Separation and Quantitation of Jasmonic Acid Using HPTLC

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Abstract

A rapid, simple, and stringent protocol for the detection and quantitation of jasmonic acid (JA) is designed using high-performance thin-layer chromatography. Acidified culture filtrate of *Lasiodiplodia theobromae* is extracted with an equal volume of ethyl acetate and spotted on silica gel 60 F_{254} foil using Linomat-5 spray-on applicator. Standard JA is also spotted either internally or adjacent to the sample, and the foils are developed with isopropanol–ammonia–water [10:1:1 (v/v)] as the mobile phase. A quantitative estimation of the separated JA is performed by measuring the absorbance at 295 nm in the reflective mode. The sensitivity of the method is improved by adding internal standard to obtain a detection limit of 1 µg. The limit of quantitation is found to be 80 µg with this method. The method is shown to have selectivity, accuracy, precision, and high sample throughput, making it useful for the routine analysis of JA in basic science and perfumery industries.

Introduction

Jasmonic acid (JA) and other similar compounds (referred to as jasmonates) are potent regulatory molecules found in plants (1) and some fungi (2). Several physiological roles have been described about these compounds during plant growth and in response to biotic and abiotic stress. Recently, JA has also been found to play a role in signaling plant defense systems (3,4). Analysis of JA from various plant extracts has become routine laboratory procedure for the study of plant physiology, defense responses, and fruit ripening (5–8).

The potential of *Lasiodiplodia theobromae* as a source of JA is analyzed. *Lasiodiplodia theobromae* was the first Deuteromycotina class of fungus reported to produce JA (9).

A number of reports have been published on the quantitative determination of JA (10). In biological samples, jasmonates can be measured by radio immuno assay and enzyme linked immuno sorbent assay; however, more or less all antibodies have cross reactivity with related compounds (stereoisomers, precursors, and metabolites), which limits the specificity of these techniques. A majority of the past works have used gas chromatography (GC)–mass spectrometry (MS); some of them have used GC with the modification of a detector (11–13). In GC, JA has to be methylated prior to separation so that the simultaneous determination of JA and methyl jasmonates in the same sample does not occur (14); moreover, other compounds/impurities with the same retention time may interfere; also, retention time varies with the concentration of methyl jasmonate in packed columns like SE-30 and OV-1 (15). Measurement by absorbance in UV spectrophotometry has also been tried. Because of the poor UV absorption of JA, its derivatization with fluroscent hydrazides like densyl hydrazide and dimethylaminobenzohydrazide was used to develop a sensitive method for the quantitation of jasmonates. Although it gave sensitive results, the dimethylaminobenzohydrazide is very toxic, and it requires great care in handling; moreover, it takes over 12 h for the derivatization of jasmonates. Thin-layer chromatography (TLC) has been frequently used for the purification and identification of jasmonates. This is the first report of quantitation of JA using high-performance thin-layer chromatography (HPTLC). Fluorescent hydrazides of JA were separated from JA by TLC using chloroform–methanol (1:3) as the mobile phase; however, the JA did not move from the origin (16). Because TLC was proven to separate JA from its derivatives, HPTLC can also be employed for the same purpose.

This paper describes an HPTLC procedure for the rapid, simple, and accurate separation as well as the quantitative determination of JA.

Experimental

Materials

Standard JA and solvents

JA (±) was purchased from Sigma (Banglore, India). Various organic solvents were procured from Qualigens (India). Ammonia was used fresh (25% w/v).

Instrumentation

The HPTLC instrument used in this study was purchased from Camag (Muttenz, Germany), and it consisted of two parts:

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an automatic applicator, Linomate 5, for accurate loading of samples onto TLC foils; and a Scanner 3, for the scanning of HPTLC foils after running in the appropriate solvent system.

