SIMULTANEOUS DETERMINATION OF ABSCISIC ACID AND JASMONIC ACID IN PLANT EXTRACTS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic assay for the simultaneous determination of abscisic acid and jasmonic acid from plant extracts is described. The method is based on the derivatization of the plant growth regulators with a fluorescent hydrazide to give stable fluorescent products. The use of fluorescent derivatives allowed detection of abscisic acid and jasmonic acid at 5 pmol and provided a linear response to 15 nmol. The method is selective and reproducible, and yields single peaks for each compound regardless of isomer. The identity of the dansyl hydrazone plant growth regulator peaks was supported by chromatography of the N-dimethyl-anthranilohydrazones of the compounds. The use of two fluorescent hydrazides makes routine confirmation of peak identity a simple matter. The presence of abscisic acid and jasmonic acid in tissue extracts was also supported by chromatographic identification of corresponding methyl and ethyl esters following esterification. Chromatographic peaks corresponding to the dansyl hydrazones of abscisic and jasmonic acid were obtained from extracts of several different tissues of soybean [*Glycine max* (L.) Merr.], snap beans (*Phaseolus vulgaris* L.), lima beans (*Phaseolus lunatus* L.), and broccoli (*Brassica oleracea* L.).

INTRODUCTION

The chromatographic determination of abscisic acid (ABA) from plant tissues has traditionally used gas chromatography coupled with electron-capture or mass spectrographic detection[1–4]. The methods give reliable identification of ABA but require extensive sample preparation. ABA can be separated by high-performance liquid chromatography (HPLC), but plant extracts contain many interfering substances that absorb at 254 nm making quantification using HPLC risky[1]. Extensive purification of tissue extracts can yield good results[5,6], but the method can be tedious[1,7,8]. The development of monoclonal antibodies to ABA has yielded the most sensitive assay to date, but the antibodies are not yet commonly available[9,10].

Jasmonic acid and methyl jasmonate have been determined routinely using gas

chromatography[11-14]. The jasmonic acid is generally methylated prior to chromato-
graphy so that determination of methyl jasmonate and jasmonic acid in the same
sample is impossible[13,14]. Liquid chromatography has not generally been used be-
cause the low UV absorbance of jasmonate makes detection difficult[14,15].

Dansyl hydrazine (1-dimethylaminonaphthalene-5-sulfonylohydrazide) has
been used to obtain fluorescent hydrazone derivatives of carbonyl compounds[16], and
these derivatives have been used for HPLC[17-19]. Because both ABA and jasmonic
acid are ketones, they should form dansyl hydrazone derivatives. In this report, a
method is presented for the production of fluorescent hydrazones of ABA and jas-
monic acid, and their separation by HPLC.

EXPERIMENTAL*

Materials

Soybean tissue *Glycine max* (L.) Merr. cv. Ransom were collected from green-
house plants when pods were at about 1/2 podfill. All other plant material was pur-
chased at local markets. Immature seeds from *Phaseolus vulgaris* L. were obtained
from fresh material, whereas seeds from *P. lunatus* L. were from frozen material.
Cauliflower immature flower head (*Brassica oleracea* L.) was from fresh material.

Reagents

All solvents were from Fisher Scientific and acetonitrile was HPLC grade.
Dansyl hydrazine and ABA were from Sigma. The DL-*cis,trans*-[G-^{3}H]abscisic acid
was from Amersham. Authentic methyl jasmonate was the generous gift of Dr.
Gunther Ohloff (Firmenich SA, Geneva, Switzerland). Jasmonic acid was prepared
by hydrolysis of methyl jasmonate[9] with subsequent purification on C_{18} Sep-Pak
(Waters Assoc.)[20]. Dihydrojasmonic acid was prepared by hydrogenating 100 mg
methyl jasmonate in methanol with 20 mg platinum oxide catalyst for 12 h at 1 atm
hydrogen followed by hydrolysis to dihydrojasmonic acid[9]. Dimethylanthranilohy-
drazide (2-dimethylaminobenzohydrazide; DMA-hydrazide) was prepared by mixing
1 g ethyl-*o*-dimethylaminobenzoate (Aldrich) with a 100-fold excess of anhydrous
hydrazine in 10 ml dry methanol at 60°C for 3 h. The resultant DMA-hydrazide was
separated from excess hydrazine by partitioning between water and chloroform. The
chloroform phase was dried under vacuum. The resultant DMA-hydrazide was pur-
ified on preparative silica gel thin-layer plates, using chloroform–methanol (70:30).

