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Improved methodology for analysis of multiple phytohormones using sequential magnetic solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry

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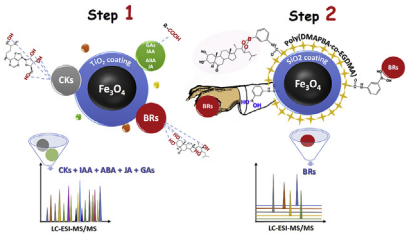
HIGHLIGHTS

- A simple and efficient method was developed for simultaneous analysis of multiple phytohormones.
- IAA, ABA, JA, GAs, BRs and CKs were purified by sequential magnetic solid-phase extraction.
- 16 endogenous phytohormones could be detected in 100 mg (fresh weight) flower of *Brassica napus* L.

GRAPHICAL ABSTRACT



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ABSTRACT

Phytohormones are special small molecules that play important role in plant growth and development at trace levels. Quantification of multiple phytohormones will be great helpful for researches about cross-talks that plant hormones regulate the responses of plants against both biotic and abiotic stresses by means of synergistic or antagonistic interactions. In the current study, we developed a method for profiling of phytohormones in one small sample, including indole-3-acetic acid, abscisic acid, jasmonic acid, gibberellins, cytokinins and brassinosteroids. These phytohormones mentioned above were firstly purified and separated by sequential magnetic solid-phase extraction (MSPE) and then analyzed by ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-MS/MS). Under the optimized extraction conditions, good linearity was obtained with correlation coefficients (r) ranging from 0.9961 to 0.9998. The limits of detection (LODs, S/N = 3) were ranged from 0.45 to 126.1 pg mL^{-1}. The recoveries were between 85.0% and 116.2%. The relative standard deviations (RSDs) were ranged from 2.7% to 16.1%. With the proposed strategy, 23 phytohormones could be purified and analyzed from a single plant extract. Finally, 16 phytohormones could be detected in 100 mg (fresh weight) flower of *Brassica napus* L., including IAA, ABA, JA, 4 GAs, 3 BRs and 6 CKs with the concentration ranged from 0.09 to 305.23 ng g^{-1}.

1. Introduction

Phytohormones are of vital importance at trace amount level for the growth and development of plants and protective response against stress [1,2]. They are grouped into several classes, including

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abscisic acid (ABA), auxins, gibberellins (GAs), cytokinins (CKs), ethylene (ET), brassinosteroids (BRs), jasmonates and salicylates according to their structures and physiological functions [3,4]. Although each class of phytohormones has been identified for their specific biological functions, they usually collectively regulate the plant responses against biotic and abiotic stress by means of synergistic effect or antagonistic effect [5–7]. It is referred to as cross-talk of phytohormones which is universally present in plant physiological activities [5]. Because the cross-talks are complex and refined, the molecular mechanisms of them remain elusive. For better understanding the cross-talk mechanisms of phytohormones, simultaneous determination of multi-class phytohormones is needed.

The challenges to simultaneous determination of multi-class phytohormones are originated not only from their low abundance in plants (pmol per gram fresh weight, pmol g^{-1} FW) and the complex matrix of plants, but also from the difficulties of simultaneous extraction and purification of multi-class phytohormones due to their diverse chemical properties. Although a variety of analytical methods have been developed for single-class phytohormones by GC-MS or LC-MS [8–16], few methods were reported for the simultaneous determination of multi-class phytohormones [17–19].

Up to now, many sample preparation methods have been developed for enrichment and purification of multiple phytohormones, such as immune affinity purification (IAP) [20–22], liquid-liquid extraction (LLE) [23,24], solid-phase extraction (SPE) [19,25–27], polymer monolith microextraction (PMME) [28], magnetic solid-phase extraction (MSPE) [29] and so on. Thanks to these sample preparation methods, Kojima *et al.* developed a multi-step strategy for determination of 43 phytohormones in rice including auxins, ABA, gibberellins and CKs [17]. Liu *et al.* described a method for simultaneous analysis of CKs and acidic phytohormones (auxins, ABA and GAs), in which a binary SPE was employed for purification and enrichment of phytohormones [19]. CKs and acidic phytohormones were eluted from different SPE cartridges respectively. And the two fractions of elution were combined for UHPLC-MS/MS analysis. More recently, a TiO_2-based MSPE was developed for profiling of multiple phytohormones, including indole-3-acetic acid (IAA), ABA, JA, GAs and CKs through hydrophilic interaction and coordination [18]. All target analytes mentioned above were assigned to acidic phytohormones (IAA, ABA, JA and GAs) and CKs. Nevertheless, none of the methods could be used for simultaneous analysis of acidic phytohormones, CKs and BRs.