Fungal culture

An 8-mm mycelial mat was cut with a sterile cork borer from two-day-old *L. theobromae* (MTCC 3068), grown on a potato dextrose agar plate (Hi-media, Mumbai, India) and transferred into a 250-mL Erlenmeyer flask containing 100 mL of basal salt broth (18) (Composition (g/L): sucrose 50, $NaNO_3$ 7.5, KH_2PO_4 2.0, KCl 0.3, $MgSO_4.7H_2O$ 0.6, $FeSO_4.7H_2O$ 0.6, $ZnSO_4.7H_2O$ 0.03, $MnSO_4.2H_2O$ 0.003, $CuSO_4.7H_2O$ 0.003, Na_2MoO_4 $2H_2O$ 0.003 Yeast Extract 2.0). Flasks were incubated for 7 days at 27°C ± 1°C in static submerged condition and broth was filtered using muslin cloth.

Standard solution

JA (±) (100 mg) was dissolved in methanol, and the volume was made to 5 mL with methanol in a volumetric flask.

Extraction of JA (sample)

One hundred milliliters of 7-day-old fungal cultural filtrate (FCF) was acidified to pH 3.0 with 6 N HCl, and extracted with an equal volume of ethyl acetate. The organic phase was separated using a separating funnel, evaporated to dryness under a flow of N_2, and dissolved in 1 mL of ethyl acetate.

TLC

Pre-conditioning of TLC foils

Aluminum-backed silica gel 60 F_{254} TLC foils (20 × 10 cm) of 0.25-mm thickness (Merck, Darmstadt, Germany) were run with methanol as the mobile phase and then dried in an oven at 120°C for 20 min before sample loading.

Sample loading

Sample loading onto TLC foils was performed with a Linomat 5 applicator (CAMAG, Muttenz, Germany) using a 100-µL syringe. The operational settings were: band length, 6 mm; application rate, 150 nL/s; table speed, 10 mm/s; distance between bands was set automatically, distance from edge of plate to origin on the *X*-axis was 15 mm and on the *Y*-axis 10 mm.

Mobile phase

Five different mobile phases were compared for the effective separation of JA from the FCF: isopropanol–ammonia–water (I–A–W) [10:1:1 (v/v)]; benzene–ethyl acetate [1:1 (v/v)]; chloroform–ethyl acetate [1:1 (v/v)]; chloroform–methanol [9:1 (v/v)]; and diethyl ether. Ten or 20 mL of the mobile phase was used to pre-saturate the 10 × 10 cm or 20 × 10 cm twin trough chamber (Camag), depending upon the size of the TLC foil, for 30 min before running TLC. Twenty, 40, 60, 80, 100, 120, 160, and 200 µg of standard JA was loaded onto a 10 × 10 cm TLC foil as single loading, and a 20 µL sample was loaded on a separate foil. Development time was 90 min for I–A–W and approximately 15 min for the other four mobile phases. After running, the foils were dried by nitrogen flow in an oven at 50°C. The JA band was detected in comparison to standard JA in various solvent systems. The separated band of JA was subjected to spectral scanning (200 to 700 nm) to find out its absorption maxima.

Densitometric evaluation of chromatogram

After development, the plate was air dried for 10 min, and the peak area of the JA band in the sample and standard were quantitated by linear scanning at 295 nm using the Camag TLC Scanner 3 with deuterium source at a scanning rate of 20 mm/s. The slit dimension setting was 6-mm length × 0.45-mm width, and data resolution was 100 µm per step using filter factor Savitsky-Golay 7. A calibration plot of standard JA was produced using the winCATS software version 1.2.2 and subjected to linear regression. The concentration of JA in samples was automatically calculated from the calibration curve by comparing the peak area of standard JA and the peak area of JA in the sample. The recovery (%) was calculated by comparing the mean concentration of JA in the replicate sample with the theoretical (known) concentration.

LOD and LOQ determination

Based on the separation pattern and the number of compounds separated, I–A–W [10:1:1 (v/v)] was used to analyze the sensitivity of the technique by measuring the limit of detection (LOD) and limit of quantitation (LOQ). Ten, 20, 40, 60, 80, 100, 500, and 1000 ng and 2, 6, 10, 20, 40, 60, 80 and 90 µg of JA were loaded in duplicate on two different 20 × 10 cm TLC foils. The LOD and LOQ were determined using polynomial regression.

Accuracy of method

To determine the accuracy of the method, 20, 40, 60, and 80 µg of standard JA were loaded in triplicate along with a control sample (standard JA) onto 20 × 10 cm TLC foil, and the concentration of JA separated on foil was measured by scanner 3 as described later. The percentage recovery of JA was the average JA measured in four independent experiments, considering the loaded (expected) µg of JA as 100%.

