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RESEARCH ARTICLE

# Analysis of Brassinosteroids in Soybean Seeds and Leaves by Liquid Chromatography-Tandem Mass Spectrometry

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**Abstract:**

***Objective:***

A simple and fast high performance liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) method has been developed for the analysis of brassinosteroids (BRs) in plants without derivatization.

***Materials:***

The BRs (including castasterone, 24-epicastasterone, brassinolide and 24-epibrassinolide) have been extracted with ice cold 80% aqueous methanol solution.

***Method:***

Five different purification strategies have been tested for the purification and enrichment of BRs.

***Conclusion:***

This analytical method was sensitive, reliable, rapid and applicable to trace analysis in complex plant samples.

**Keywords:** Brassinosteroids, HPLC-ESI-MS/MS, Castasterone, Brassinolide, 24-Epibrassinolide, 24-Epicastasterone.

## 1. INTRODUCTION

Brassinosteroids (BRs) are a class of naturally occurring polyhydroxylated steroid plant hormones with wide occurrence across the plant kingdom. More than 60 natural BRs have been reported in the plant kingdom since BRs were first discovered from rape pollen in the 1970s [1]. BRs play an essential role in the growth and the development of plants, including cell elongation, vascular differentiation, reproductive development and stress tolerance, *etc* [2].

The trace amounts of BRs in complex plant matrixes and their lack of chromophore for detection make it challenging for the analysis of BRs. Many different techniques have been developed for the analysis of BRs in plant tissues. Bioassays and gas chromatography-mass spectrometry were the most widely used techniques for the estimation of BRs in plant tissues in 1990s. Due to the low volatility of BRs, they must be derivatized, *i.e.* methaneboronated and/or trimethylsilylated, prior to GC/MS analysis [3]. High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) or fluorometric detectors is one of the most frequently used analytical methods for the separation and analysis of BRs which were derivatized with boronic acid such as naphthaleneboronic acid, 9-phenanthreneboronic acid, or (dansyl-3-amino) phenylboronic acid [3]. Recently, liquid chromatography-tandem mass spectrometry (LC-

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MS/MS) has been widely used for analyzing BRs due to its high sensitivity and selectivity. Gamoh *et al.* [4] developed an LC–MS method for analyzing BRs as their naphthaleneboronates using Atmospheric Pressure Chemical Ionization (APCI). Svatos *et al.* [5] reported a highly sensitive and selective LC-(ESI)-MS method for analyzing BRs as dansyl-3-amino phenylboronates in plant extracts. Huo *et al.* [6] and Tarkowska *et al.* [7] reported an ultra HPLC-ESI-MS/MS method for the analysis of BRs as 2-bromopyridine-5-boronate in plants, and in minute samples of plant tissue respectively. A relatively fast and highly sensitive method reported by Wang *et al.* [8] employed on-line solid phase microextraction with polymer monolith coupled to liquid chromatography-spectrometry (SPME-LC-MS), and Wang *et al.* [9] quantified small quantities of endogenous BRs using MSPD-MAX-MCX-HPLC-MS/MS. Due to the extremely low concentration of BRs and complicated matrix, the purification and enrichment of endogenous BRs from plant tissues are required prior to derivatization with boronic acid followed by instrumental analysis. Liquid-liquid extraction [10], solid phase extraction [6, 11], Magnetic Solid Phase Extraction (MSPE) [12], Solid Phase Microextraction (SPME) [13] and HPLC purification or combinations thereof were extensively employed in BR pre-treatment [14]. In the current study, a fast, simple and direct LC-ESI-MS/MS method, without derivatization, has been developed for the analysis of BRs in plants including: castasterone, 24-epicastasterone, brassinolide and 24-epibrassinolide.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Materials

Brassinosteroid standards, including castasterone, 24-epicastasterone, brassinolide and 24-epibrassinolide (Fig. **1**) were purchased from Chemiclones Inc. (Waterloo, Ontario, Canada) with a purity of 95%. The stock solutions of BRs were prepared at a concentration of 100 mg/L in methanol and stored at -18 ºC in the dark. Standards of lower concentrations were prepared weekly by the serial dilution of the stock solution with methanol and were stored at 4 ºC in the dark.

