Journal of Chromatography A, 1297 (2013) 56–63

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quantification of endogenous brassinosteroids in plant by on-line two-dimensional microscale solid phase extraction-on column derivatization coupled with high performance liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 29 November 2012
Received in revised form 27 March 2013
Accepted 16 April 2013
Available online 22 April 2013

Keywords:
On-line two-dimensional microscale solid phase extraction (2DμSPE)
On-column derivatization (OCD)
Endogenous brassinosteroids (BRs)
Tomato leaf sample

ABSTRACT

An on-line two-dimensional microscale solid phase extraction (2DμSPE)-on column derivatization (OCD)-high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) method was developed for quantification of brassinosteroids (BRs) in plant tissues. Five BRs with widest distribution in plant species and high bioactivity (24-epibrassinolide, 24-epicastasterone, 6-deoxo-24-epicastasterone, teasterone and typhastero) were selected as target analytes. 2DμSPE column packed sequentially with phenyl boronic acid silica sorbent (the first dimension) and C18 silica sorbent (the second dimension) was used to selectively extract and enrich BRs by 110–146 times. OCD was carried out on the second dimension of 2DμSPE column with *m*-aminophenylboronic acid (*m*-APBA) as a derivatization reagent, enhancing the sensitivity of MS/MS to BRs by 13–8437 times. It was also found that pre-trap of derivatization reagent on the C18 section of 2DμSPE column could increase reaction efficiency by 3–10 times. The whole process time of the on-line system was less than 30 min. The detection limits of the method for five BRs were between 1.4 and 6.6 pg with RSDs less than 10%. Endogeneous BRs in tomato leaves were analyzed by using this method. Owing to the high selectivity of this on-line 2DμSPE system, BRs in plant extracts could be quantified using matrix-free standard calibration method with relative recoveries in the range of 80–124%.

1. Introduction

Brassinosteroids (BRs) are a class of plant polyhydroxysteroids that have been recognized as a new kind of phytohormones with 69 naturally occurring analogues identified. They have unique and obvious biological effects on plants growth and regulations, such as stem elongation, synthesis of nucleic acid and proteins, carbohydrate assimilation and allocation [1].

Detection of endogenous BRs in plant has always been a challenge due to their extremely low concentration (1–100 $\mu g\,kg^{-1}$ fresh weight in pollen and immature seeds, 0.01–0.1 $\mu g\,kg^{-1}$ fresh weight in shoots and leaves [2]) in complex matrix, and few unique structural features for detection. The most widely used detection methods in conventional botany are bioassays and gas chromatography–mass spectrometry (GC–MS) [3]. Bioassay is sensitive and able to detect as low as 0.05 $ng\,mL^{-1}$ brassinolide (BL) [4]. However, it is generally semi-quantitative, costs a long time, and is carried out at strictly controlled conditions, which can be hardly fulfilled in most laboratories [5]. Currently, GC–MS is the standard method for quantification of BRs with limit of detection (LOD) to $ng\,g^{-1}$ fresh mass level, but tens or even hundreds of grams plant samples have to be collected and purified prior to analysis [3,4]. While other methods such as immunochemical approaches, show low LOD (5 fmol for BL and castasterone (CS)) [5], the development of specific antibodies with low cross-reactivity is prerequisite to this method, which is still a challenge for the detection of some BRs [5,6].

Recently, BRs analysis with high performance liquid chromatography coupled with mass spectrometry (HPLC–MS) has gradually prevailed. Separation at room temperature, high loading volume and few requirements for cleanup make it suitable for plant sample analysis. However, direct analysis of BRs with HPLC–MS is still difficult due to its high LOD. The LOD for naphthalenoboronate derivatives of BRs was 2–4 ng by using atmospheric pressure chemical ionization (APCI) MS [7], and that

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http://dx.doi.org/10.1016/j.chroma.2013.04.043

for five BRs was nearly 30 pg by using electro spray ionization (ESI) MS [5].

Due to the low sensitivity of direct HPLC–MS analysis for BRs, sample preparation prior to analysis becomes critical. Among limited references about sample preparation for HPLC analysis of BRs [8–11], it was found that boronic acid compounds were widely used to enhance the sensitivity of detection. All bioactive BRs possess a vicinal 22R,23R diol structural functionality [2], and boronic acids react selectively with 1,2-diols groups to form cyclic complexes in an aprotic or alkaline protic condition [8,12]. In earlier works, boronic acids with special groups were used to derivatize BRs for ultraviolet (UV), fluorimetric, or electrochemical detection after HPLC [8,9]. Recently, dansyl-3-aminophenylboronate derivatization was utilized to increase the ionization efficiency of BRs in ESI source for standard sample, which resulted in sub-femtomolar range of LOD for standard sample [11]. However, in real sample analysis, the LOD was ∼10 ng because of the low selectivity of the sample preparation method. Phenylboronic acid (PBA) polymer gel had been used to extract BL and CS off-line prior to HPLC-UV analysis [10], but 5% hydrogen peroxide in the eluent was harmful for HPLC, and inhibited on-line BRs analysis. Therefore, a rapid, selective and efficient sample preparation method for direct analysis of BRs in plant extracts is urgently needed for HPLC–MS.