Calibration of the method and determination of JA from FCF of L. theobromae

For calibration of the method, 5 and 10 µL of extracts from 7-day-old FCF of *L. theobromae* were loaded in triplicate adjacent to 6, 10, 20, 40, 60, and 90 µg of standard JA in duplicate on 20 × 10 cm TLC foils. To estimate the JA in samples containing less than 6 µg (LOD), 70, 140, 280, and 420 ng were loaded, as well as 1.36 and 5.44 µg of JA obtained from FCF of *L. theobromae* and with 10 µg of JA as an internal standard. The JA in samples was calculated by subtracting the internal standard amount. The percentage recovery of total JA was measured in triplicate.

Results and Discussion

Selection of mobile phase

Five mobile phases were selected based on earlier reported

work on JA separation and empirical observation in our lab (15). Out of five different mobile phases used, I–A–W [10: 1: 1 (v/v)] was found to be the best mobile phase for the separation of JA because in this mobile phase, the maximum number of bands were obtained and the separation of JA was achieved in the middle of the plate (R_f 0.63) with a run time of 90 min. The separated band of JA in sample and standard was sharp up to 20 µg of JA; however, as the concentration of JA was increased, diffusion of the band was observed (Figure 1). The standard deviation of peak area in this mobile phase was less and the regression coefficient was the highest (Table I). Selective baseline separation of standard JA and JA in the sample using I–A–W [10:1:1 (v/v)] mobile phase is shown in Figures 2 and 3, respectively.

Linearity and range

A wide range of calibration was initially investigated by spotting 20–200 µg of JA. Using linear regression analysis, linearity was obtained in the range of 20 to 80 µg of JA. The R_f value of JA, linear detection range, regression, and relative standard deviation (RSD) obtained using various mobile phases are listed in Table I. Using polynomial regression analysis, it was found that in I–A–W [10:1:1 (v/v)] mobile phase, the LOD of JA was 6 µg and the LOQ was 90 µg. Although the scanner could detect as low as 10 ng of JA, quantitation was not possible below 6 µg. By adding a known amount of internal standard to a lesser amount of JA and then subtracting the internal standard amount, the sensitivity of the technique could be improved so that correct quantitation was made possible up to 1 µg of JA.

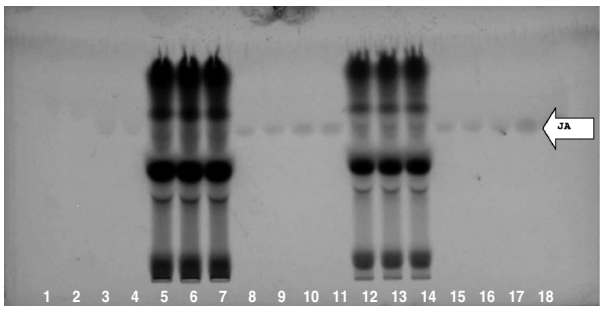

Figure 1. Sample and standard JA were loaded onto silica gel 60 F_{254} TLC foils and run in I–A–W [10:1:1 (v/v)] and the foil was exposed to UV light. Track 1 and 2 = 6 µg of JA; Track 3 and 4 = 10 µg of JA; Track 5, 6, and 7 = 10 µL sample; Track 8 and 9 = 20 µg of JA; Track 10 and 11 = 40 µg of JA; Track 12,13, and 14 = 5 µL sample; Track 15 and 16 = 60 µg of JA; Track 17 and 18 = 90 µg of JA.

Scanning of standard JA and sample

Standard JA and JA separated from the FCF of *L. theobromae* showed the same R_f value (R_f 0.63). JA (±) separated on silica gel 60 F_{254} TLC foils showed absorption maxima at 295 nm. Spectral scanning of JA separated in the sample and standard JA showed the same absorption pattern when scanned at 200–700 nm, signifying an effective separation of JA (Figure 4).