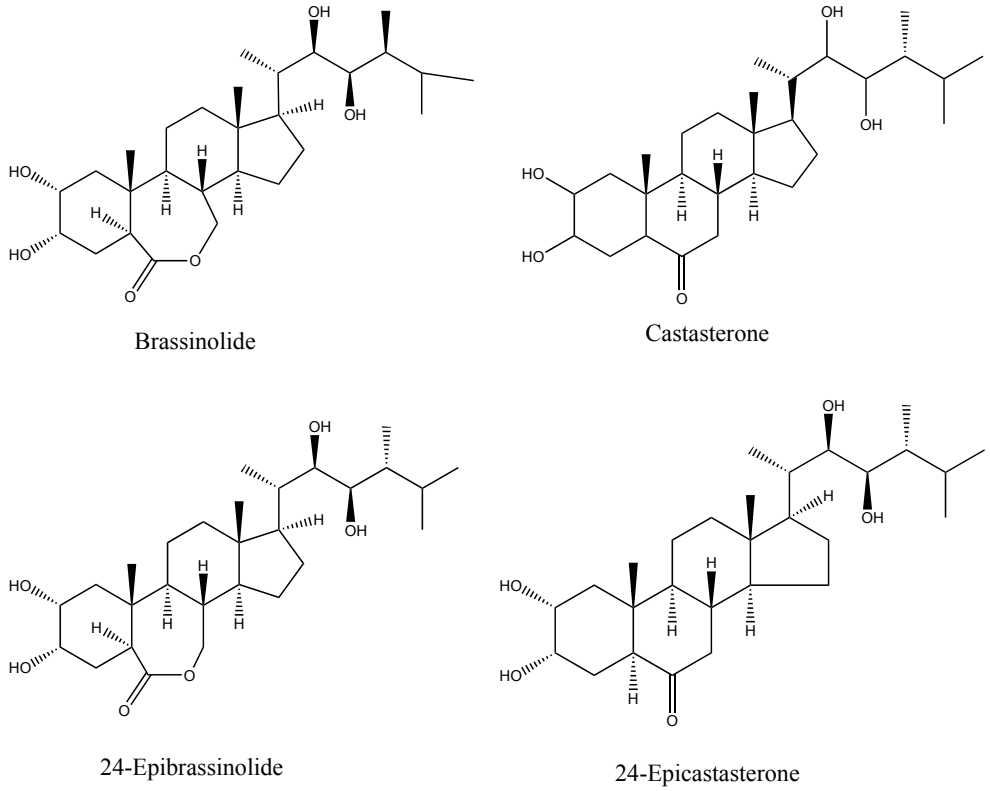


**Fig. (1).** The structures of BRs studied in the present work.

High performance liquid chromatography (HPLC grade or equivalent) methanol, acetone, acetonitrile, ACS reagent grade dichloromethane (DCM), hydrochloride acid (37%), sulphuric acid (96%), formic acid (88%), and ammonium hydroxide (30%) were purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Reversed-phase C18 SPE cartridges (C18, 6cc, 150 mg. Canadian Life Sciences, Peterborough, Ontario, Canada).

The soybean plants were grown in a green house where they were exposed to continuous white light at 20 ºC. The young leaves were harvested around 1 month old (~9 cm tall). The developing and relatively mature seeds were harvested at various time periods. The soybean leaves and seeds were harvested, immediately frozen in liquid nitrogen, and stored at -80 ºC until use.

### 2.2. Stock and Working Solution Preparation

Stock solutions of the standards were prepared individually in methanol and stored in amber vials at -20 ºC. The working solution of the four hormones contained 1.0 µg/mL of each plant hormone. Calibration solutions were prepared by combining the working solutions of castasterone, 24-epicastasterone, brassinolide and 24-epibrassinolide and diluting the mixture with methanol. The calibration solutions were prepared at concentrations ranging from 2 to 500 ng/mL for each BR. Calibration curves were constructed by plotting the obtained peak areas of analytes *versus* their concentrations.

Instrumental LOD and LOQ were determined for each standard from a chromatogram of standard solutions based on signal to noise (S/N) ratios of 3:1 and 10:1, respectively.

