DETERMINATION OF PHYTOHORMONES IN RAPE TISSUES BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY TANDEM MASS SPECTROMETRY

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Abstract

(Background) Phytohormones are important regulators and play a crucial role at different stages of rape development, such as growth, differentiation, metabolism, and morphogenesis. Different phytohormones genetically modified rapes are highly interconnected in a complex dynamic network and a change in one component of the hormonal system may affect other parts. Additionally, synthetic plant growth regulators were most generously used in rape production, and they may make possibility to decrease rapeseed quality. Owing to trace amount of phytohormones present and active in minor concentration in rape tissues, the determination of plant hormone concentration is normally considered very difficult. Therefore, it is very necessary to develop an accurate analysis method for phytohormones in rape tissue.

(Objective) The aim of this study was to establish an analysis method with high accuracy for trace hormone in rape tissue.

(Method) In the experiment, a high-performance liquid chromatography and electrospray ionization-tandem mass spectrometry with selected reaction monitoring was used to simultaneous determine of various classes phytohormones in rape tissues. The rape tissues were involved ultrasound-assisted extraction and purified by dispersive solid-phase extraction, and then transferred into the liquid chromatography system.

The analysis was carried out using a reverse phase HPLC gradient elution, the mobile phase using methanol with 0.1% formic acid and water with 0.1% formic acid. The technology was applied to analyze rape tissues, including roots, stems, leaves, flowers, immature pods and rape seeds. The quantification limit for each plant hormone was defined by the ratio of signal/background noise (S/N) 10.

(Result and Conclusion) The results showed perfect linearity (R^2 values of 0.9996-1.0000) and reproducibility of elution times (relative standard deviations, RSDs, < 1%) and peak areas (RSDs, < 4%) for all target compounds. The separation of the seven phytohormones, including different classes of phytohormones, was completed within 30 min with a simple extraction procedure.

Keywords: rape tissues, phytohormones, liquid chromatography-tandem mass spectrometry

1. Introduction

Phytohormones play a crucial role at different stages of rape development, such as growth, differentiation, metabolism, and morphogenesis [1]. Different phytohormones genetically modified rapes are highly interconnected in a complex dynamic network and a change in one component of the hormonal system may affect other parts [2]. Additionally, synthetic plant growth regulators were most generously used in rape production, and they may make possibility to decrease rapeseed quality. Phytohormones are typically active in minor concentration in rape tissues, the determination of plant hormone concentration is normally considered very difficult [3]. Therefore, it is very necessary to develop an accurate analysis method for phytohormones in rape tissue. In recent study, LC-MS/MS has been used to simultaneously determine different classes of phytohormones for its excellent selectivity and sensitivity [4]. In this experiment, we developed a LC-ESI-MS/MS method to simultaneous analysis seven phytohormones in rape tissues, including indole-3-acetic acid (IAA), α-

naphthaleneacetic acid (α-NAA), gibberellic acid (GA_3), abscisic acid (ABA), 2,4-dichlorophenoxyacetic acid (2,4-D), paclobutrazol (PA) and 2,4-epibassinolide (2,4-EP).

2. Materials and methods

2.1 Materials and sample preparation

Fresh rape plants in pod bearing period were obtained from experimental field of Oil Crops Research Institute Chinese Academy of Agricultural Science. Roots, stems, leaves, flowers and the immature pods were collected respectively. The materials were cold-freezing dried at Refrigerant charge (Thermo savant, England). The rape seeds were got from a local market.

2.2 Extraction and purification of plant tissues

Ultrasound-assisted extraction was used in the experiment. Powder of rape tissues were weighed and placed in 50-mL centrifuge tube along with 80% methanol in water containing 0.1% formic acid, vortexed, ultrasound-assisted extraction for 15 min at room temperature. The supernatants were filtered, the filtrate was evaporated using rotatory evaporator at 30°C. Dispersive solid-phase extraction was used as purification method [5]. 2 mL supernatant was removed into 5-mL centrifuge tube with 300 mg Magnesium sulfate and 100 mg Primary secondary amine (PSA) (Agilent Technologies, USA) which was one kind of bulk sorbent and centrifuged at 4500 rpm, at room temperature, 10 min. Then the supernatant was filtered using a 0.45 μm PTFE filter (Waters, Milford, MA, USA). Then the filtered fluid was transferred into sample vial and send to the chromatography system.