Extraction procedure

Tissue was added to methanol containing 100 mg l^{-1} butylated hydroxyto-
luene (BHT)[21] at a ratio of 1 g to 5 ml. Radiolabeled ABA ([^{3}H]ABA 1.4 GBq/μmol;
1.7 kBq to about 20 g fresh weight tissue) was added prior to homogenization as an
internal standard[1]. Tissue was chopped with a razor blade to about 0.5-cm^2 pieces,
followed by homogenization with a Polytron PT 20st. The homogenate was centri-
fuged at 9200 *g* for 15 min and the resulting pellet was re-homogenized in 5 ml of

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methanol–water (8:2) containing 100 mg l^{-1} BHT. After recentrifugation, methanol was removed from the combined supernatants under vacuum and the resultant aqueous solution was frozen in liquid nitrogen. Following thawing, the solution was centrifuged at 26 000 g for 20 min and the supernatant (A) saved.

For samples to be derivatized with dansyl hydrazine, the supernatant A was adjusted to pH 9.0 with 10 mM Tris–HCl and was extracted twice with 0.5 volume of diethyl ether containing 100 mg l^{-1} BHT. The aqueous phase was adjusted to pH 2.0 followed by extraction with three 0.3 volumes of chloroform. The chloroform was evaporated under vacuum. The residue was dissolved in methanol and stored under nitrogen at $-20°C$.

For samples to be derivatized with DMA-hydrazide, the supernatant A was adjusted to pH 2 with 20 mM phosphate followed by extraction with chloroform. The chloroform extract was concentrated by evaporation to 5 ml and loaded on a Florisil Sep-Pak column. The Sep-Pak was washed with methanol–chloroform (32:68) followed by elution of sample with methanol–chloroform (65:45). The eluent was evaporated and the resultant sample stored as described above.

Derivatization

Samples were derivatized at a molar ratio of carbonyl–hydrazide–HCl of 1:5:5.5 in HPLC-grade methanol or ethanol. When samples were from tissue extracts, hydrazide was added at a ratio of about 1 mg hydrazide to 14 g fresh weight seed. Dansyl hydrazine or DMA-hydrazide stocks of 10 to 15 mg ml^{-1} (in solvent system used) were made fresh daily. The volume of the derivatization mixture was kept below 100 μl by using conical serum vials. The sample was purged with argon and allowed to react overnight in the dark at 25°C. Conversion of ABA and jasmonic acid to their respective methyl and ethyl esters was done by derivatizing samples at a molar ratio of carbonyl–hydrazide–trichloroacetic acid of 1:5:25 in the presence of molecular sieves 4A (Linde) in the appropriate alcohol.

Analytical HPLC

A Hewlett-Packard HPLC system Model 1084B with auto injector model 79841A was used throughout the study. Chromatographic separation was performed with a Waters Assoc. radial compression module fitted with a Radial-Pak C$_{18}$ 10-μm cartridge. A C$_{18}$ 5-μm cartridge was used to separate the DMA-hydrazones. The hydrazones were separated using step gradients of acetonitrile in 20 mM phosphate buffer, pH 4.0. Details for each separation are given in the figure legends. Fluorescence was detected using a Perkin-Elmer fluorometer Model LS-5 fitted with an HPLC flow cell Model 5212-3126. Peak areas were determined by feeding the detector signal back into the HPLC controller. Excitation for dansyl hydrazones was at 255 nm with 1.5-mm slit width. Emission was set at zero order with a 2.0-mm slit width and a 505-nm cut-off filter. Excitation for DMA-hydrazones was at 354 nm with 1.5-mm slit width. Emission was set at 450 nm with a 2.0-nm slit width and a 420-nm cut-off filter.

