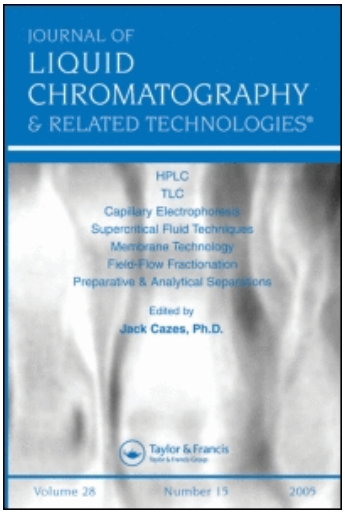

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REVERSED-PHASE CLEANUP AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ABSCISIC ACID IN *CERCOSPORA rosicola* LIQUID CULTURE MEDIA

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ABSTRACT

A high performance liquid chromatographic method is described for measuring abscisic acid produced by the mold *Cercospora rosicola* Passerini in liquid culture media. The acidified liquid medium was passed through a C_{18} reversed-phase cartridge, impurities and ABA were eluted separately, and the ABA fraction was chromatographed on a reversed-phase column. Detection was by UV absorbance at 268 nm. No significant difference was found between ABA recoveries obtained by solvent partition and reversed-phase cartridge cleanup.

INTRODUCTION

Cercospora rosicola Passerini is the first mold reported to produce the secondary metabolite (+)-abscisic acid (ABA) (1). A quantitative method to measure production of ABA by this fungus is necessary in our studies of ABA biosynthesis and regulation.

High-performance liquid chromatography (HPLC) has been used to quantitatively measure ABA in plant extracts. ABA in leaves, stems, and roots of soybeans, pinto beans, cotton, apple seedlings and orange peel was measured after cation exchange separa-

tions (2) and in buds and nodes of grapes after strong anion exchange separations (3,4). Reversed-phase separations were used to measure ABA in leaf and peel of citrus (5), soybean plants (6), sorghum leaves (7), soybean exudates (8), and potato roots (9). In the above methods, detection was based on the absorbance of underivatized ABA. p-Nitroylbenzyl ester derivatives and polar bonded-phase separations were used in methods to measure ABA in lettuce seed extracts (10). All of the above analyses, which involved higher plants, required rigorous cleanup procedures because of high concentrations of interfering compounds and low concentrations of ABA.

The chemically defined liquid media used to culture C. rosicola and the metabolic products of the fungus are far less complex than plant systems, and the amount of ABA produced is large in comparison. C. rosicola can produce as much as 30 µg ABA/ml of medium. This report describes a rapid, reversed-phase cleanup, and a quantitative chromatographic method for measuring ABA in the culture medium of this fungus.

EXPERIMENTAL

Instrumentation

The liquid chromatograph consisted of a 6000 A pump (Waters Associates, Milford, Massachusetts), Model 7120 injector with a 100 µl injection loop (Rheodyne Inc., Berkeley, California), Model LC-75 spectrophotometric detector (Perkin Elmer Corp., Norwalk, Connecticut), Model A-25 recorder (Varian Instruments, Walnut Creek, California), and Supergrator Model 3A programmable computing integrator (Columbia Scientific Industries, Austin, Texas). All analyses were at ambient temperature. Eluants were degassed by sonification before start-up and stirred with a magnetic stirrer during use. Detection was by UV absorbance at 268 nm and 0.16 AUFS.

We confirmed the identities of t-ABA and ABA produced by the fungus by combined gas chromatography and mass spectrometry of the methyl esters. GC-Mass spectra were obtained at 70 eV on VG Micromass 70/70F with glass jet separator at 200° C and VG data system and software for total ion current and mass spectral acquisition and reprocessing. Silanized glass columns packed with 2% QF-1 were temperature programmed from 100° to 230° C at 4°/min. Methyl derivatives of ABA peaks collected from HPLC were prepared with diazomethane in diethyl ether.