### 2.3. Extraction and Purification of Brassinosteroids from Soybean Plant Tissues

The extraction and purification of BRs from soybean plants are based on previously developed methods [2, 11] with some modifications. The fresh soybean leaves or seeds (0.5-1g) were homogenized using a ball mill grinder (30 Hz, Retsch MM300) and zirconium oxide grinding beads with 2-3mL 80% ice-cold methanol for 5 min at 4 °C. The homogenized samples were transferred from the ball mill cylinder to glass tubes, and the cylinder was rinsed with 1.0 mL of 80% ice-cold methanol three times. The combined extracts were sonicated for 10 min, then centrifuged (Thermo Scientific; Model Sorvall ST16, Ottawa, Canada) for 10 min at 5000 rpm. The supernatants were transferred to a clean glass tube. The residue was extracted with 3 mL of ice-cold methanol and sonicated two more times.

Five methods were tested for the purification of BRs from the methanol extracts.

#### *2.3.1. Method 1: Purification with DCM*

The combined methanol extracts were concentrated to aqueous phase in a speed vacuum concentrator (Savant SPD111V, UVS400, Thermo Fisher Scientific, Waltham, MA) at 30 °C, and subsequently partitioned three times with 3 mL of DCM. The combined DCM-soluble extracts were evaporated to dryness under vacuum at 30 °C. The samples were reconstituted in 0.4 mL of 80% methanol and stored at -20 °C before LC-MS/MS analysis.

#### *2.3.2. Method 2: Purification with DCM Followed by C18 Solid-Phase Extraction*

The combined methanol extracts were partitioned with DCM as previously mentioned in Method 1. The DCM extracts were evaporated to dryness under vacuum and the residue was reconstituted with 10 mL HPLC water followed by purification with C18 Solid-Phase Extraction (SPE) columns. The aqueous samples were loaded on the SPE cartridge at a speed of 1.0 mL/min, and the cartridge was washed with 10 mL of 40 mM ammonium acetate (pH 6.5). Brassinosteroids retained on the cartridge were eluted with 3×3 mL 100% methanol (HPLC grade), and the eluates were dried under vacuum and stored at -20 °C until analysis. Before use, the C18 cartridge was activated and equilibrated with 6 mL of 80% methanol, 6 mL of water and 6 mL of 40 mM ammonium acetate (pH 6.5) in series.

#### *2.3.3. Method 3: Purification with Ethyl Acetate*

The methanol extracts were partitioned with ethyl acetate instead of DCM as described in Method 1.

#### *2.3.4. Method 4: Purification with Ethyl Acetate Followed by C18-SPE*

The combined methanol extracts were partitioned with ethyl acetate as previously stated in Method 1. The ethyl acetate extracts were evaporated to dryness under vacuum and the residue was reconstituted using 10 mL HPLC water followed by C18 Solid-Phase Extraction (SPE) as per Method 2.

#### *2.3.5. Method 5: Hexane Wash Followed by Ethyl Acetate Partitioning*

The methanol extracts (80% methanol) were partitioned with 2 mL of hexane to remove the fat fraction. The hexane

fraction was washed with 2 mL of 80% methanol two more times. The combined 80% methanol extracts were concentrated to aqueous phase under vacuum at 30 °C, and then partitioned three times with 3 mL of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness under vacuum at 30 °C. The samples were reconstituted in 0.4 mL of 80% methanol and stored at -20 °C until use.

### 2.4. Linearity and Recovery Test

The recovery tests were conducted to evaluate the recovery during extraction and each purification procedure. The least rigorous method with the highest recovery was chosen for the sample preparation. The mature soybean seeds were used for recovery test due to low or negligible BRs normally found in mature seeds. A volume of 100 µL of 500 ng/mL mixture of brassinolides and castasterones was added to the methanol extracts of the mature seeds before sonication. The mature seeds, without addition of BRs standards, were extracted at the same time to determine the naturally existing BRs, using the aforementioned processes. The concentration of BRs in mature seeds was subtracted from that of the spiked seeds, and recovery of the whole method was calculated according to the linear curve generated from the standards without matrix.

### 2.5. Instrumental Analysis

The compounds were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Shimadzu HPLC coupled to an API 3000 tandem mass spectrometer (ABSciex, Concord, ON, Canada) equipped with an electrospray (ESI) source.

The separation of samples was achieved on a Genesis C18 column (150 × 2.1 mm i.d., 4 µm particle size; Chromatographic Specialties, Brockville, ON, Canada) coupled with a guard column with the same packing material (4 mm × 2.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase was the following binary gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) at a flow rate of 0.24 ml/min: 0 min, 20% B; 0-1.5 min, 65% B; 1.5-3 min, 90% B; held constant at 90% B for 5 min, then back to the initial 20% B within 0.5 min. The column was equilibrated to initial conditions for 5 min. The injection volume was 25 µL.