3. Results and discussion

3.1 Development of LC-MS/MS method

A liquid chromatography-tandem mass spectrometry (Thermo scientific Triple Stapes Quadrupole Quantum Ultra EMR) coupling with an electrospray ionization (ESI) interface was used. HPLC was performed by gradient using a binary solvent system comprising methanol with 0.1% formic acid, water with 0.1% formic acid, the gradient conditions were showed in Table 1. Solvent were mixed and delivered to the analytical column (C18 ODS-3, 5μm, 150 mm × 2.1 mm I.D., Inertsil, Japan) at flow rate of 0.20mL/min.

Table 1. Chromatographic gradient conditions for the analysis of phytohormones

Time(min)	Methanol with 0.1% formic acid (%)	Water with 0.1% formic acid (%)
0	30	70
3	30	70
10	70	30
15	70	30
20	95	5
25	95	5
28	30	70
33	30	70

The selected reaction monitoring (SRM) mode was used to monitor the precursor-to-product ion transitions. IAA, α-NAA, GA_3, ABA and 2, 4-D were analyzed in negative scan mode, and the precursor ions were selected as $[M-H]^-$ ions; while PA and 2, 4-EP were analyzed in positive scan mode, and the precursor ions were selected as $[M+H]^+$ ions. The collision energy of each phytohormone was confirmed through Compound Optimization. All data were showed in Table 2.

Table 2. Optimized MS/MS conditions, Precision, calibration curves and other quantitative data for thirteen phytohormones

Analytes	Retention times (min)	Precision (RSD, %)		Equation of calibration curve[a]	Correlation coefficient (R^2)	Limit of Detection (μg mL^{-1})	Linear range (μg mL^{-1})
		Retention time	Peak areas				
IAA	11.59	0.36	3.02	Y=-11278.3+5.15982X	0.9996	0.01	0.05-80
α-NAA	14.83	0.69	2.21	Y=-3891.15+12.1394X	0.9999	0.01	0.25-120
GA_3	10.14	0.58	3.55	Y=-4592.38+13.6715X	0.9999	0.01	0.05-20
ABA	12.54	0.37	2.68	Y=-3096.65+6.41597X	0.9997	0.01	0.025-20
2,4-D	18.40	0.61	1.57	Y=-3713.93+19.3779X	1.0000	0.01	0.05-40
PA	18.26	0.90	2.43	Y=-46840.4+4957.08X	0.9999	0.001	0.0025-20
2,4-EP	21.59	0.42	3.51	Y=-6206.94+232.401X	1.0000	0.02	0.1-40

3.2 Quality parameters of the LC-MS/MS method

Under the optimized LC-MS/MS conditions described above, a group of five standards with different concentrations were determined. The peak area of the diagnostic product ion under optimized conditions was used for quantification. According to the relationship between the peak area and concentration, a curve was built. The dose-response curves were liner in the concentration ranges selected for various compounds (R^2 values of 0.9996-1.0000). The detection limit for each plant hormone was defined by the ratio of signal/background noise (S/N) 3.The results were showed in Table 2.

The precision of machine was expressed by relative standard deviation (RSD). The RSDs of the retention time and peak areas for all analytes were in the range of 0.36-0.90%, 1.57-3.55%, respectively. The standards recovery of rape root, stem, leaf, flower, immature pod and seed were in the range of 90.0-99.8%, 86.7-99.7%, 82.1-99.9%, 82.3-99.9%, 87.4-99.9%, 81.5-99.2%, respectively.

3.3 Sample analysis

Under the optimum operating conditions, both the developed method and the compared method were used to determine rape tissues. The LC chromatogram of rape tissues determined by our developed method was seen in Figure 1. Each sample had triplicate analysis, and all RSDs were less than 10%.

4. Conclusion

In the experiment, an LC-ESI-MS/MS method was developed to determine seven phytohormones in rape tissues and precision of the chromatogram system and recovery of the extraction process were estimated. Ultrasound-assisted extraction was adopted and the dispersive solid-phase extraction as the purification method. The separation of the seven phytohormones, including different classes of phytohormones, was completed within 30 min.

