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Simultaneous determination of plant hormones in peach based on dispersive liquid–liquid microextraction coupled with liquid chromatography–ion trap mass spectrometry

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ABSTRACT

Fruit development is influenced greatly by endogenous hormones including salicylic acid (SA) and abscisic acid (ABA). Mass spectrometry with high sensitivity has become a routine technology to analyze hormones. However, pretreatment of plant samples remains a difficult problem. Thus, dispersive liquid–liquid microextraction (DLLME) was used to concentrate trace plant hormones before liquid chromatography–ion trap mass spectrometry (LC–ITMS) analysis. Standard curves were linear within the ranges of 0.5–50, 0.2–20 ng/mL for SA and ABA, respectively. The correlation coefficients were greater than 0.9995 with recoveries above 87.5%. The limits of detection were 0.2 ng/mL for SA and 0.1 ng/mL for ABA in spiked water solution, respectively (injection 20 μL). The successful analysis of SA and ABA in fruit samples indicated our DLLME–LC–ITMS approach was efficient, allowing reliable quantification of both two compounds from very small amounts of plant material. Moreover, this research revealed the relationship between SA and ABA content and development of peach fruit at different growth stages.

1. Introduction

Fruit development and maturation is a normal physiological phenomenon in plants which is affected by external environmental conditions (including light, moisture, and temperature) as well as internal factors (such as plant hormones). Plant hormones are a group of trace endogenous signal molecules [1,2]. Abscisic acid (ABA) is known to trigger various biological functions involving the acceleration of abscission, induction of dormancy, inhibition of seed germination and increasing of stress resistance, etc [3]. Salicylic acid (SA), which was confirmed as a new plant hormone in 1992, is also an endogenous signal substance. Many physiological processes such as seed development, fruits ripening, horticultural products preservation and environmental stress responses are stimulated and controlled by SA [4].

The role of SA or ABA in influencing fruit development is well documented [5–11]. Endogenous SA content increased sharply at the early stage of fruit development in Ya-li pear [5], apple [6] and banana [7], followed by marked augmentation of fruit size and weight. Additionally, the contents of ABA changed continuously in Roxburgh rose [8] and Nai [9] at the different developing stages of above seeds and fruits. When the amount of ABA reached its peak, serious physiological fruit dropping began in young loquat [10] and sweet cherry fruit [11]. Since endogenous SA and ABA are involved in fruit development, exogenous application is employed to increase fruit yield and quality in agriculture. There is little research on the relationship between content change of endogenous plant hormones and peach development. A recent study reported on content change of ABA in peach fruit development [12]. Nevertheless, research on simultaneous analysis of SA and ABA is lacking.

Under physiological conditions, SA and ABA, like other plant hormones, are present at very low concentrations against a background of abundant primary and secondary metabolites. Therefore, analytical approaches to detect SA and ABA must be extremely selective

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and sensitive. Compared with conventional methods involving high performance liquid chromatography, gas chromatography, and gas chromatography–mass spectrometry, liquid chromatograph-mass spectrometry (LC–MS) has become increasingly popular for plant hormones detection [13–16], especially liquid chromatography/electrospray ionization tandem mass spectrometry such as ion trap MS (LC/ESI–ITMS). This technique combines the efficient separation capability of LC with the great power of structural characterization of MS. Furthermore, the distinct MS/MS function possessed in IT can isolate the desired signal from possible interfering peaks and improve both analytical sensitivity and specificity.

Sample preparation is required before LC–MS analysis, especially for the determination of ultra-trace plant hormones in a complex plant matrix. An ideal cleanup technique will isolate target compounds from the matrix, reduce or even eliminate the background interferences coexisting in the sample and concentrate trace analytes to facilitate their quantitative analysis. Therefore, sample preparation can be the bottleneck in modern analyses. In recent years, a variety of new microextration techniques have gradually replaced the traditional ones (i.e., liquid-phase and solid-phase extraction). For instance, plant hormones analysis using solid-phase microextraction (SPME) has been reported [17,18]. Hollow fiber-based liquid-phase extraction (HF-LPME) has been used to measure several plant hormones in coconut [19]. Dispersive liquid–liquid microextraction (DLLME) has been proposed by Assadi and his co-workers in 2006 [20]. In contrast with SPME or HF-LPME, DLLME requires no special interface when coupled with HPLC, and its simple operation makes it a popular technique. Moreover, this method has been reported to extract hormones from plant material in previous research with satisfactory results [21,22].

The aims of the present work were: (1) To adopt DLLME for extraction and enrichment of SA and ABA, then to simplified plant hormone pretreatment and enlarge application range of DLLME. (2) To reveal the content changes of SA and ABA in development of peach fruit. We aim to understand better the crosstalk of these two hormones in fruit physiological processes.