RESULTS

The plant growth regulators, ABA, jasmonic acid and methyl jasmonate, all

formed stable hydrazones with dansyl hydrazine and DMA-hydrazide in acidic methanol. Reaction times in excess of 12 h were necessary to obtain quantitative derivatization. The amount of derivatization for ABA was determined for each sample by use of a [³H]ABA internal standard. Derivatization averaged 90 ± 10% and samples that derivatized at less than 80% were not used. The amount of derivatization for jasmonic acid was determined for each sample by use of a dihydrojasmonic acid internal standard. Derivatization of dihydrojasmonic acid always gave higher yields than derivatization of ABA. The resultant hydrazones were completely separated on the C_{18} column (Fig. 1). Dansyl hydrazine is contaminated with several fluorescent compounds (Fig. 1B), but none of the major contaminants interfered with the detection of the plant growth regulator hydrazones. However, large excesses of dansyl hydrazine were avoided so that the contaminant peaks were insignificant. With tissue extracts, the amount of dansyl hydrazine used was determined empirically for each tissue source because of differences in derivatizable compounds in the different tissues.

Derivatization with dansyl hydrazine in acidic methanol or ethanol converted up to 20% of the jasmonic acid to the corresponding ester (Fig. 1A). Esterification was not a problem with formation of DMA-hydrazones and ABA did not form esters under normal derivatization conditions. The same concentration of jasmonic acid was found in tissue extracts when the jasmonic acid–dansyl and methyl jasmonate–dansyl hydrazone peaks were added together, or when derivatization was done with DMA-hydrazide. The loss of jasmonic acid due to esterification could easily be accounted for by use of a dihydrojasmonic acid internal standard. The elution times of the dansyl hydrazones of jasmonic acid and dihydrojasmonic acid were separated by over 8 min (Fig. 1).

Commercial ABA is normally a mixture of two stereoisomers which can be resolved into separate peaks by HPLC[1]. The ABA–dansyl hydrazone, however, always eluted as a single peak (Fig. 1). Methanolic extracts of P. lunatus contained material that formed hydrazones that had the same elution time as the corresponding authentic jasmonic acid and ABA-hydrazones made with dansyl and DMA (Fig. 1). Addition of authentic jasmonate and ABA-hydrazones to extracts of either P. lunatus or soybean showed coincidence with the corresponding peaks from the extracts (data not shown). If [³H]ABA was added to the extract prior to derivatization, all of the radiolabel could be recovered in the ABA peak. The overall yield of the assay from homogenization through HPLC varied between 75 and 95%, with the average of 5 samples at 87 ± 7% for soybean seeds as determined by loss of [³H]ABA.

The addition of trichloroacetic acid and molecular sieves to the derivatization mixture increased the formation of esters so that the elution times for the methyl ABA and ethyl ABA-hydrazones could be determined. Extracts of P. lunatus derivatized in the presence of molecular sieves in either methanol or ethanol yielded dansyl hydrazones with elution times corresponding to the methyl and ethyl esters of ABA and jasmonic acid with a corresponding loss in the ABA and jasmonic acid peaks (data not shown).