The mass spectrometer was operated in positive ionization mode. Data was acquired by the Multiple Reaction monitoring (MRM) mode. The optimized MRM mass spectrometric parameters for all standards are summarized in Table **1**. To obtain the optimal ESI source conditions, a solution containing four BRs at 1.0 µg/mL was employed and injected into the MS system using a syringe pump at 10 µL/min. The optimized source operating parameters were as follows: Curtain gas (CUR), 12 psi; Nebulizer gas (NEB), 15 psi; Ion Spray voltage (IS), 5000 V; Temperature, 400 ºC. Analyst (ver. 1.5) software (AB Sciex, Concord, ON, Canada) was used to control instruments and acquire and process data.

## 3. RESULTS AND DISCUSSION

### 3.1. Optimization of Mass Spectrometry Conditions for BR Ionization

The mass spectrometric parameters were all optimized including: ion spray voltage, source temperature, curtain gas and nebulizer gas flow (see details in Section 2.5). The MRM parameters of four BRs are shown in Table **1**.

According to the electrospray positive-ion full scan spectra (Figs. **2**, **3**), the peak at m/z 465.5 corresponds to the $[M+H]^+$ of castasterone and 24-epicastasterone, and m/z 487.5 and m/z 503.5 corresponds to their sodiated and potassiated adduct ions, respectively. The peaks at m/z 481.5, 503.5 and 519.5 correspond to $[M + H]^+$, $[M + Na]^+$, and $[M+K]^+$, respectively for brassinolide/2,4-epibrassinolide. The competition between protonation and sodium/ potassium–adduct formation is very common during the electrospray positive ionization [2]. The protonated ions were chosen as the precursor ions for product scan of the target brassinosteroids. The presence of formic acid in the standard solution increases the protonation and suppresses the sodium- and potassium –adduct formation. When optimizing the compound dependent parameters using direct infusion with syringe pump, one drop of concentrated formic acid (80%) was added in the individual standard solution (1µg/mL). The successive losses of $H_2O$ molecules from $[M+H]^+$ of castasterone/24-epicastasterone give rise to the ions of m/z 447.3 $[M+H-H_2O]^+$, m/z 429.3$[M+H-2H_2O]^+$, and m/z 411.4 $[M+H-3H_2O]^+$ . The successive losses of $H_2O$ molecules from $[M+H]^+$ of brassinolide/2,4-epibrassinolide give rise to the ions of: m/z 463.4 $[M+H-H_2O]^+$, m/z 445.3$[M+H-2H_2O]^+$, and m/z 427.3 $[M + H - 3H_2O]^+$ . The fragment ion, $[M+H-2H_2O]^+$ was the most abundant of the product ions for branssinosteroids (Figs. **4**, **5**). Of the investigated

compounds, their isomers are found to exhibit nearly the same fragmentation patterns and this is consistent with reported studies [6].