Accuracy

Twenty to 80 µg of standard JA was loaded in triplicate onto pre-coated TLC plates, run in I–A–W [10:1:1 (v/v)] mobile phase, and scanned at 295 nm using in-system calibration to prove the accuracy of the method. The average of recovery rate obtained from four different experiments is shown in Table II.

Quantitative determination of JA

JA was identified by the HPTLC scanner in the ethyl acetate extract of culture filtrate of *L. theobromae* at 0.63 R_f value based on the standard JA (Sigma). In 5 and 10 µL of samples, JA was found to be 14.157 and 30.357 µg, respectively. JA produced by *L. theobromae* on the seventh day was 30.357 mg/L of FCF. As quantitative analytical results are influenced by the quality of calibration, two aliquots of doubling volume of samples were loaded in triplicate and calibrated with six different concentrations of standard JA. It was found that as the sample volume was doubled, the JA concentration in the sample was also doubled (14.157 µg of JA in 5 µL and 30.357 µg in 10 µL of sample). For detection of nanogram quantities of JA in samples, 10 µg of JA was added as internal standard and then the absorbance was measured at 295 nm, and up to 1 µg of JA was

Table I. Separation Profile of Standard JA in Various Mobile Phases

Mobile phase	R_f value	Linear detection range (µg)	Regression equation	Regression coefficient	Standard deviation (%)
Isopropanol–ammonia–water [10:1:1 (v/v)]	0.61–0.68	20–80	$Y = 312.85 + 112.456 \times X$	0.9956	4.03
Benzene–ethyl acetate [1:1 (v/v)]	0.43–0.48	20–80	$Y = 1840.64 + 78.961 \times X$	0.9769	9.43
Chloroform–ethyl acetate [9:1 (v/v)]	0.22–0.27	20–60	$Y = 1463 + 88.617 \times X$	0.9808	23.91
Chloroform–methanol [9:1 (v/v)]	0.55–0.58	20–80	$Y = 624.40 + 724.714 \times X$	0.9926	7.03
Diethyl ether	0.70–0.76	20–60	$Y = 412.233 + 91.193 \times X$	0.9851	11.08

found in the sample. The recovery rate was near the expected value; however, the recovery rate was not found consistent by putting internal standard to ng quantities of JA (Table III). Use of the internal standard also confirmed that the separated band of JA was indeed JA.

Comparison with the other methods

A number of reasons can be cited for preferential use of HPTLC over the other methods for the quantitation of JA. Sample preparation for HPTLC analysis is simple in comparison to GC–MS or HPLC. Moreover, using GC–MS, only 10 samples per day (17) can be analyzed, whereas using HPTLC, more than 100 samples per day can be analyzed. Also, simultaneous chromatography of samples and standards under identical conditions in the same foil leads to results with excellent accuracy and precision, and solvent usage per sample basis is also very low. Some incomparable advantages of HPTLC over other methods are that once the separation is achieved, the sample can be re-extracted from the solid support, or each band can be scanned with absorbance. The same plate can also be derivatized and rescanned. Even after development of the plate, it can be stored for many days before scanning without a significant decrease in concentration.

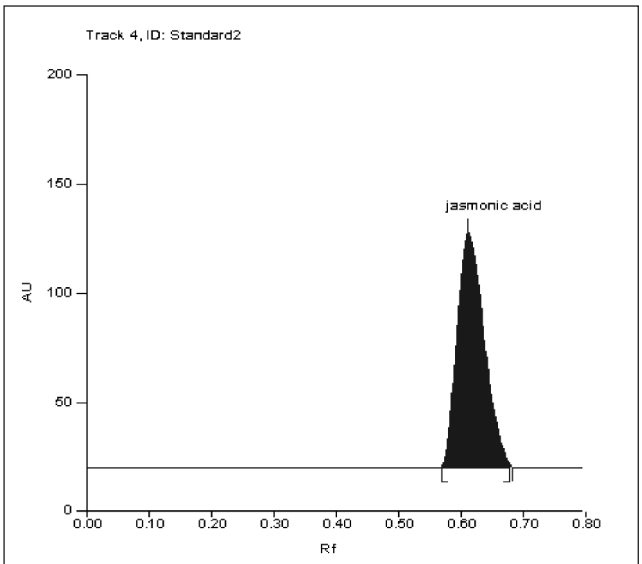

Figure 2. Densitogram of standard JA, run in I–A–W [10:1:1 (v/v)] mobile phase, obtained by scanning the TLC foil at 295 nm.

