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Comparison of sample pretreatment methods for the determination of multiple phytohormones in plant samples by liquid chromatography–electrospray ionization-tandem mass spectrometry

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ABSTRACT

Sample preparation is often the bottleneck in the analysis of phytohormones in plant samples. In the present study, sample pretreatment involving extraction by acidified and non-acidified organic solvents and extract purification using solid-phase extraction (SPE) and/or liquid–liquid extraction (LLE) has been optimized and compared for the determination of multiple phytohormones, including auxins, cytokinins (CKs), abscisic acid (ABA), gibberellins (GAs), jasmonic acid (JA) and salicylic acid (SA) in biological samples by liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) under multiple-reaction monitoring (MRM). A novel and efficient sample pretreatment method for multiple phytohormones in acidified acetonitrile extracts of plant samples using polymer-based mixed-mode cation-exchange solid phase extraction (SPE) with Oasis MCX cartridges combined with ethyl acetate LLE has been developed. The method detection limits (MDLs) for the target phytohormones ranged from 0.0013 to 0.021 $ng \cdot mL^{-1}$, and the recoveries were obtained by spiking the target phytohormones in the two-month-old leaves of oilseed rape (*Brassica napus* L.) at concentrations of 0.20, 2.0 and 10 $ng \cdot g^{-1}$, which ranged from 75.1 to 111%, 79.6 to 113% and 89.2 to 111%, respectively. The intra-day precisions were in the range of 1.15 to 10.2%, and the inter-day precisions ranged from 2.92 to 12.4%. Additionally, matrix effects were substantially decreased. The proposed sample pretreatment method has been successfully applied to the analysis of multiple phytohormones in the leaf samples of oilseed rape (*B. napus* L.).

1. Introduction

Phytohormones are a structurally unrelated collection of small molecules derived from various essential metabolic pathways [1]. The early-discovered phytohormones auxin, gibberellin (GA), cytokinin, abscisic acid (ABA) and ethylene are generally referred to as the five 'classic' plant hormones, whereas brassinosteroid (BR), jasmonate (JA), salicylate (SA), strigolactone (SL), nitric oxide (NO), polyamine, and peptide are recognized as new families of phytohormones. These compounds are important regulators that mediate plant growth, differentiation, development and responses to both biotic and abiotic stresses by means of synergistic or antagonistic actions [2]. Many studies have revealed the role of each class of phytohormone, and in recent years, molecular genetic studies have been elucidating complicated crosstalk among phytohormones [3,4]. To elucidate the hormonal signaling networks, functions, and dynamics, the simultaneous and reliable determinations of multiple phytohormones at the organ, cellular, and sub-cellular levels are required [5,6].

The traditional determination methods for phytohormones are bioassay and immunoassay [7]. Gas chromatography–mass spectrometry (GC–MS) was once popular for the analyses of multiple phytohormones [8,9]. Recently, liquid chromatography–electrospray ionization (ESI)-tandem mass spectrometry (LC–ESI-MS/MS) under multiple-reaction monitoring (MRM) has emerged as an effective method for the analysis of multiple phytohormones [10–18]. To date, LC–ESI-MS/MS has been applied to the determination of cytokinins [10–12], GAs [13,14], IAA [15,16] and ABA [17,18] in plant samples. Unfortunately, the determination of multiple phytohormones is still a challenging issue for plant biologists [5,9].

Phytohormones are often present in plant tissues at ultra-trace levels, normally in the $pg \cdot g^{-1}$ to $ng \cdot g^{-1}$ fresh weight (FW) range, depending on the tissue. Many phytohormones have similar core structures and vary only by their substituents. Some phytohormones are even isomers, such as *cis*- and *trans*-zeatin and *cis*- and *trans*-ABA [4,9]. The extraction of multiple phytohormones with organic solvents is widely used, and ethyl acetate, acetonitrile and methanol are the most commonly used

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solvents [9,12]. Ideally, the extraction solvent should efficiently extract the multiple phytohormones, and the quantity of the interfering substances extracted should be as low as possible. However, the extraction efficiency of multiple phytohormones in biological samples depends on the extent to which they are associated with other substances such as lipids and other lipophilic matrix substances. The co-extraction of lipids and other lipophilic matrix substances can alter the ionization efficiency of LC–ESI-MS/MS, which may lead to serious matrix effects [14]. Therefore, the purification of phytohormones before extraction is a key procedure in the determination of multiple phytohormones by LC–ESI-MS/MS [9,13].

Many efforts have been made to remove lipids and other lipophilic matrix substances in complex plant matrices [5,9,10,12,13]. In the early years, liquid–liquid extraction (LLE) was a fairly common technique [5,9]. In recent years, dispersive liquid–liquid microextraction (DLLME) [9,19], vapor-phase extraction (VPE) [9,20], solid-phase microextraction (SPME) [9,21], and molecularly imprinted solid-phase extraction (MISPE) [22] have been applied to purify phytohormones from complex plant samples. Compared with other techniques, SPE including polymer-based reversed-phase SPE (i.e., Oasis HLB, Waters) [9,21], silica-based reversed-phase sorbents (e.g., HyperSep C_{18}, Thermo Fisher Scientific) [9,21,23], polymer-based mixed mode anion-exchange sorbent (i.e., Oasis MAX, Waters) [23–25], and polymer-based cation-exchange SPE (i.e., Oasis MCX, Waters) [9,24] are more widely used. Nevertheless, a single SPE process has typically been insufficient to remove lipids and other lipophilic matrix substances, preventing multiple phytohormones from being detected by HPLC–ESI-MS/MS [9,25]. Thus, two or more SPE processes have been employed to extract and purify phytohormones in complex plant matrices, but some of them suffer from low recovery and reproducibility, e.g., IAA and ABA [6,26,27]. The objectives of this study were to (1) optimize and compare, in terms of efficiency, the sample pretreatment involved in the extraction and purification of multiple phytohormones and (2) develop an efficient analytical method for their simultaneous determination in plant samples.