Chemicals

Reverse osmosis-deionized water (Milli-Q System, Millipore Corp., Bedford, Massachusetts) was used throughout. Acetonitrile (UV grade) was from Burdick and Jackson Laboratories, Inc., Muskegon, Michigan. Reagent grade methanol and ethyl acetate were redistilled in an all-glass distillation apparatus. H_3PO_4 was reagent grade (J. T. Baker Co., Phillipsburg, New Jersey).

ABA standards

(±)-Abscisic acid, a 50% mixture of cis, trans (ABA) and trans, trans (t-ABA) isomers (R. J. Reynolds Tobacco Co., Winston-Salem, North Carolina) was used as a standard. A solution of the standard was prepared in HPLC eluant (10 µg/ml) and a calibration run was made daily. The integrator used peak areas of the standards and sample unknowns to calculate t-ABA and ABA concentrations in µg/ml.

Cultures

The C. rosicola Pass. culture (strain No. 138.35) used was obtained from Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. Cultures were maintained on potato dextrose agar (Difco Laboratories, Detroit, Michigan), and liquid cultures were

grown in synthetic media for 1 to 4 weeks on reciprocating shakers at 24-26° C under continuous fluorescent lighting (11). The cultures were filtered (Whatman No. 3 paper) under reduced pressure. The filtrates were adjusted to pH 2.5 with 1 N HCl, transferred quantitatively to 200-ml volumetric flasks, and made to volume with 0.1 N HCl. Aliquots (2 to 20 ml) were taken for the cleanup procedures.

Cleanup procedures

Solvent partitioning: The liquid culture medium (pH 2.5) was extracted 3 times with ethyl acetate, the combined ethyl acetate fraction was partitioned 3 times against 8% sodium bicarbonate, and the acidified (pH 2.5 with 1 N HCl) sodium bicarbonate partitioned 3 times against ethyl acetate. The final combined ethyl acetate fraction was dried over sodium sulfate and evaporated to dryness on a flash evaporator. The residue was then dissolved in the HPLC eluant for analysis.

Disposable cartridge: An aliquot of the culture medium was forced through a disposable C_{18} reversed-phase cartridge (Sep-Pak™, Waters Associates, Milford, Massachusetts), washed with 2 ml of 10% methanol in 0.002 M H_3PO_4, and eluted from the cartridge with 2 ml of 75% methanol in 0.002 M H_3PO_4.

HPLC separation

The cleaned ABA sample was placed on a Partisil PXS 5/25 ODS column, 4.6 mm I.D. x 25 cm, (Whatman Inc., Clifton, New Jersey), and the column was eluted with 50% methanol in 0.004 M H_3PO_4 at a rate of 1 ml/min.

RESULTS AND DISCUSSION

Cleanup

Most cleanup methods for ABA analyses of plant extracts involve solvent partition to isolate the acid fraction, followed by

treatment with polyvinylpolypyrrolidone and one or more types of chromatographic separations for further cleanup. Cleanup of the culture media of C. rosicola was necessary to remove sugars, salts, and some of the metabolic products of the fungus. Solvent partition was adequate for this purpose. However, this method was time consuming and necessitated evaporation of the final ethyl acetate fraction so that it could be dissolved in the HPLC eluant. We therefore tested alternative methods and found that using a C_{18} disposable cartridge enabled fast effective cleanup and direct transfer of the cleaned sample to the HPLC colum for quantitative analysis. The sample and eluant for the cleanup had to be acidic for maximum recovery; the recoveries were 95 ± 3% and 96 ± 3% (n=28) for t-ABA and ABA standards, respectively.

ABA recoveries after C_{18} reversed-phase cleanup and solvent partition cleanup were compared for 22 experimental cultures (Table 1). Standard solutions passed through the reversed-phase cartridge or solvent partition were used as reference for ABA quantitation by HPLC. According to a t-test for paired observations, there was no significant difference between values obtained by the two cleanup procedures.

