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CHOICE OF METHODS FOR THE DETERMINATION OF ABSCISIC ACID IN PLANT TISSUE

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SUMMARY

Two standard procedures are described for the determination of abscisic acid in plant tissue. One includes partial but rapid clean-up followed by semi-preparative high-performance liquid chromatography on a 5-μm reversed-phase C_{18} column in preparation for quantitative analysis by electron-capture gas–liquid chromatography (GLC). The alternative method employs Clin Elut tubes plus pre-adsorbent and reversed-phase thin-layer chromatography plates. The need for freezing precipitation, separating funnels, polyvinylpyrrolidone slurries or glass extraction columns has been eliminated from both methods. Improved resolution during GLC has been achieved by using a column packing with an ultra-thin phase of Carbowax 20M and by careful choice of the injection solvent. In addition to electron-capture detection and UV isomerization, further confirmation of identity is obtained by flame-ionization detection after injection of the sample into a fused-silica capillary column.

INTRODUCTION

The determination of abscisic acid (ABA) in plant tissue involves a number of time-consuming steps and may require several working days in order to process a set of samples through extraction, quantitation and confirmation of identity[1–5]. Although a few reports have described procedures that significantly reduce analysis times[6–9], the benefits obtained must be weighed against the requirement for well defined peaks, good recovery and the need to minimize experimental variation among replicate samples. After testing a range of methods and modifications in our laboratory, we have developed two standard procedures that have significantly reduced experimental error but still provide an acceptable yield of data per unit of time invested. The faster and more convenient procedure includes semi-preparative high-performance liquid chromatography (HPLC) followed by gas–liquid chromatography (GLC). The alternate procedure relies on thin-layer chromatography (TLC) followed by GLC, but it includes the use of several products that provide greater convenience, speed and uniformity of results than previous TLC methods[2–4].

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EXPERIMENTAL

High-performance liquid chromatography

Gradient elution chromatography is carried out with a Spectra-Physics (Santa Clara, CA, U.S.A.) 8000 three-solvent system using an SP 8400 variable-wavelength UV detector operating at 269 nm. Samples are introduced via a nitrogen-driven auto-injector fitted with a 100-μl loop and then separated on a semi-preparative column (250 × 10.0 mm I.D.) packed with 5-μm Ultrasphere-ODS (Beckman, Berkeley, CA, U.S.A.).

Chemicals

Ultra-high-purity solvents (HPLC grade; Caledon Labs, Georgetown, Ontario, Canada) are used in all HPLC and GLC procedures and all water is glass distilled and filtered through 0.2-μm MF Millipore filters (Millipore, Bedford, MA, U.S.A.) immediately prior to use. Authentic ±-*cis–trans*-abscisic acid is obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

Unless fresh material is specificially required, all samples are frozen in liquid nitrogen and immediately lyophilized for 48 h before storage at −20°C. The dry tissue is ground through a 40-mesh sieve in a Wiley mill and replicate samples of 0.1–1.0 g are taken for analysis. Each sample is extracted for 1 h on a shaker in a 50-ml test-tube with 20 ml of 80% (v/v) aqueous methanol containing 0.5% (v/v) glacial acetic acid. The methanolic extract is centrifuged at 2570 *g* for 8 min and the supernatant is decanted into a round-bottomed flask. The tissue is extracted (30 min) and centrifuged (8 min) two more times. The combined supernatant is reduced to the aqueous phase *in vacuo*, transferred to a 15-ml vial and then adjusted to pH 2.5 with 4 *M* hydrochloric acid. The acidified extract is loaded onto Clin Elut tubes (No. 1020; Analytichem International, Lawndale, CA, U.S.A.) and solvent partitioned against 4 × 20 ml of ethyl acetate. These tubes eliminate the need for separating funnels and, in addition to solvent partitioning, they provide effective filtration through an inert matrix[2,4]. The combined ethyl acetate fractions are reduced to dryness and the flask then rinsed three times with 2 ml of ethyl acetate, and the rinsings are transferred to a 15-ml vial and blown dry under nitrogen in preparation for further purification via procedure I or II (Fig. 1).