2. Materials and methods

2.1. Reagents and chemicals

ABA standard (purity >98%) was purchased from J&K Chemical (Shanghai, China). SA standard (purity >99.5%) was obtained from Fuchen Chemical Reagents (Tianjin, China). The above standards were dissolved individually in acetonitrile (ACN) at a stock concentration of 2.0 mg/mL and stored at 4 °C. Working standard solutions of lower concentrations were prepared by diluting stock solutions with ACN prior to use. HPLC-grade ACN and methanol were obtained from Merck (Darmstadt, Germany). Other reagents used were of analytical reagent grade (Shanghai Chemical Reagents Corp., Shanghai, China). Distilled water was sourced from a Milli-Q SP Reagent water system (Millipore, Bedford, USA). All the solvents were passed through a 0.45 μm cellulose filter (Xinya Purification Apparatus Factory, Shanghai, China) before use.

Peach samples (Prunus persica cv. NinhBinh) were kindly provided by Sciences Research Institute of Pomology of Fujian Academy of Agricultural (Fujian, China). During the fruit growth period, samples were picked at regular time for hormone determination, and each sample was tested in triplicate. And the fruits were obtained from the same five trees and stored quickly at −80 °C for following experiments.

2.2. LC/ESI–ITMS analysis

Plant hormones analyses were performed with a LC/ESI–ITMS system containing an 1100 Series LC (Agilent, USA) consisting of an autosampler, a quaternary pump and a degasser. Separation of SA and ABA was performed on an Eclipse XDB-C_{18} reversed-phase column (5 μm, 3.0 mm × 250 mm, Agilent) with a flow rate of 0.6 mL/min and column temperature of 25 °C. Binary solvent system consisting of ACN/water (40:60, v/v, %) was adopted for an isocratic chromatographic separation. The injection volume was 20 μL for each analysis. An Esquire 3000 Ion Trap MSn (Bruker Daltonik GmbH, Germany) was equipped with an ESI source and operated in the negative ion mode. The ESI conditions were as follows: capillary voltage 3.5 kV; end plate offset voltage −500 V; capillary exit voltage 100 V; nebulizer pressure 40 psi; drying gas flow 8 L/min and temperature 350 °C. Nitrogen was used as nebulizer and drying gas. The ITMS was operated in full scan and multiple reaction monitoring (MRM) modes, scanning at 50–280 m/z range. ChemStation software was used for instrument control, data acquisition and data processing.

2.3. Procedure for sample preparation

Fresh peaches were divided into two parts of pulp and core immediately after collection, and the former was stored at −80 °C. Before analysis, frozen pulp material was pulverized with liquid nitrogen using a pestle and mortar. 250 mg fine powdered sample was weighed accurately and transferred to a 7 mL-microcentrifuge tube. An aliquot of 1.5 mL of methanol–water–acetic acid extraction solution (80:19:1, v/v/v, %, stored at 4 °C prior to use) containing 1 mmol/L 3, 5-di-tert-butyl-4-hydroxytoluol as an antioxidant was added. Subsequently, the extracting mixture was kept at 4 °C overnight. Then the mixture was centrifuged at 8000 rpm for 5 min at 4 °C and the supernatant was transferred to a 4 mL vial. The residue was re-extracted once with 0.5 mL of above extractant for 1 h. Both supernatants were combined together and the methanol was removed under a stream of N_2 at ambient temperature. Finally, the solution was diluted and adjusted to 5.0 mL with acidized water (pH 3.0 with 0.1 mol/L HCl) for DLLME.

2.4. DLLME enrichment

The above 5.0 mL water solution (pH 3.0 adjusted with 0.1 mol/L HCl) was placed in a conical test tube, spiking with 0.1 μg/mL analytes of interest. The extraction solvent (30 μL $CHCl_3$) and the disperser solvent (800 μL tetrahydrofuran, THF) were mixed and added to the sample solution. Very quickly, a cloudy solution consisting of many dispersed fine droplets of $CHCl_3$ was observed. After the mixture was shaken gently for 0.1 min and centrifuged at 4500 rpm for 3.0 min, the sedimented phase (about 25 μL) in the bottom of the conical tube was withdrawn with a 50 μL LC syringe (Shanghai Gaoge Industrial and Trading Co. Ltd., China) and then placed into a vial insert fitted with polymeric feet (Agilent, USA). The $CHCl_3$ phase containing target plant hormones were immediately subjected to LC–MS analysis.

3. Results and discussion

3.1. Optimization of chromatographic conditions

As is known, HPLC separation is affected greatly by a number of variables including the mobile phase, proportion of organic solvent, pump flow rate, etc., therefore, preliminary studies were carried out to obtain optimum chromatographic conditions with 0.1 μg/mL standard mixture. Based on our experiments, ACN and H_2O were selected as the mobile phase due to the well-shaped peaks and better chromatographic behavior. After examining three kinds of chromatographic columns (2.1 mm × 150 mm, 3.0 mm × 250 mm, 4.6 mm × 150 mm, C_{18}, particle size 5 μm), we found that SA and