HPLC separations

In developing the HPLC separation of t-ABA and ABA from other components of the liquid media, three columns were tested. Eluants for all three columns had to be acidic to reduce tailing. First, a Bondapak NH_2 column (Waters Associates, Milford, Massachusetts) was used with a solvent system (acetonitrile, chloroform, and acetic acid) suggested by Chia et al. (6) for soybean extracts. This system separated t-ABA, ABA, and other media constituents effectively and could be used with cleaned ABA samples in nonaqueous media; however, detection at low UV wavelengths was not possible.

The second column tested was a LiChrosorb C_{18} reversed-phase column (Altex Scientific Inc., Berkeley, California),

TABLE 1

Comparison of Abscisic Acid Recoveries in Cercospora rosicola Liquid Media that had been Subjected to Cleanup by Solvent Partition and by C_{18} Reversed-phase Cartridges.

	Solvent Partition		C_{18} Reversed-phase	
Sample No.	t-ABA µg/ml	ABA µg/ml	t-ABA µg/ml	ABA µg/ml
1	0.15	0.97	0.17	1.40
2	0.14	0.01	0.15	1.30
3	0.09	0.92	0.08	0.77
4	0.11	0.94	0.06	0.46
5	0.86	10.58	1.08	13.93
6	0.28	1.80	0.26	1.97
7	0.14	0.88	0.15	1.20
8	0.29	2.95	0.25	2.51
9	0.09	0.61	0.08	0.70
10	2.70	14.69	2.00	11.22
11	0.02	0.30	0.07	0.68
12	0.03	0.31	0.01	0.22
13	0.11	0.22	0.17	0.22
14	0.17	1.84	0.25	3.27
15	0.20	2.07	0.25	4.06
16	0.08	0.43	0.03	0.29
17	0.04	0.21	0.07	0.27
18	0.03	0.19	0.03	0.20
19	0.03	0.29	0.05	0.38
20	0.04	0.34	0.01	0.29
21	0.17	0.54	0.08	0.54
22	1.47	11.84	1.50	12.86
mean	0.33	2.45	0.31	2.67
Std. dev.	0.63	4.15	0.52	4.22

Paired t-test $t=0.73$ [$t_{0.05}$ (21 d.f.) = 2.08]

eluted with acetonitrile in dilute H_3PO_4. This column also clearly separated t-ABA, ABA, and other media constituents.

The Partisil column eluted with methanol in H_3PO_4 retained t-ABA and ABA longer than the other two columns, but it was the best in isocratically resolving the media components, which became more complex as the culture aged. Of the acids tested, H_3PO_4 was the best in controlling pH; also, it allowed the use of low UV wavelengths for detection. Ternary mixtures of acetonitrile, methanol, and tetrahydrofuran were tested with the C_{18} column but decreased resolution.

Figure 1 is a high-performance liquid chromatogram (Partisil column) of an ABA standard after it had been passed through a C_{18} cartridge. Light catalyzes the isomerization of ABA to establish a 1 : 1 ratio of t-ABA and ABA (13); hence a 50% mixture of the two isomers provides a stable standard. If the cultures are incubated in light, isomerization of ABA is likely but probably minimal. Under the conditions we used to grow C. rosicola, ABA was by far the most predominant component of the culture media (Fig. 2).

Because little is known about the other metabolic products of this fungus, we chromatographed a two-week-old culture on the Partisil C_{18} column. The eluant was changed from 30% methanol in 0.003 M H_3PO_4 to 98% methanol in 1 M H_3PO_4 over 35 minutes according to a linear gradient: the eluate was monitored at three different wavelengths (Fig. 3). Nearly 30 peaks were detected at 206 nm, half as many at 268 nm, and about 12 peaks at 456 nm. C. rosicola produced several red and yellow secondary metabolites, which absorbed at 268 and 456 nm in the acidic eluant. Two of the red compounds have been isolated and identified by Assante et al. (12) as two epimers, dothistromin and 2-epidothistromin. We have begun work to identify some of the other components in the liquid medium.