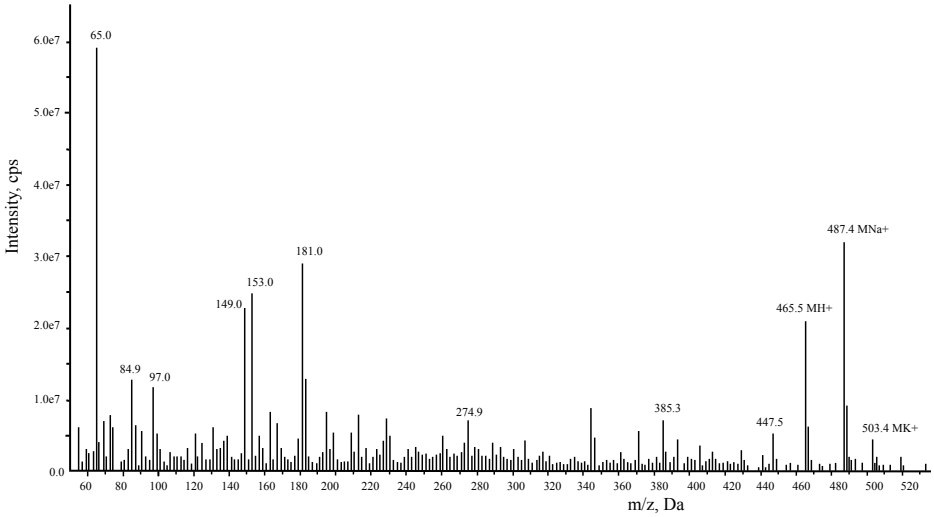


**Fig. (2).** The full scan mass spectrum of brassinolide.

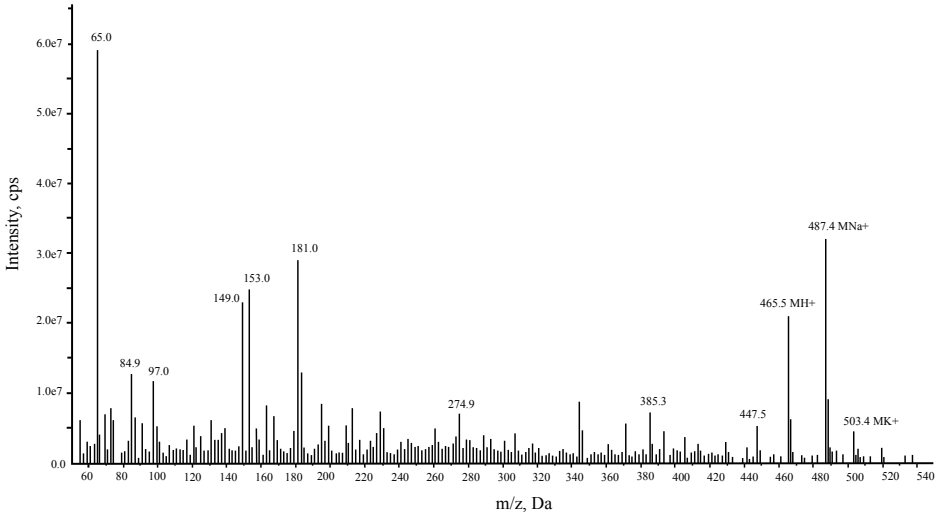


**Fig. (3).** The full scan mass spectrum of castasterone.

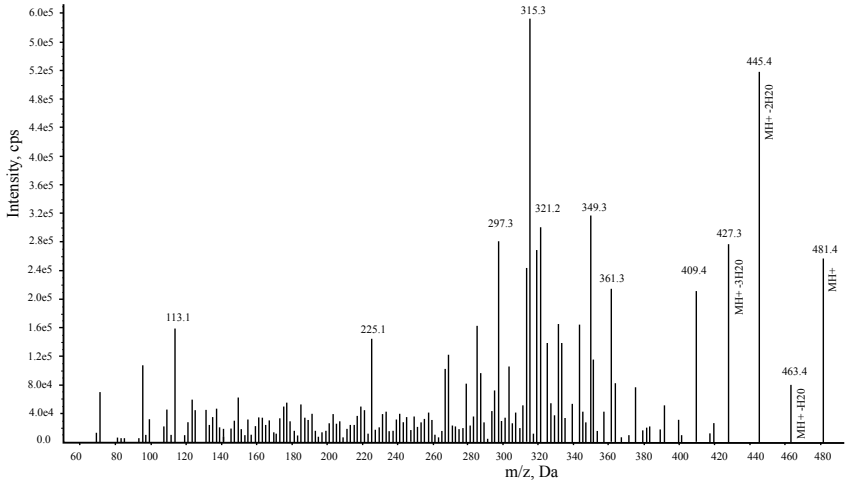


**Fig. (4).** The MS/MS spectra of the $[M + H]^+$ ion of brassinolide.

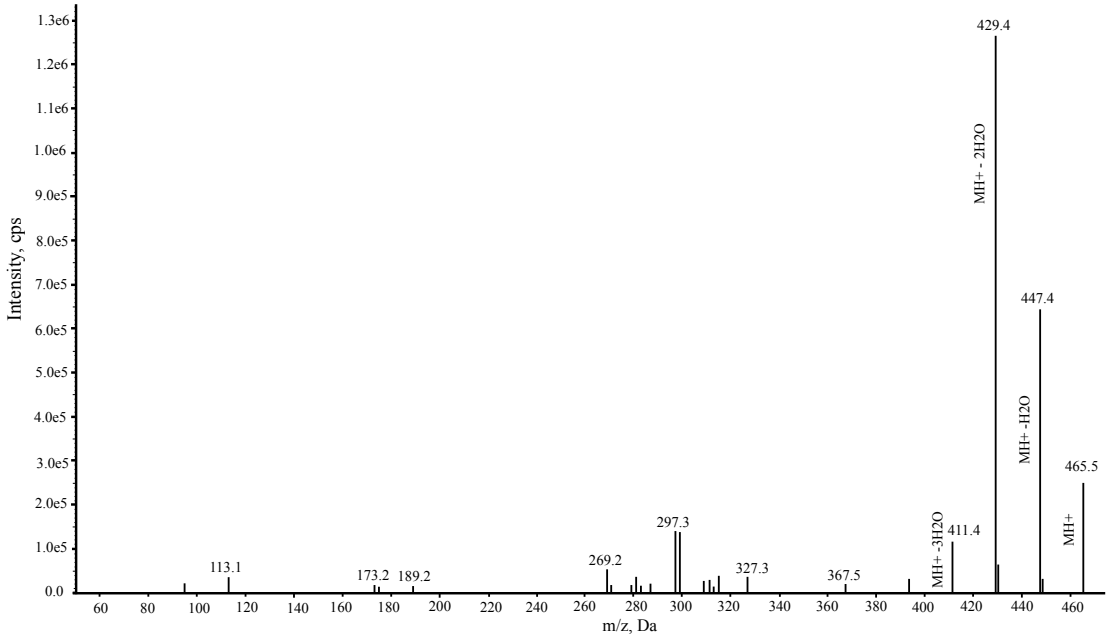


**Fig. (5).** The MS/MS spectra of the $[M + H]^+$ ion of castasterone.

The protonated molecule was chosen as the precursor ion and the most intensive product ion was selected for the quantification. The selected quantification ion plus another specific product ion $[M + H - H_2O]^+$ were chosen for the confirmation. MRM mass spectrometric parameters of the target BRs were summarized in Table **1**.

**Table 1. Summary of tandem mass spectrometry parameters used for multiple reaction monitoring (MRM) for target compounds analyzed by LC-ESI-MS/MS. DP: declustering potential; FP: focusing potential; EP: entrance potential; CEP: collision cell entrance potential; CE : collision energy; CXP : collision exit potential.**

| Analyte | Q1 | Q3 | Dwell (ms) | DP | FP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|---|
| Brassinolides (quantification) | 481.4 | 445.5 | 100 | 40 | 185 | 10 | 14 | 15 |
| Brassinolides (confirmation) | 481.4 | 463.4 | 100 | 40 | 185 | 10 | 14 | 15 |
| Castasterones (quantification) | 465.5 | 429.3 | 100 | 46 | 194 | 9 | 23 | 14 |
| Castasterones (confirmation) | 465.5 | 447.4 | 100 | 46 | 194 | 9 | 23 | 14 |

