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Membrane-supported liquid–liquid–liquid microextraction combined with anion-selective exhaustive injection capillary electrophoresis-ultraviolet detection for sensitive analysis of phytohormones

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ABSTRACT

A novel method based on off-line membrane-supported liquid–liquid–liquid microextraction (MS-LLLME) combined with on-column anion-selective exhaustive injection (ASEI) capillary electrophoresis–ultraviolet (CE–UV) detection was established for the analysis of seven phytohormones (abscisic acid (ABA), jasmonic acid (JA), 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), salicylic acid (SA) and gibberellic acid (GA)). In MS-LLLME, the target phytohormones were extracted from the acid donor phase to the alkaline acceptor phase, and the acceptor solutions were directly analyzed by ASEI-CE–UV. Under the optimal experimental conditions, the analytical performance of the method was evaluated. The limits of detection (LODs) of ABA, JA, 2,4-D, NAA, IAA, SA and GA were determined to be 1.00, 2.21, 0.33, 0.17, 0.67, 0.05 and 16.5 ng/mL, respectively. The relative standard deviations (RSDs, $n = 7$) ranged from 4.7% to 12.9%, and the enrichment factors were in the range of 307 to 20,160. The proposed method was successfully applied for the determination of multiple phytohormones in banana, cabbage and cucumber extracts, and ABA, IAA and SA were detected in these samples. The recoveries for the spiked samples were in the range of 79.0 to 116.4%. The proposed method was demonstrated to be suitable for the simultaneous quantification of multiple phytohormones with high sensitivity and good sample cleanup ability.

1. Introduction

Phytohormones, a group of naturally occurring substances detected at extremely low concentrations, play important roles in a variety of regulation processes, such as growth, metabolism, cell division, cell differentiation and leaf and organ senescence, as well as the protective response to biotic and abiotic stresses [1–3]. The most widely recognized phytohormones include auxins, cytokinines, gibberellins, abscisic acid, salicylic acid and jasmonates [4]. Different phytohormones exert their functions additively, synergistically or antagonistically, and their regulation is greatly dependent on their levels in plants. Based on their physiological functions, different phytohormones can be utilized in agriculture to achieve various enhanced agricultural characteristics during critical growth stages. Phytohormones can also be found in mammals [5], stimulating the human body [6] or affecting immune cells [7]. Obviously phytohormones have its potential application value on agriculture and biomedicine, while a better understanding of the functions and interactions among them relies on the accurate quantitative method. Therefore, it is necessary to develop a fast, simple and sensitive method for the simultaneous analysis of multiple classes of trace phytohormones.

To date, several methods have been developed for the determination of phytohormones, including gas chromatography (GC) [8], high-performance liquid chromatography (HPLC) [9–11], capillary electrophoresis (CE) [12–14] and chromatography coupled with mass spectrometry (MS) [15–18]. Among these methods, CE is a powerful microanalytical technique, the fast speed of analysis, high resolution and sensitivity make it an attractive method to analyze phytohormones [12–14,18]. CE with UV [12,14], laser-induced fluorescence (LIF) [13] and MS [18] as the detector has been employed for phytohormones analysis. However, MS is expensive, and the interface of CE–MS is extremely complicated, while a derivatization step is often required in LIF detection, which is

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time-consuming and can introduce error due to the complex matrix. UV is the most easily available and efficient detector for CE with low cost, and CE–UV is appropriate for the simultaneous analysis of multiple phytohormones without derivatization. However, the sensitivity of CE–UV is strictly limited by its sample injection volume (nL) and narrow optical path length. In addition, it is difficult to directly and accurately quantify trace phytohormones in complex plant matrices. Such limitations can be overcome by incorporating both on-column concentration techniques or/and off-line sample pretreatment techniques with CE–UV [19]. On-column preconcentration techniques, such as stacking, sweeping and dynamic pH junctions, are powerful approaches for substantially improving the sensitivity of CE–UV [19] but have not been fully applied to the analysis of phytohormones. Only Chen et al. [20] have developed a method involving microemulsion electrokinetic capillary chromatography (MEKC) coupled with large volume sample stacking (LVSS) for the analysis of six phytohormones. This method has a high enrichment factor but a low sample cleanup capacity. Due to the existence form of many phytohormones as anions under alkaline conditions, anion-selective exhaustive injection (ASEI) is expected to be a powerful stacking technique that can selectively, electrokinetically and efficiently inject phytohormones and enhance sensitivity simultaneously.