Procedure I

Prior to HPLC, the sample is re-dissolved in 1 ml of methylene chloride and loaded onto a disposable Sep-Pak silica cartridge (Waters Assoc., Milford, MA, U.S.A.) that has been prewashed with 5 ml of methylene chloride. After loading, the sample is washed with 10–20 ml of methylene chloride, followed sequentially by 10 ml of 5% (v/v) diethyl ether and 5 ml of 5% (v/v) acetone, both made up to volume with methylene chloride. The ABA fraction is eluted with 10 ml of 20% (v/v) methanol and then with 5 ml of 60% methanol in methylene chloride. The use of the Sep-Pak cartridges, or other concentrator products such as Amberlite XAD resins[10,11], shortens the overall analysis time, reduces solvent waste, prolongs the life of the HPLC column and effectively removes a number of interfering compounds.

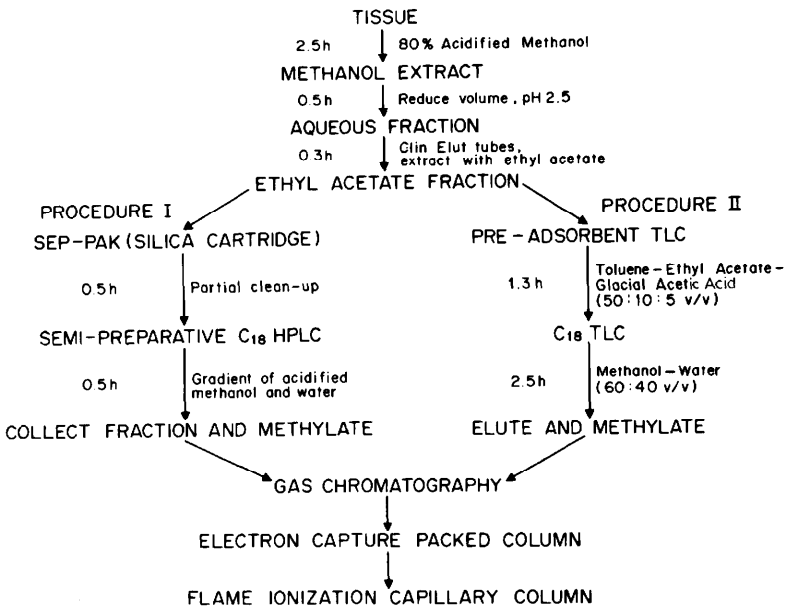

Fig. 1. Alternative procedures for the extraction and purification of ABA from plant tissues.

The methanolic eluates containing ABA are combined and reduced under vacuum to approximately 1 ml. Aliquots, equivalent to about 100 mg of dried tissue, are chromatographed on the semi-preparative reversed-phase column using a gradient of acidified methanol and water. In preparation for analysis by GLC, the ABA fraction is methylated with diazomethane[12] using an apparatus assembled in our laboratory[13].

Procedure II

In our alternative procedure, used when the HPLC equipment is committed to other work, the ethyl acetate residue is re-dissolved in 400 μl of acetone–methanol (9:1, v/v) and rapidly strip-loaded, between authentic *cis–trans*-ABA marker spots, onto Whatman LK_6F pre-adsorbent TLC plates that have been pre-washed in ethyl acetate and re-activated at 65°C. The strip-loading procedure is repeated with 300 μl and finally 200 μl of acetone–methanol (9:1) to ensure complete recovery from the 15-ml sample vial. The sample is separated (*ca.* 80 min) in a solvent system composed of toluene–ethyl acetate–acetic acid (50:10:5). The pre-adsorbent plates accept heavy sample loads, but they still yield tight bands and separate ABA from most pigments common to plant extracts.