### 3.2. Optimization of Chromatographic Separation

It has been reported that the addition of organic acid to the mobile phase greatly improves the peak sharpness and peak symmetry of acid plant hormones. Different organic solvents (methanol and acetonitrile) and additives (formic acid and acetic acid at various concentrations) were tested as mobile phases to separate the BRs. Methanol was chosen as organic mobile phase because of the better separation efficiency and higher intensities for target BRs. Formic acid provided more sensitive results than acetic acid under the ESI positive mode. A better signal response was obtained when 0.1% formic acid was added into both mobile phases (water and methanol). Gradient elution was chosen for better chromatographic resolution and higher response of ESI-MS/MS.

### 3.3. Recoveries from Different Cleanup Procedure

Extraction and purification of BRs from soybean seeds with different methods were investigated as described in 2.3. From Table **2**, the C18 cleanup did not appear to contribute a great deal in terms of sample purity and retention. Furthermore, such an SPE procedure is very time-consuming and labor intensive. The solvent partitioning with DCM is more efficient than solvent partitioning with ethyl acetate. The wash step with hexane to remove fat/lipids is not efficient for the recovery of BRs which may have been caused by the BRs transfer to hexane rather than remaining in the aqueous methanol solution. The recoveries of brassinolides are better than those of castasterones. The partition with DCM is the best cleanup method for all the BRs studied. If labeled isotopic isomers are applied to the method, better recovery efficiency should be achieved. Furthermore, this study mainly focused on LC-MS/MS instrumentation.