Recently, Tong *et al.* revealed a previously unknown mechanism underlying BR and GA cross-talk depending on tissues and hormone levels, which greatly advanced our understanding of hormone actions in crop plants, appearing much different from that in *Arabidopsis thaliana* [6]. It is indispensable to get more information of phytohormones from the same sample to illustrate the complex relationship between phytohormones. However, it is still a challenge for simultaneous analysis of multiple class phytohormones in one small sample, including acidic phytohormones, CKs and BRs. Also, it is a bottleneck that simultaneous purification of multiple phytohormones with different structures and chemical properties from one crude plant extract.

In the current study, we developed a method for simultaneous analysis of multiple phytohormones including acidic phytohormones (IAA, ABA, JA, GAs), BRs and CKs by sequential MSPE coupled with UHPLC-MS/MS. The classification, name and structures of all the targets were shown in Table S1. In our proposed strategy, the crude plant extract was firstly processed by a TiO_2-based MSPE [18]. Subsequently, the second MSPE was employed for further purification of BRs while the separation of BRs from acidic hormones and CK could be realized. Finally, the developed method was successfully applied to detect multiple phytohomones in flower of *Brassica napus* L.

2. Experimental

2.1. Chemicals and reagents

Phytohormone standards: indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), gibberellic acids (GA1, GA3, GA4, GA7, GA12, GA24, GA53), 28-norbrassinolide (28-norBL), 28-norcastasterone (28-norCS), 28-homobrassinolde (28-homoBL), brassinolide (BL), castasterone (CS), N^6-isopentenyladenine (iP), N^6-isopentenyladenine riboside (iPR), N^6-isopentenyladenine 9-glucoside (iP9G), *trans*-zeatin (tZ), *trans*-zeatin-riboside (tZR), *trans*-zeatin 9-glucoside (tZ9G), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR) and stable isotope-labeled standards: [$^2H_2$]IAA, [$^2H_6$]ABA, [$^2H_2$]GA1, [$^2H_2$]GA3, [$^2H_2$]GA4, [$^2H_2$]GA12, [$^2H_2$]GA24, [$^2H_2$]GA53, [$^2H_3$]BL, [$^2H_3$]CS, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP9G, [$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_5$]tZ9G, [$^2H_3$]DHZ, [$^2H_3$]DHZR were all purchased from Olchemim Ltd. (Olomouc, Czech Republic).

Ferric chloride ($FeCl_3 \cdot 6H_2O$), sodium acetate (NaAc), ethylene glycol (EG), 1,2-ethylenediamine (ETH), ethanol (EtOH), tetraethyl orthosilicate (TEOS), ammonia hydrate ($NH_3 \cdot H_2O$, 25%, aqueous solution), ammonium hexafluorotitanate ($(NH_4)_2TiF_6$), boric acid (H_3BO_3), pyridine, formic acid (FA, 88%), 2,2-azobis(2-methyl-propionitrile) (AIBN) were all purchased from Sinopharm Chemical Reagent (Shanghai, China). 3-(Methacryloxy)propyl trimethoxysilane (MPS) was bought from Wuhan University Silicone New Material (Wuhan, China), while ethylene glycol dimethacrylate (EGDMA) was bought from Sigma–Aldrich (St. Louis, MO, USA). 4-(N,N-dimethyamino) phenylboronic acid (DMAPBA) was purchased from J&K Scientific Ltd (Beijing, China). AIBN was recrystallized from ethanol, and other reagents were of analytical grade and used directly without further purification. Acetonitrile (ACN, HPLC grade) was obtained from Tedia Co. (Fairfield, OH, USA). Ultra-pure water used throughout the study was purified by Milli-Q system (Milford, MA, USA).

2.2. Preparation of magnetic polymer particles Fe_3O_4@SiO_2@Poly(DMAPBA-co-EGDMA)

MPS-modified Fe_3O_4 particles were prepared according to our previous method [29]. Subsequently, Fe_3O_4@SiO_2@Poly(DMAPBA-*co*-EGDMA) particles were synthesized by the distillation–precipitation polymerization method proposed by Yang *et al.* [30]. Briefly, DMAPBA (0.25 g) was dissolved in 200 mL ACN/pyridine (50/1, v/v) in a 500-mL two-necked round bottom flask equipped with a distillation apparatus and a stirring device. Then, MPS-modified Fe_3O_4@SiO_2 (0.50 g), EGDMA (2.25 g) and AIBN (0.02 g) were successively added. The mixture was heated to boiling within 30 min and kept boiling until approximately half of the acetonitrile was distilled out (occurring within 2 h). As soon as the mixture cooled down to room temperature, Fe_3O_4@SiO_2@Poly(DMAPBA-*co*-EGDMA) particles were separated by the aid of a magnet, and washed several times with water and methanol. Finally, the resultant magnetic polymers were dried in a vacuum at 60 °C.

2.3. Plant materials

Three-month-old *Brassica napus* L. flowers were provided by Dr. Jia Liu from Oil Crops Research Institute, Chinese Academy of Agricultural Sciences (Wuhan, China). The plant materials were harvested, weighted, immediately frozen in liquid nitrogen and stored at -80 °C.