The ABA zone, located under UV light, is scraped from the plate and transferred to pre-washed (9:1 acetone–methanol) Whatman cellulose extraction thimbles and eluted into a 15-ml vial with 6–8 ml of acetone–methanol (9:1). The sample is blown dry under nitrogen and, following the same loading procedure used with the pre-adsorbent plates, the sample is chromatographed for 2.5 h in 60% methanol on half of a standard size Whatman ($KC_{18}F$) reversed-phase TLC plate that has been pre-washed in absolute methanol. These plates can be rapidly strip-loaded and provide a significant amount of additional clean-up. The ABA fraction is eluted from the

appropriate R_F zone as described above and is then methylated with diazomethane[12].

Gas–liquid chromatography

The methylated extract is dissolved in 0.1–1.0 ml of hexane–ethyl acetate (9:1) and 1 μl is injected into a Hewlett-Packard 5730A gas chromatograph fitted with a ^{63}Ni electron-capture detector. Peak areas are measured with an HP 3385A digital integrator.

Three liquid phases were tested. Glass columns were packed with (A) Chromosorb W coated with an ultra-thin film (0.2%, w/w) of Carbowax 20M as described by Aue *et al.*[14], (B) Ultrabond PEGS (Alltech, Deerfield, IL, U.S.A.) and (C) Chromosorb W HP coated with OV-17 (Chromatographic Specialties, Brockville, Ontario, Canada). Identity of methyl-ABA was confirmed by co-injection with the authentic ester, by UV isomerization[15] and by capillary GC with flame-ionization detection using a Hewlett-Packard 5710 chromatograph fitted with a 12.5 m × 0.31 mm I.D. fused-silica column coated with OV-101.

RESULTS AND DISCUSSION

A comparison of the three column packings shows sharp, well defined peaks following injections of a *cis–trans*-ABA standard; however, the peaks obtained with the column prepared according to Aue *et al.*[14] are higher, sharper, more symmetrical,

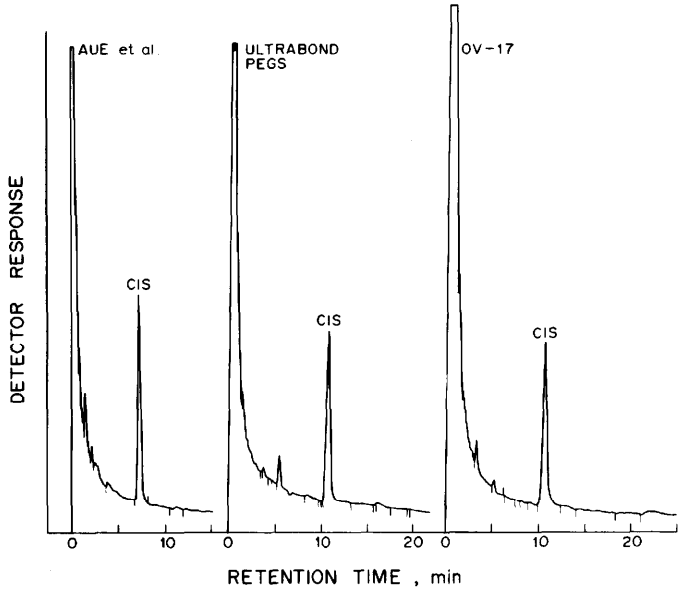

Fig. 2. Electron-capture analysis of methylated authentic *cis–trans*-ABA on three liquid phases. GLC conditions: columns, glass (180 cm × 2 mm I.D.); carrier gas, argon–methane (95:5); injector temperature, 250°C; detector temperature, 300°C; ABA concentration, 0.4 ng/μl; attenuation, 2^6. Column conditions for each liquid phase: Aue *et al.* (100–120 mesh): flow-rate, 20 ml/min; column temperature, 190°C. Ultrabond PEGS (100–120 mesh): flow-rate, 30 ml/min; column temperature, 230°C. OV-17 (80–100 mesh): flow-rate, 20 ml/min; column temperature, 200°C.