The response of the fluorescence detector was linear for ABA–dansyl hydrazones from 10 pmol to over 15 nmol. The detection limit was about 5 pmol of ABA. Sensitivity was limited by the fluorescence quantum yields of the derivatives, by dansyl hydrazine contaminants (Fig. 1) and by the fluorescence detector. The response
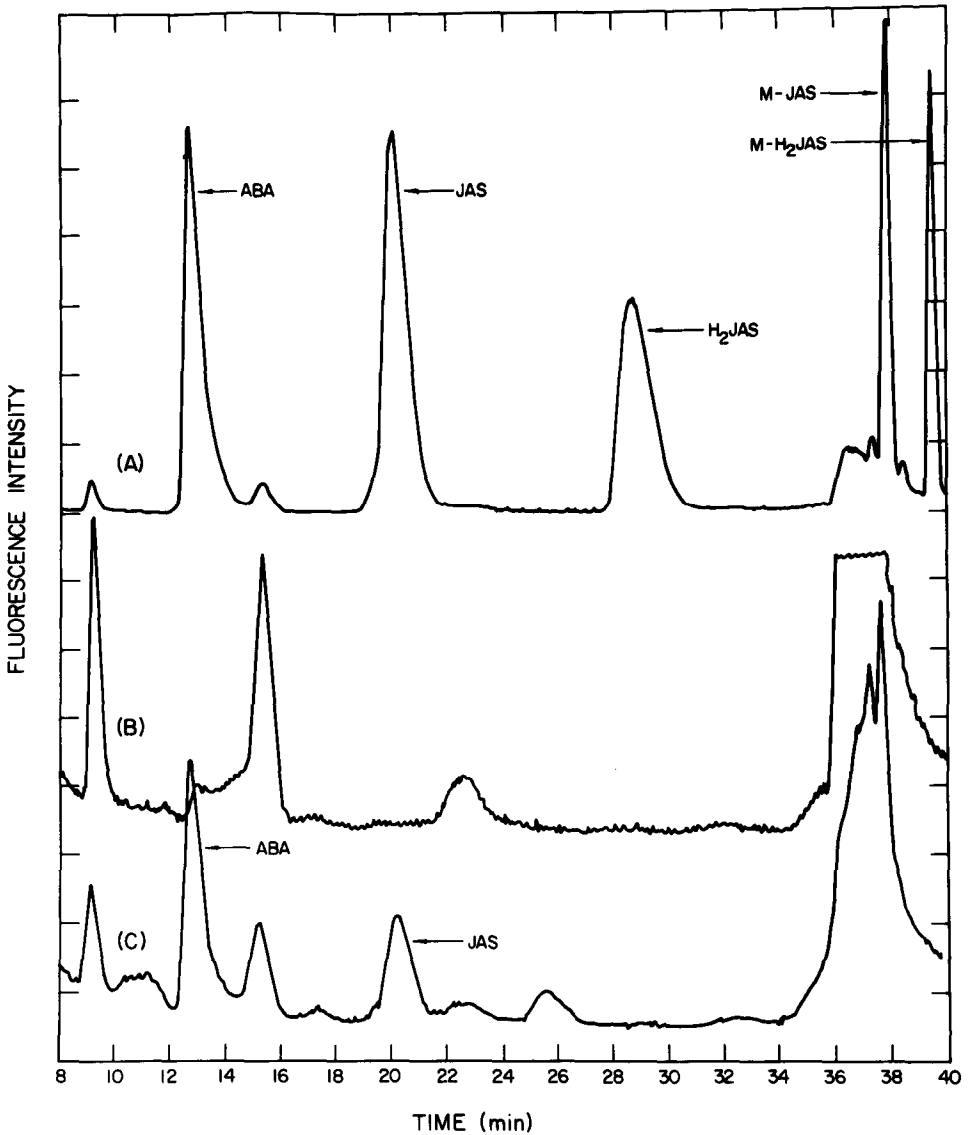
Fig. 1. Comparison of HPLC separations of (A) dansyl hydrazones of standards, (B) dansyl hydrazine blank at 10 × sensitivity, and (C) dansyl hydrazones of *Phaseolus lunatus* extract. Standards were abscisic acid (ABA), jasmonic acid (JAS), dihydrojasmonic acid (H₂JAS), methyl jasmonate (M-JAS), and methyl dihydrojasmonate (M-H₂JAS). Mobile phase was acetonitrile–20 mM phosphate (45:55) at pH 4.0 for 31 min, a gradient of 45% to 75% acetonitrile for 31–34 min, and 75% for 34–40 min. Flow-rate, 1.5 ml/min.

was also linear for jasmonic acid derivatives over the same range as ABA. Because the commercial methyl jasmonate preparation is probably a mixture of isomers, jasmonic acid concentrations are only approximate and were determined from the average of three dry weight determinations of a hydrolyzed sample of methyl jasmonic acid. Both jasmonic acid and dihydrojasmonic acid derivatized equally; therefore,

the jasmonic acid concentration was determined from the relative dihydrojasmonic acid peak area.