The cleanup method based on using a C_{18} cartridge is simple and efficient; thus, within a given period, many more samples of

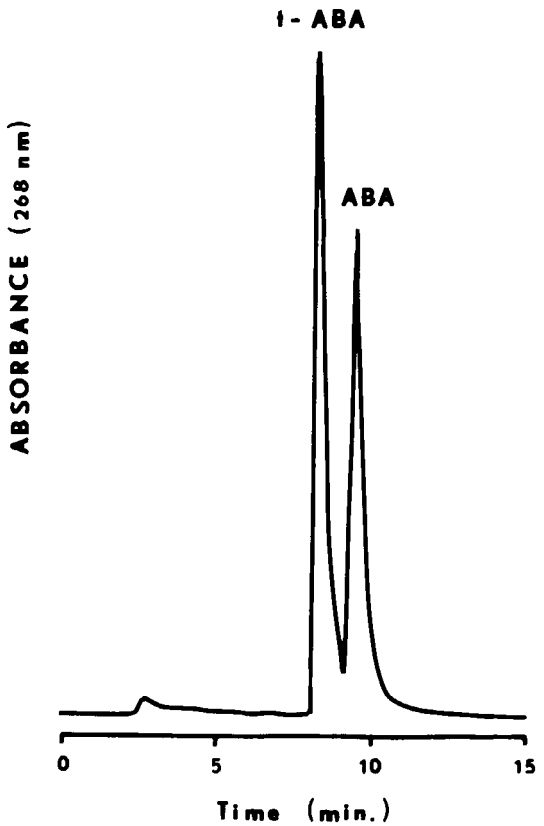

FIGURE 1. ABA standard, 5 µg/ml of each isomer. Partisil PXS 5/25 ODS column, 50% methanol in 0.004 M H_3PO_4, isocratic, 0.16 AUFS. Rt t-ABA = 8.5, ABA = 9.8 min.

growth media can be processed by that method than by solvent partition. The reversed-phase cartridge may also facilitate the cleanup of complex plant extracts. The HPLC method is a rapid and reliable means of monitoring ABA production by C. rosicola. One Partisil PXS column has been in use for 14 months without deterioration. Information concerning ABA biosynthesis by C. rosicola will be reported separately (14).