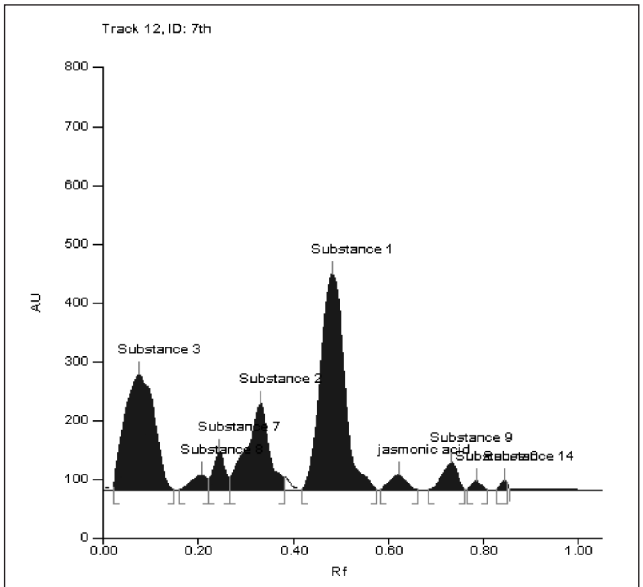

Figure 3. Densitogram of compounds separated from the sample of FCF of seven-day-old *L. theobromae* showing separation of JA. The TLC foil was run in I–A–W [10:1:1 (v/v)] mobile phase. The sample track was scanned at 295 nm.

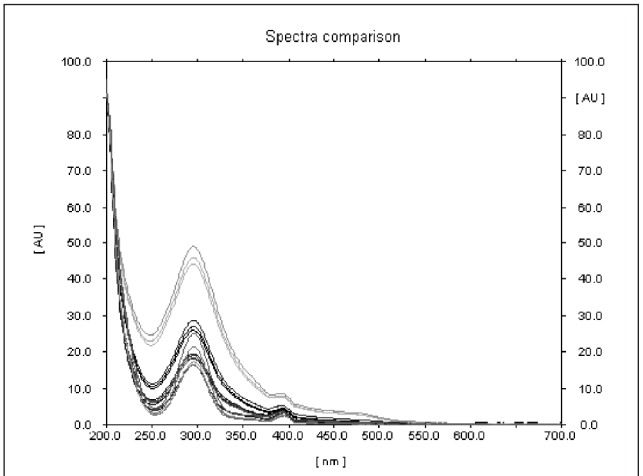

Figure 4. Standard JA and JA separated from the sample on TLC foils were scanned in the range of 200–700 nm. The spectra of JA separated from sample showed the same absorption pattern as standard JA.

Table II. Accuracy of Determination of Standard JA in I–A–W [10:1:1 (v/v)]

	1	2	3	4
Expected (µg)	20	40	60	80
Calculated* (µg)	19.07	42.96	63.73	82.94
Recovery rate	95.38	107.4	106.28	103.68

*Average from four independent experiments.

Table III. Determination of Smaller Quantity of JA by Addition of Internal Standard

	1	2	3	4	5	6
Expected (µg) (µg)	70 –	140 –	280 –	480 –	1.36 –	– 5.44
Internal standard (µg)	10	10	10	10	10	10
Calculated* (µg)	10.26	10.31	10.81	10.85	11.19	15.88
Recovery rate	101.88	101.60	105.15	101.60	98.50	102.84

* Average from three experiments.

Conclusion

This method was found to be sensitive for the quantitation of up to 1 µg of JA. The quantitation range found was 1–80 µg. The method can also be useful for measuring basal levels of JA in some plants; however, at least 10 g of the sample will be required. For measuring JA levels in fungi and induced plants, this can stand as an alternate high throughput protocol. Although there is the possibility to make this method more sensitive by derivatization of the sample, this can lead to a loss of the sample in the derivatization and recovery process. HPTLC can stand as a rapid, simple, accurate, and high throughput method for the quantitative analysis of JA in biological samples.

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