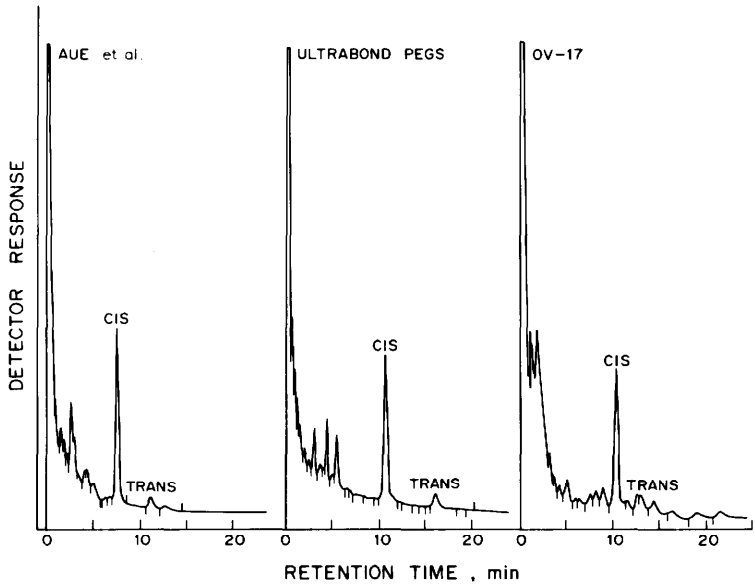

Fig. 3. GLC analysis of ABA from 0.5 g of salt-stressed tomato leaves using the TLC method (procedure II). Sample dilution, 300 μl; injection volume, 1 μl. Chromatographic conditions as in Fig. 2.

show less tailing and provide shorter retention times than either of the commercial packings (Fig. 2). Similar results were obtained after injection of ABA samples that were isolated from 0.5 g of salt-stressed tomato leaves using procedure II (Fig. 3). The Ultrabond PEGS and OV-17 gave satisfactory results, but the reduced tailing and excellent symmetry obtained with the packing prepared according to Aue *et al.* contribute to a significant reduction in variation among replicate samples during routine analysis. Although columns prepared to Aue *et al.* are readily made in the laboratory, a commercial packing with similar chromatographic characteristics is available under the trade-name Ultrabond 20M (Alltech). Both ultra-thin phases require the removal of oxygen and moisture from the carrier gas to prevent rapid deterioration of the columns at high temperatures.

It is necessary to choose an appropriate injection solvent in order to optimize the results obtained with any stationary phase. In this study, we evaluated several solvents and solvent mixtures, including acetone, ethyl acetate–methanol (9:1) and hexane–ethyl acetate (9:1), and found that hexane–ethyl acetate had highly satisfactory solvent-peak characteristics when used with a range of liquid phases.

Following electron-capture detection and quantitation of ABA, the methylated samples must be concentrated from 10 to 50 times prior to further confirmation of identity by capillary GC with flame-ionization detection. This method of detection yields sharp and symmetrical peaks when used with ABA standards (Fig. 4a), but its low specificity is clearly emphasized by the presence of several extraneous peaks on the chromatogram of the tomato-leaf extract (Fig. 4b). In spite of its shortcomings, however, the method is useful as an adjunct to electron-capture detection, *i.e.*, to provide additional confirmation of identity in conjunction with the UV isomerization procedure[4,16]. A specific bioassay[17] or analytical HPLC of a methylated ABA