2. Experimental

2.1. Chemicals and reagents

N^6-benzyladenine (6-BA), N^6-(isopentenyl) adenine (iP), indol-3-acetic acid (IAA), (±) abscisic acid (ABA), *trans*-zeatin (*t*-Z), salicylic acid (SA) and jasmonic acid (JA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gibberellin A_1 (GA_1), gibberellin A_3 (GA_3) and gibberellin A_4 (GA_4) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The deuterium-labeled internal standards d_5-indol-3-acetic acid (d_5-IAA), d_7-N^6-benzyladenine (d_7-BA) and d_6-abscisic acid (d_6-ABA) were obtained from OlChemIm (Olomouc, Czech Republic). Ethyl acetate (EtOAc), acetonitrile (ACN), and HPLC-grade methanol were obtained from Merck (Darmstadt, Germany). Formic acid (FA), dichloromethane (DCM), *n*-hexane (Hex) and acetic acid (HAC) were purchased from Fisher Scientific (Hanover Park, IL, USA). Ultra-pure water was obtained from a Milli-Q purification system (Millipore Corporation, Billerica, MA). HyperSep C_{18} (3 cm^3, 200 mg) SPE cartridges were obtained from Thermo Fisher Scientific (Waltham, USA). Oasis HLB (6 cm^3, 150 mg), Oasis MAX (6 cm^3, 150 mg) and Oasis MCX (6 cm^3, 150 mg) SPE cartridges and 0.45-μm PTFE filters were purchased from Waters (Milford, MA, USA).

2.2. Preparation of standard solutions

IAA, ABA, GA_1, GA_3, GA_4, SA, JA, *t*-Z, 6-BA, iP, d_5-IAA, d_7-BA and d_6-ABA were dissolved in MeOH to prepare 0.1 $mg \cdot mL^{-1}$ stock solutions. A mixed stock solution containing 10 $\mu g \cdot mL^{-1}$ of IAA, ABA, GA_1, GA_3, GA_4, SA, JA, *t*-Z, 6-BA, iP, d_5-IAA, d_7-BA and d_6-ABA was prepared in MeOH. All solutions were stored in darkness at −20 °C, and the working solutions were prepared from these stock solutions and serially diluted with a combined solution of MeOH/H_2O (50/50, v/v) immediately before use.

2.3. Biological material

Oilseed rape (*Brassica napus* L.) plants were grown in a greenhouse at 22 °C under 16 h light/8 h dark photoperiods. Two-month-old leaves were used for the experiments. All of the collected leaves were weighed, immediately frozen in liquid nitrogen, and stored at −80 °C until sample extraction.

2.4. LC–MS/MS analysis

An Agilent 1260 series HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a degasser, binary pump, autosampler and column oven coupled to an Applied Biosystems-Sciex API 4500 (Applied Biosystems, Concord, Ontario, Canada) triple–quadrupole mass spectrometer was employed for the separation and quantification of the target plant hormones.

Separation of the target plant hormones was achieved on a Thermo Hypersil ODS-2 column (150 × 2.1 mm i.d., 5 μm) with a security guard precolumn (10 × 2.1 mm i.d., 5 μm; Thermo Scientific, Bellefonte, PA, USA). The column was equilibrated at 25 °C. The mobile phase consisted of water with 0.15% FA (A)/MeOH (B) at a flow rate of 0.6 mL min^{-1}. The gradient profile started with 10% of eluent B for 1.0 min and then increased linearly to 90% within 9.0 min; this composition was held for 10.0 min before being returned to 10% of eluent B within 0.10 min, followed by a re-equilibration time of 2.0 min. The injection volume was 5 μL.

The MS parameters were optimized with mixtures of standard solutions. Nitrogen was used as the nebulizer, drying, and collision gases. The nebulizer gas pressure, drying gas pressure, curtain gas pressure, source voltage and source temperature were set at 60 psi, 50 psi, 30 psi, −4.5 kV and 500 °C, respectively. Quantification was performed in the MRM acquisition mode. SA, JA, CKs (*t*-Z, iP, and 6-BA) and their corresponding internal standards were analyzed in the positive-ion mode. ABA, IAA, GA_1, GA_3, GA_4 and their corresponding internal standards were analyzed in negative-ion mode.

Data were acquired and processed using Analyst 1.4.1 software (AB SCIEX, USA).

2.5. Sample extraction

The plant hormones were extracted from plant samples according to the method of Dobrev [24] with modifications.

Plant samples were ground in liquid nitrogen and then transferred into a 5.0-mL centrifuge tube. IAA, ABA, GA_1, GA_3, GA_4, SA, JA, *t*-Z, 6-BA, iP, d_5-IAA, d_7-BA and d_6-ABA were added and then extracted with 2.0 mL of the extractants overnight at −20 °C. The extract was separated by centrifugation at 10,000 ×*g* under 0 °C for 20 min. The residue was re-extracted three times for 60 min. The combined extracts were evaporated to near dryness. The following experiments were tested at the variants of purification protocols.

2.6. Sample purification

2.6.1. Liquid–liquid extraction (LLE)

The purification method was employed as described previously [9,23] with minor modifications.

The extracted supernatants or the standard solutions were re-dissolved in 2.0 mL Milli-Q water and sequentially extracted with 2 × 3 mL EtOAc, Hex and DCM. The target phytohormones were collected, dried, and reconstituted in 50 μL of mobile phase for LC–ESI-MS/MS analysis. Prior to analysis, this reconstituted extract was filtered through a 0.45-μm PTFE filter.