The DMA-hydrazones of ABA, jasmonic acid and methyl jasmonate (Fig. 2B) also could be separated by HPLC and the fluorescence response was linear with concentration (data not shown). Using the DMA-hydrazones instead of dansyl hydrazones, the same concentration of ABA and jasmonic acid was found in an extract of *P. lunatus* seeds. The fluorescence yield of DMA-hydrazide was increased following derivatization with jasmonic acid (Fig. 2B). The fluorescence increase was specific for jasmonic acid derivatives and was not observed for ABA–DMA hydrazones or the contaminates.
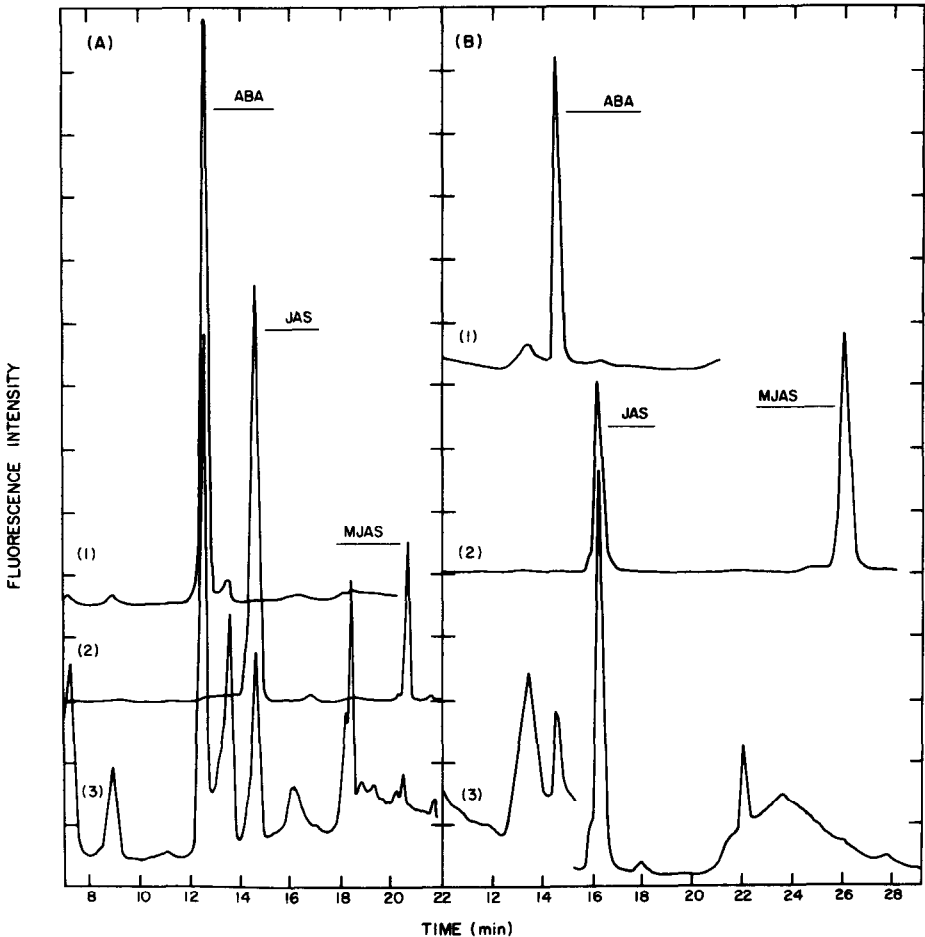

Fig. 2. Comparison of HPLC separations of ABA–jasmonic acid standards and immature seed extract from *Phaseolus lunatus* derivatized with dansyl hydrazine (A) and N-methylanthranilohydrazide (B). Abscisic acid (ABA) standard; (2) jasmonic acid (JAS) and methyl jasmonate (MJAS) standards; (3) *P. lunatus* seed extract purified using Florisil Sep-Pak. The chromatogram for the DMA derivative of the seed extract has a 5-fold decrease in sensitivity at 15.3 min. Hydrazones separated with acetonitrile–20 mM phosphate pH 4.0. (A) At 0–9.5 min 50% acetonitrile; at 9.5–16.5 min 65%; at 16.5–17.5 75%; and at 17.5–24 min 85%. (B) A linear gradient from 30% to 80% acetonitrile. Flow-rate, 3 ml/min.

Extracts were made from cauliflower immature flower head, *P. vulgaris* immature seeds, soybean immature seeds, and *P. lunatus* immature seeds (Table I). All of the extracts contained materials that formed dansyl hydrazone derivatives corresponding to the three plant growth regulators. Three different lots of *P. vulgaris* seed contained between 0.83 and 2.7 nmol/gram fresh weight jasmonic acid as determined using dansyl hydrazine; previous values found were 3.4 nmol/gram fresh weight[14].