Therefore, the cleanup procedures presented were designed to be simple and fast while taking advantage of the high sensitivity and selectivity of LC-MS/MS. As a result of this simple approach, it was determined that BRs solubilized in organic solvents without labour-intensive sample cleanup could still be identified by LC-MS/MS. Future studies could explore other methods to increase recovery and reduce the complexity of the sample matrix.

**Table 2. Recoveries of BRs spiked in the plant at 25 ng/g (n=3).**

| Purification Method | Castasterone | Brassinolide |
|---|---|---|
| DCM | 59.2 ± 4.1 | 80.4 ± 10.3 |
| DCM+C18 | 42.9 ± 9.4 | 60.4 ± 3.6 |
| ethyl acetate | 25.4 ± 2.0 | 58.8 ± 5.1 |
| ethyl acetate+C18 | 9.7 ± 2.7 | 43.3 ± 4.3 |
| Hex+ethyl | 8.8 ± 4.1 | 32.6 ± 2.5 |

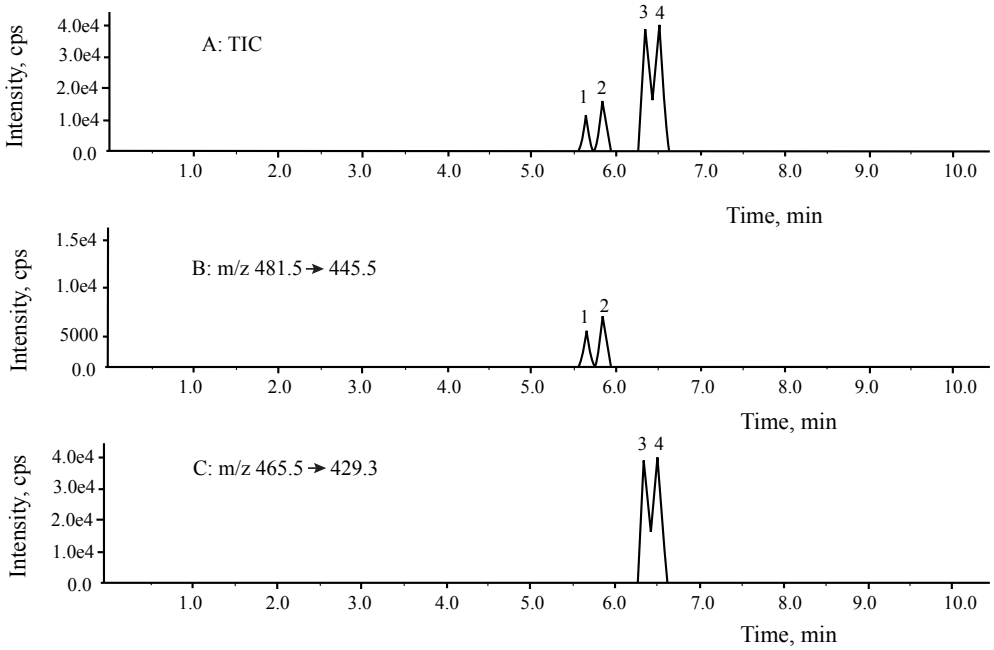


**Fig. (6).** Total ion chromatogram (TIC) and reconstructed ion chromatogram from BR standards.

### 3.4. LC-MS/MS Analysis of BRs

Brassinolide/24-epi- brassinolide, and castasterone/24-epi-castasterone are two pairs of diastereomers. It is very challenging to separate diastereomers using a regular C18 column. Usually chiral column is the method of choice for the separation of diastereomers. However, the chiral column is very expensive and the maintenance of the column is complicated. Considering both resolution and sensitivity, the combination of HPLC with tandem MS may potentially differentiate two pair of diastereomers. Fig. (**6**) shows the MRM chromatogram of the four BRs; The peaks at 5.63 min, 5.88 min, 6.38 min and 6.63 min correspond to 24-epi- brassinolide, brassinolide, 24-epi-castasterone and castasterone, respectively. Quantification was performed using the external standard method. A series of standard mixtures of BRs (2, 10, 50, 100, 200, 500 ng/mL) were prepared for the method validation. The calibration curve was constructed by plotting the peak area of analytes versus the concentration of the analyte. Good linearity for these BRs was obtained in the range of 2.0-500 ng/mL with correlation coefficients ($R^2$) of 0.95-0.99 Table **3**. Limits of Detection (LODs) of the proposed method were determined as the analyte concentrations corresponding to signal-to-noise ratio (S/N) of 3 from BRs spiked in soybeans extract matrixes. In this respect, the LODs of target BRs are 0.2 ng/g for brassinolide and 24-epibrassinolide, and 0.02 ng/g for castasterone and epi- castasterone. The reproducibility and accuracy of this method were evaluated with extractions of five samples over a day. The relative standard deviations (RSDs) of 5 samples were below 20%, indicating good reproducibility and accuracy of the method. Considering the low concentrations of BRs present in plant tissues, it is of great importance that efforts are focused on decreasing the LOD in future studies. LOD