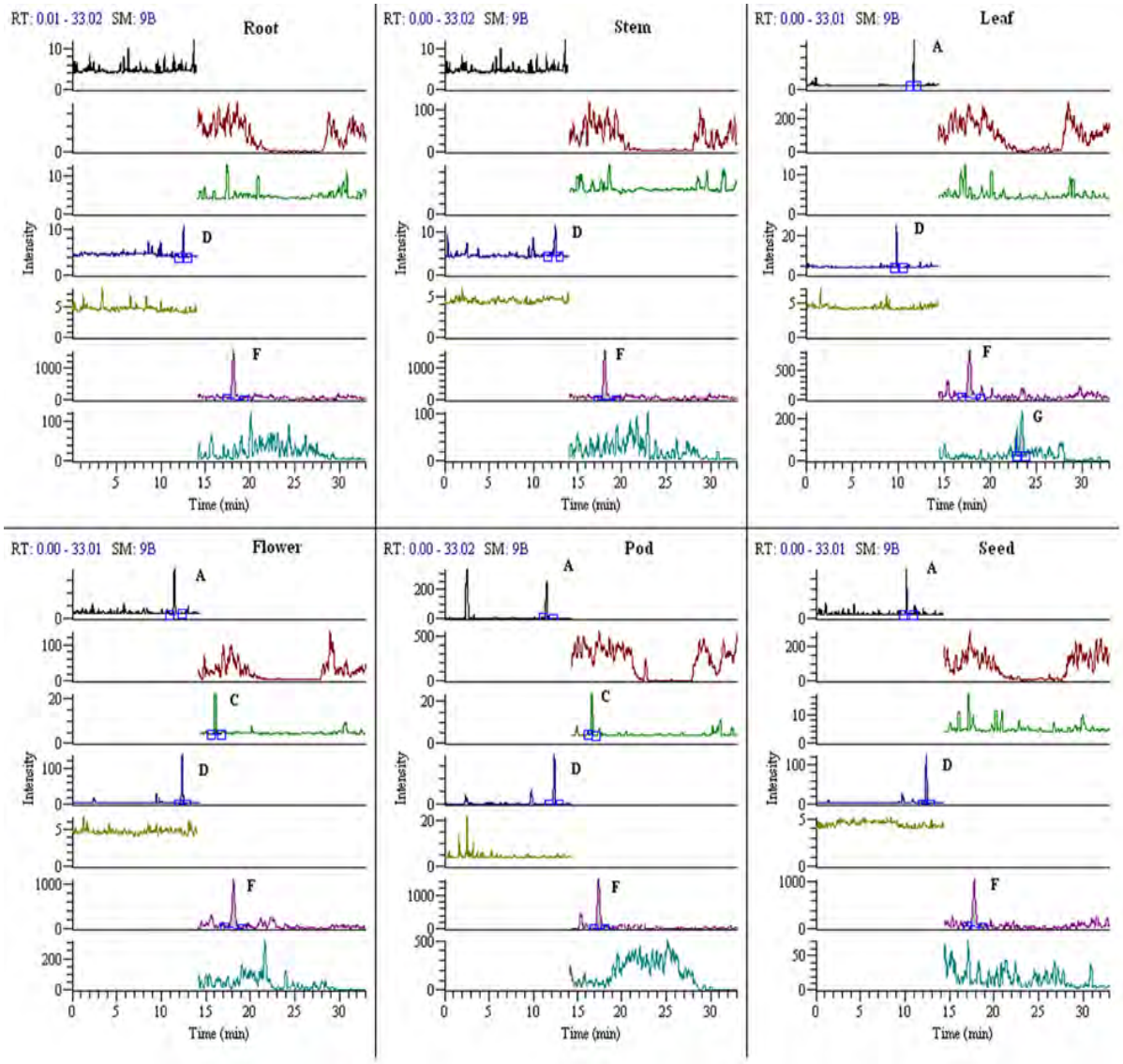

Figure 1. The LC chromatograms of rape root, stem, leaf, flower, immature pod and rape seed. A~G represent the chromatogram of IAA, α-NAA, 2, 4-D, ABA, GA_3, PA and 2, 4-EP, respectively.

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