TABLE I

LEVELS OF GROWTH REGULATORS EXTRACTED FROM VARIOUS PLANT TISSUES

Data represent single determinations except *Phaseolus vulgaris* which was repeated four times.

Tissue	Amount (pmol per g fresh weight)	
	ABA	Jasmonic acid
Glycine max L. (Merr) var Ransom, immature seed	50	18
Phaseolus lunatus L. immature seed	2	2
Phaseolus vulgaris L. immature seed	5.1 ± 0.5	2.7 ± 0.3
Brassica oleracea L. immature flower head	1	4

The purification procedure for tissue extracts could be abbreviated for tissues with moderate amounts of the plant growth regulator of interest. An example was *P. lunatus* seed where the aqueous supernatant following the second centrifugation was extracted directly with chloroform at pH 2.0. The use of the abbreviated purification did not change the resultant chromatograph appreciably (data not shown). Also, tissues with low concentrations of the plant growth regulators such as soybean leaf tissue could be assayed if the extract was purified using Florisil Sep-Paks.

DISCUSSION

The use of dansyl or DMA-hydrazone derivatives allows the simultaneous quantitation of ABA and jasmonic acid from plant extracts using HPLC. Little purification of most tissue extracts is needed prior to derivatization, although sugars must be removed[17]. Both ABA and jasmonic acid can be determined at a sensitivity of about 20 pmol per extract. Also, the hydrazones give a linear fluorometric response over a large range of concentrations. Because the final step in the purification brings the sample to dryness, almost all of the sample can be injected into the HPLC[1] so that the theoretical limit of detection is close to the actual limit for samples. The major disadvantage of the technique is the long reaction times for the formation of the dansyl derivatives. Heating the samples to 80°C increased the reaction rate[22], but also increased the formation of interfering contaminants. The spectrofluorometer used was not ideal for HPLC work; hence, the sensitivity obtained in this report could be improved.

Identity of dansyl hydrazone peaks from extracts of plant tissues as ABA and

jasmonic acid could have been in error if the extracts contained other compounds whose hydrazones gave similar elution times. Therefore, the DMA-hydrazones of ABA and jasmonic acid were also routinely made. Dimethylanthranilohydrazide is a smaller compound than dansyl hydrazine; consequently, elution times of the DMA-hydrazones should be more dependent on the derivatized compound than with the dansyl hydrazones. The same concentration of ABA and jasmonic acid in plant extracts was found with either the dansyl or DMA derivatives.

Finally, by adding molecular sieves and trichloroacetic acid to the derivatization mixture of extracts of *P. lunatus* seeds, a large proportion of both the ABA and jasmonic acid could be shifted to the elution position of the corresponding methyl and ethyl esters. The HPLC data strongly supports the identification of ABA and jasmonic acid in the tissue extracts but does not provide conclusive identification. Application of the HPLC method to a particular tissue should probably be supported by mass spectroscopy of the derivatives. However, the HPLC technique easily quantified authentic ABA, jasmonic acid, and dihydrojasmonic acid added to plant tissue extracts.

Some tissue extracts required more extensive purification prior to derivatization with DMA-hydrazide in order to get results similar to those obtained with dansyl hydrazine. However, the increase in fluorescence yield, which was specific for DMA-hydrazones of jasmonic acid, made DMA-hydrazide the better reagent for determining jasmonic acid when ABA was not quantified.

Simultaneous determination of jasmonic acid and ABA was complicated by the esterification of jasmonic acid in acidic alcohol during derivatization. However, native methyl jasmonate was removed from extracts during purification, and therefore, jasmonic acid could be determined by adding together the acid and ester peak areas. However, the separation of the ester peak from contaminants requires HPLC runs in excess of 60 min (data not shown), and therefore, lost jasmonic acid was normally accounted for by using dihydrojasmonic acid as an internal standard. The DMA derivatives of ABA and jasmonic acid could be quantified because esters were not formed with DMA-hydrazide.

The technique should also work for ABA metabolites which could be derivatized directly (phaseic acid) or derivatized following oxidation[19].

ACKNOWLEDGEMENTS

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