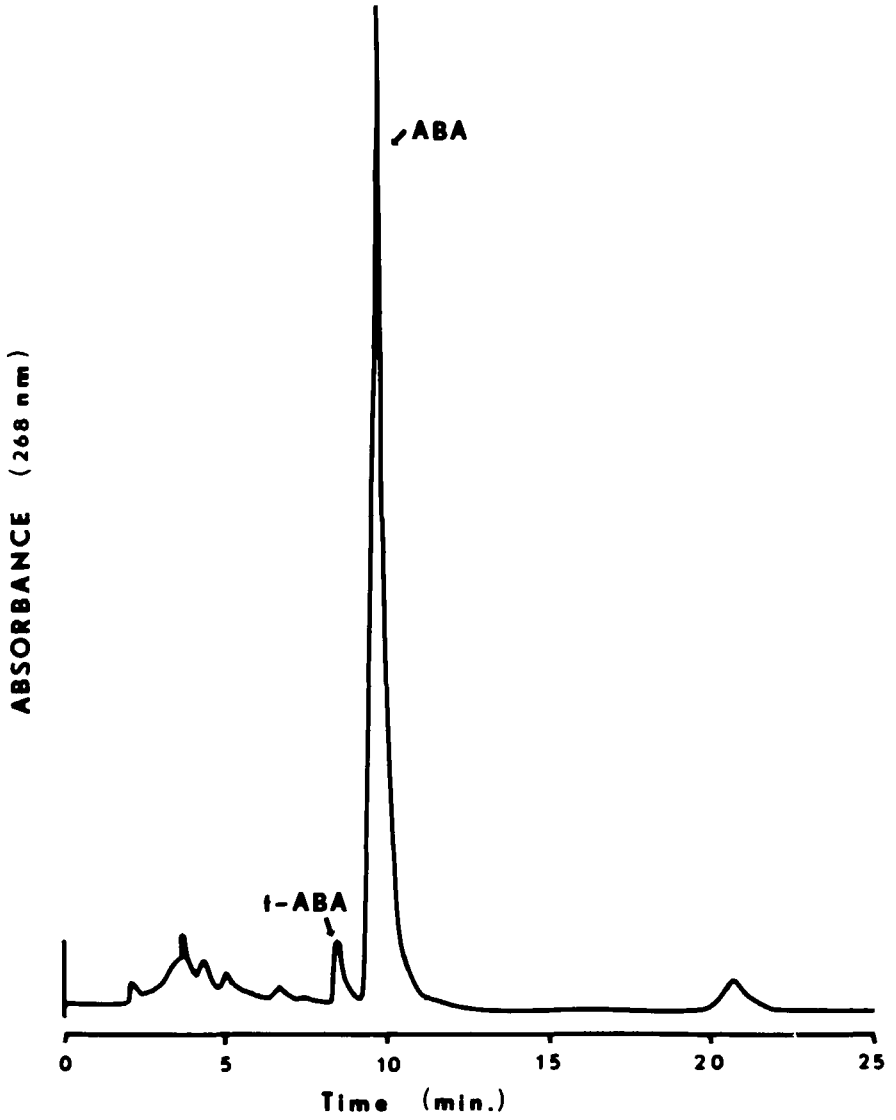

FIGURE 2. Chromatogram of a 1-ml aliquot of culture medium that had been cleaned with a C_{18} cartridge. The aliquot contained 0.75 µg t-ABA/ml and 19.65 µg ABA/ml. Partisil PXS 5/25 ODS, 50% methanol in 0.004 M H_3PO_4, isocratic, 0.16 AUFS.

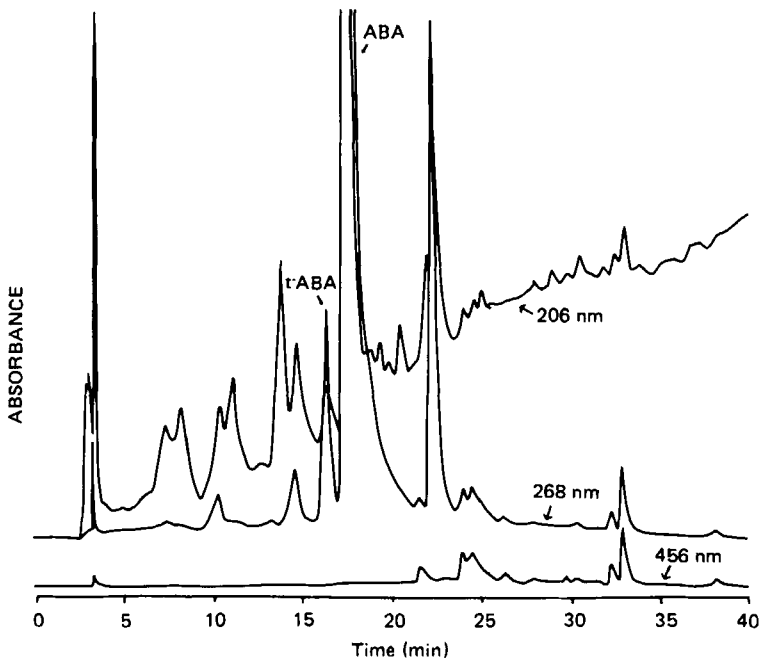

FIGURE 3. Chromatograms of C. rosicola culture medium containing 0.5 µg t-ABA/ml and 30.0 µg ABA/ml. Detection at 206, 268, and 456 nm. Partisil PXS 5/25 ODS column, 30% methanol in 0.003 M H_3PO_4 changed to 98% methanol in 2% 1 M H_3PO_4 in 35 minutes according to a linear gradient.

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