, 1H), 7.73 (d, $J$ = 8.0 Hz, 2H), 7.43 (d, $J$ = 8.0 Hz, 2H), 5.86 (s, 2H) ppm. $^{13}$C NMR ($D_2O$, 100 MHz): 64.0, 126.5, 127.1, 127.6, 128.2, 130.1, 131.4, 133.8, 134.5, 135.3, 137.2, 137.5, 149.1 ppm. [M-Br]$^+$: 264.1190 Da Calc. 264.1190 Da found.

### 2.3. Preparation of BBII modified MCX material

The preparation of BBII modified MCX material (MCX@BBII) was pretty simple. Briefly, 1.0 g commercial MCX SPE sorbent was put into a 15-mL tube containing 7 mL of BBII solution (6 mg mL$^{-1}$ in ACN/$H_2O$, 6/4, v/v). 60% ACN aqueous (v/v) was used to avoid instability adsorption resulting from hydrophilic interaction and guaranteed the fully dissolution of BBII. After being vigorously stirred for 3 min, the mixture was centrifuged at 10,000 × $g$ for 3 min. The supernatant was discarded, and the material was washed by 7 mL of ACN/$H_2O$ (6/4, v/v). Finally, the material was dried in a vacuum at 60 °C for use.

To optimize preparation time of MCX@BBII, 1.0 g commercial MCX SPE sorbent was put into a 15-mL tube containing 7 mL of BBII solution (6 mg mL$^{-1}$ in ACN/$H_2O$, 6/4, v/v), and the adsorption time from 1 min to 60 min was evaluated.

### 2.4. Plant materials

*Brassica napus* L. flowers in full-bloom stage were kindly provided by Oil Crops Research Institute, Chinese Academy of Agricultural Sciences (Wuhan, China). *Oryza sativa* shoots and roots in seedling stage were grown in greenhouse at 30 °C under 16 h light/8 h dark photoperiods according to a previously described method with some modifications [31]. First, the seedlings were soaked in water overnight and germinated on moistened gaze for 4 days. Then, the germinated seeds were transferred to a 2L-container with nutrient solution and grew for 10 days. The Hoagland's nutrient solution contained $NH_4NO_3$ (1.425 mM), $NaH_2PO_4$ (0.323 mM), $K_2SO_4$ (0.513 mM), $CaCl_2$ (0.998 mM), $MgSO_4$ (1.643 mM), $MnCl_2$ (0.019 mM), $(NH_4)_6Mo_7O_{24}$ (0.075 mM),

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