To improve the matrix resistance and sensitivity of the method, an appropriate pretreatment technique is required prior to instrument analysis. Solid phase extraction (SPE) [21–23] and liquid–liquid extraction (LLE) [24,25], have been extensively utilized for phytohormones, while poor selectivity and lengthy SPE time as well as solvent consumption and low enrichment factors in LLE are their drawbacks. To develop a more economical and automated analytical method, green and miniaturized sample pretreatment techniques, such as solid phase microextraction (SPME) and liquid phase microextraction (LPME), have rapidly evolved in recent decades. LPME is an attractive pretreatment technique for combining extraction and enrichment into a single step and is a fast and simple method with a large enrichment factor and good cleanup ability. A hollow-fiber-based liquid–liquid–liquid microextraction system for the extraction of several phytohormones was developed in our previous study [9], and this system has been successfully applied to the analysis of complex samples. A method that combines single drop liquid–liquid–liquid phase microextraction (SD-LLLME) with direct analysis in real-time mass spectrometry (DART-MS) was proposed by Bai et al. [4], but the stability of the method needs improvement. It can be concluded that three-phase liquid–liquid–liquid microextraction can efficiently remove the sample matrix while simultaneously enriching the target phytohormones. To improve the enrichment factor and the stability of LPME, a membrane-supported liquid–liquid–liquid microextraction (MS-LLLME) [23] technique with a larger sample volume and good reproducibility is a very promising method for extracting target phytohormones from acidic sample solutions via organic solvent extraction into an alkaline acceptor solution using a pH gradient and distribution in three phases. In this system, the acceptor phase volume matches the CE injection volume well.

The aim of this work is to develop a rapid, inexpensive, sensitive, efficient method based on CE–UV for the simultaneous quantification of multiple phytohormones in fruits and vegetable samples. For this purpose, an MS-LLLLME system with strong matrix resistance and high extraction efficiency was combined with an automatic, sensitive ASEI-CE–UV system for the analysis of seven target phytohormones. The factors that affect extraction and ASEI were optimized and the analytical performance was evaluated. The proposed method was applied for the determination of multiple phytohormones in banana, cabbage and cucumber extracts for validation.

2. Experimental

2.1. Reagents and materials

Table 1 shows the chemical structures of the target phytohormones as well as some of their chemical properties. Indole-3-acetic acid (IAA) and (±) jasmonic acid (JA) were purchased from Sigma-Aldrich (Sigma-Aldrich Company, USA), and gibberellic acid (GA), (±) abscisic acid (ABA), salicylic acid (SA), 1-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were obtained from Aladdin Reagent Database Inc. (Shanghai, China). Stock solutions (1 mg/mL of each analyte) were prepared by dissolving each phytohormones in methanol. All of the stock solutions were stored at 4 °C in a refrigerator. The working standard solutions were prepared daily by stepwise dilution of the corresponding stock standard solutions with high-purity water. High-purity deionized water was obtained from a Milli-Q water purification system (18.2 MΩ/cm, Millipore, Bedford, MA, USA).

2.2. CE system

All of the separation experiments were performed by a G1600AX CE system (Agilent, Waldbronn, Germany) equipped with a programmable, multiwavelength UV–Visible detector. The wavelengths employed for the determination of target phytohormones were 200 nm for GA, JA, 2,4-D and SA; 220 nm for IAA and 2,4-D; and 254 nm for SA, respectively. The capillary temperature for the separation of target phytohormones was maintained at 25 °C. The new capillary was conditioned by successive washings with 1 mol/L NaOH (30 min), water (10 min), 0.1 mol/L HCl (10 min) and water (30 min). Between runs, the capillary was rinsed with running buffer for 5 min.

The separation conditions for the CE standard injection were as follows: fused-silica capillary with dimensions of 58.5 cm (50 cm to the detector) × 50 μm i.d. ×360 μm o.d. (Yong-nian Optical Fiber, Hebei, China), background electrolyte (BGE) containing 35 mmol/L sodium borate buffer and 0.1% (m/V) β-CD (pH 10.0, adjusted by NaOH and filtered through a membrane filter with a pore size of 0.45 μm), separation voltage of 25 kV and hydrodynamic injection of the samples at 50 mbar for 5 s. A typical electropherogram of the separation of the seven phytohormones is shown in Fig. S1.

2.3. MS-LLLLME procedure

The MS-LLLME unit (shown in Fig. 1) was set up as indicated in our previous study [26]. The porous-nylon-membrane-supported extraction tip is shown in Fig. 1b. Briefly, the nylon membrane (0.8 μm pore size, Xingya purification material company, Shanghai, China) was sealed on the slightly flamed larger end of pipette tip (200 μL scale). Then, the smaller end of pipette tip was manually cut to a proper length so that the flat-end tip of a 25-μL microsyringe (Gaoge, Shanghai, China) could be inserted into the pipette tip tightly. In the MS-LLLME system, the sample solution volume was 9.1 mL, containing an appropriate amount of NaCl and HCl. This solution was transferred to a flask, and 250 μL of an organic solvent (phenetole:octanol = 6:4) was carefully added to the sample solution to form a supernatant solvent layer by pipetting. Then, a 25-μL microsyringe with a flat-end needle tip filled with 15 μL of ammonia acceptor solution was tightly inserted from the small end of the flamed pipette tip until the flat needle tip was in close contact with the nylon membrane, and 5 μL of the acceptor solution was injected to wet the nylon membrane. Next, the microsyringe along with the porous-nylon-membrane-supported extraction tips were immersed in the organic solvent layer, and the remaining acceptor solution was pushed out from the microsyringe to form a large droplet under the nylon membrane. An