may be decreased by scheduled MRM to improve instrumental detection limit, using high efficiency HPLC columns with small particle sizes in the packing medium, and more efficient sample preparation procedures during extraction and cleanup. However, the current study provides preliminary results that clearly outline LC-MS/MS capabilities for the analysis of BRs.

**Table 3. The linearity and detection limit (LOD) of LC-MS/MS method for the determination of BRs in plants.**

| Compound | Linear Range | Square of Correlation ($r^2$) | LOD (ng/g) |
|---|---|---|---|
| | (ng/mL) | | |
| Castasterone /epi- Castasterone | 2-500 | 0.95-0.99 | 2 |
| Brassinolides/epi-Brassinolides | 2-500 | 0.95-0.99 | 0.1 |

### 3.5. The BRs in Soybean Seeds and Leaves

The established method was applied to the determination of four endogenous BRs in young soyabean seeds and young leaves. A low level of BRs was found in seeds, and no castasterone was found in either seeds or leaves. Fig. (**7**) shows the LC-MS/MS chromatogram from the seeds of soybeans spiked with standards BRs mixture. The four target BRs are well separated from the bean matrix.

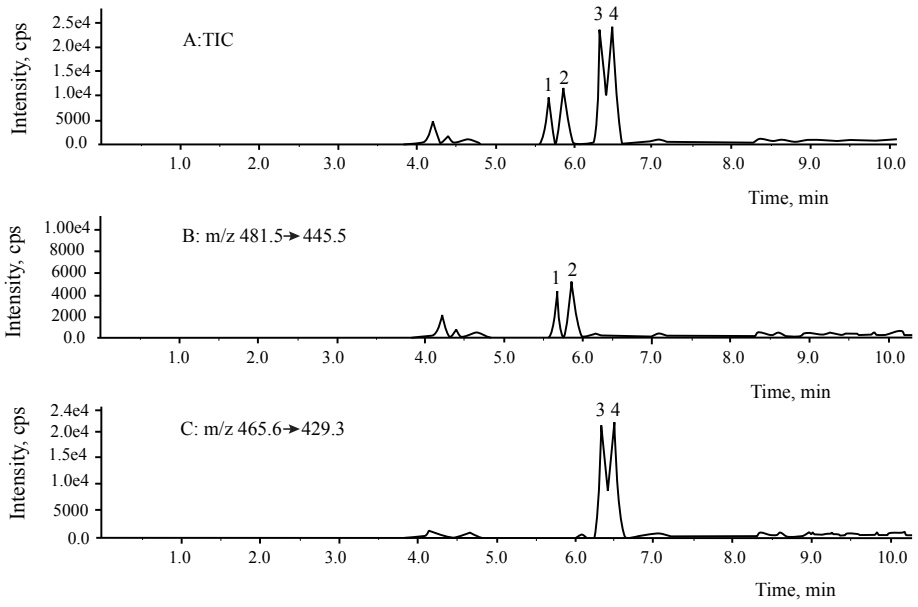


**Fig. (7).** Total ion chromatogram (TIC) and reconstructed ion chromatograms from soybeans spiked with BR standards.

## CONCLUSION

A rapid and simple HPLC-ESI-MS/MS method for the sensitive analysis of endogenous BRs in plant tissues without derivatization was developed in the present study. As compared to previously reported methods, the method proposed in this study is simple, fast and selective. In addition, the endogenous BRs can be detected in 0.5-1 g (Fresh weight) tissues. The sensitivity can be further enhanced with scheduled MRM for the acquisition and quantification using LC-MS/MS. Improved recoveries should be easily achieved if the use of stable isotope-labeled standards is applied to the method.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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