In this work, we presented an on-line two-dimensional microscale solid phase extraction (2DμSPE)-on column derivatization (OCD)-high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) method to simultaneously quantify five BRs from plant tissues. The selected five BRs (24-epiBL, 24-epiCS, 6-deoxo-24-epicastasterone (6-deoxo-24-epiCS), teasterone (TE) and typhastero (TY)) are most widely distributed in plant species, and are representative for natural 69 BRs in both chemical and biological properties. 2DμSPE was prepared by packing phenyl boronic acid silica sorbent and C18 silica sorbent into two continuous segments. The broad elution band of BRs from PBA sorbent could be compressed to narrow elution band from C18 sorbent, and the selectivity of μSPE to BRs could be enhanced. Then, after BRs was enriched on C18 sorbent, OCD was carried out with *m*-amino PBA (*m*-APBA) as the derivatization reagent to increase the ionization efficiency of BRs in ESI source. Finally, the on-line, selective and rapid sample preparation method coupled with HPLC–MS/MS detection was validated, and applied to analysis of endogenous BRs in crude plant tissue extracts.

2. Experimental

2.1. Chemicals and materials

24-epiBL ((22R,23R,24R)-2,3,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5-cholestane-6-one) of purity >98% was purchased from Toronto Research Chemicals Inc. (North York, Canada). 24-epiCS ((22R,23R,24R)-2,3,22,23-tetrahydroxy-24-methyl-5-cholestane-6-one), 6-deoxo-24-epiCS (22R,23R,24R)-2,3,22,23-tetrahydroxy-24-methyl-5-cholestane), TE ((22R,23R)-3β,22,23-trihydroxy-5α-campestan-6-one) and TY ((22R,23R)-3α,22,23-trihydroxy-5α-campestan-6-one) of purity >98% were obtained from OlChemIm Ltd. (Olomouc, Czech Republic). Stock solutions of all the BRs were prepared at 100 μg mL^{-1} in acetonitrile under −20 °C. *m*-APBA monohydrate (purity of 97%) was purchased from Aladdin (Shanghai, China). Pyridine and hydrochloric acid aqueous solution (36–38%) of analytical grade were purchased from Kermel Chemical Reagent Co. (Tianjing, China). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Deionized water used in this experiment was Wahaha purified water (Hangzhou, China). The real plant samples were provided by Institute of Plant Physiology & Ecology (IPP&E), Shanghai Institutes for Biological Science, Chinese Academy of Sciences.

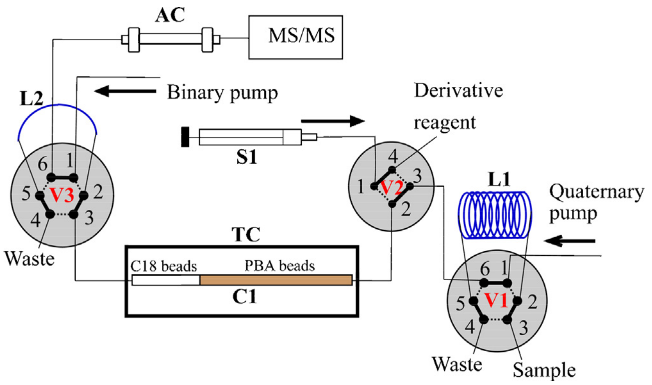

Fig. 1. Schematic illustration of 2DμSPE-OCD–HPLC–MS/MS for BRs determination. V1, high-pressure six-port rotary valve for plant sample loading; V2, high-pressure four-port rotary valve for on-column derivatization; V3, high-pressure six-port rotary valve for injection of HPLC; C1, 2DμSPE column; AC, analytical column; S1, syringe pump; TC, temperature controller; L1, 10 mL sample loop; L2, 30 μL sample loop.

PBA silica sorbent (50 μm) was purchased from Baseline Chromtech Research Center (Tianjing, China), and C18 silica sorbent (40 μm) was purchased from Fuji Silysia Chemical Ltd. (Kasugai, Japan).

2.2. HPLC–MS/MS system

HPLC–MS/MS analysis were performed on an Agilent 1200 HPLC gradient system coupled with an Agilent 6460 triple quadrupole mass spectrometer. Manual injections were conducted with a cheminert high-pressure six-port rotary valve (Valco, TX, USA) equipped with a 30 μL loop. This valve was also used as the interface between the extraction system and analytical column.

A C18 column (Agilent ZORBAX SB-C18, 50 mm × 2.1 mm 1.8 μm) was used for separation. The mobile phase included solvent A (aqueous solution with 0.05% formic acid) and solvent B (acetonitrile). The gradient was kept at 30% B for 3 min, and then to 100% B in 15 min at the flow rate of 0.4 mL min^{-1}. The parameters of MS/MS detection are summarized in Table S1.

2.3. On-line 2DμSPE-OCD system

A schematic illustration of the sample preparation setup is depicted in Fig. 1. A quaternary high pressure pump (Agilent 1200, Agilent Technologies, USA) was used to deliver solvents for on-line SPE operations except for the derivatization reagent. Derivatization reagent was delivered by a homemade syringe piston pump (Method S-1). A Cheminert high-pressure six-port rotary valve (Valco, TX, USA) equipped with a 10-mL stainless steel sample loop (valve 1) was used as injector. It was connected via a 400 mm × 0.25 mm i.d. poly(ether-ether-ketone) (PEEK) transfer line to a four-port rotary valve (valve 2), which was used to switch flow between quaternary high pressure pump and syringe pump. One port of valve 2 was connected to the 2DμSPE column. The 2DμSPE column was a 150 mm × 0.5 mm i.d. transparent Kel-F fluoroplastic tube containing PBA (20 mg) silica sorbent and C18 silica sorbent (10 mg). Both ends of the column were fitted with PEEK 10-32 to 1/4-28 adapters and 2-μm stainless steel frit to hold the packing materials (Upchurch Scientific, WA, USA). The packing procedure is described in Method S-2. The column was kept in a home-made thermostat (Method S-3). It was connected to the injector (valve 3) of HPLC–MS/MS system mentioned above via a 200 mm × 0.25 mm i.d. PEEK transfer line.