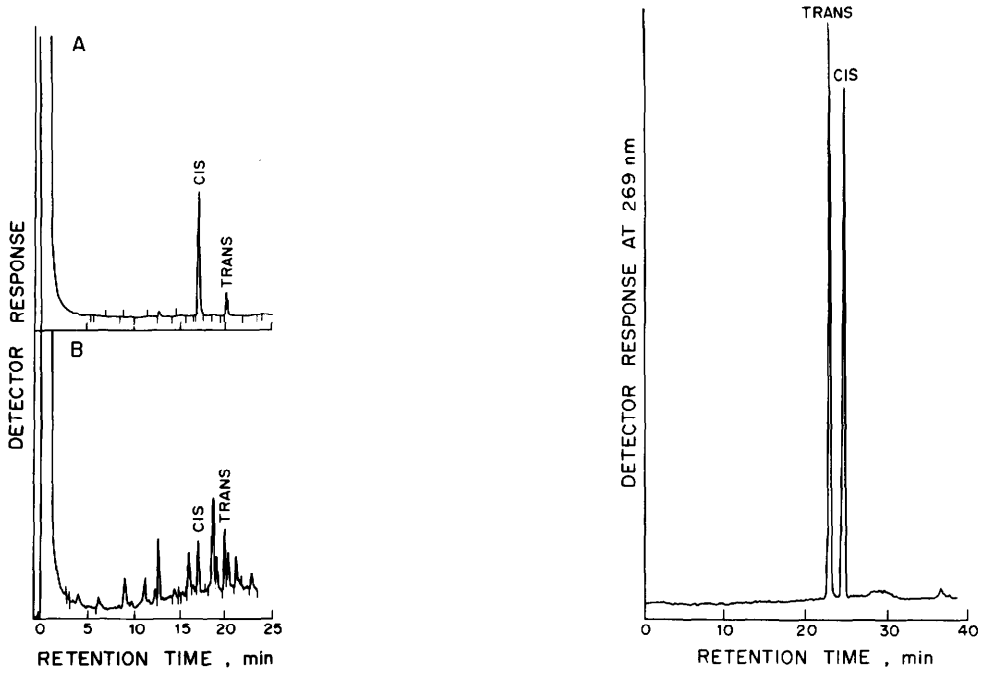

Fig. 4. Capillary GLC analysis using flame-ionization detection for additional confirmation of identity of (A) the methylated ABA standard and (B) the methylated ABA extract from salt-stressed tomato leaves. Column, OV-101 on fused silica (12.5 m × 0.31 mm I.D.); carrier gas, helium; velocity, 20 cm/sec; injector temperature, 250°C; detector temperature, 300°C; concentration of ABA standard, 100 ng/μl; sample dilution, 100 μl; injection volumes, 1 μl; attenuation, 2^3; temperature program, 160°C (4 min hold) to 260°C at 2°C/min.

Fig. 5. Separation of the geometric isomers of authentic underivatized ABA by reversed-phase HPLC. Column, Ultrasphere-ODS, 5 μm (250 × 10.0 mm I.D.); flow-rate, 2 ml/min; temperature, 40°C; concentration of ABA standard, 100 ng/μl; injection volumes, 100 μl. A two-solvent mobile phase with a linear gradient: (A) 30% methanol with 0.1 *M* formic acid; (B) 70% water with 0.1 *M* formic acid, held for 2.0 min, then programmed to 90% A, 10% B over 28 min.

sample[16] may also be used for confirmation, but positive identification by mass spectrometry is, of course, the preferred alternative when a suitable instrument is available.

The 5-μm reversed-phase HPLC column used in procedure I gives excellent resolution of the geometric isomers of authentic underivatized ABA (Fig. 5); and when used in the semi-preparative mode with a tissue sample, resolution of the isomers is retained and significant purification of the ABA fraction is achieved (Fig. 6). However, much of the material in the *trans*-peak proved to be a closely associated contaminant and not *trans*-ABA.

Damage to the Ultrasphere column is minimized by using a Brownlee RP-18 (Technical Marketing Assoc., Mississauga, Ontario, Canada) guard column and by rapid clean-up of the crude extracts with Clin Elut tubes and Sep-Paks as described under Experimental. This eliminates the use of PVP slurries[2,4] and the preparation of glass extraction columns[1,4,18,19] during the early stages of purification. More importantly, it allows the use of just one HPLC column, thus minimizing costs, equilibration

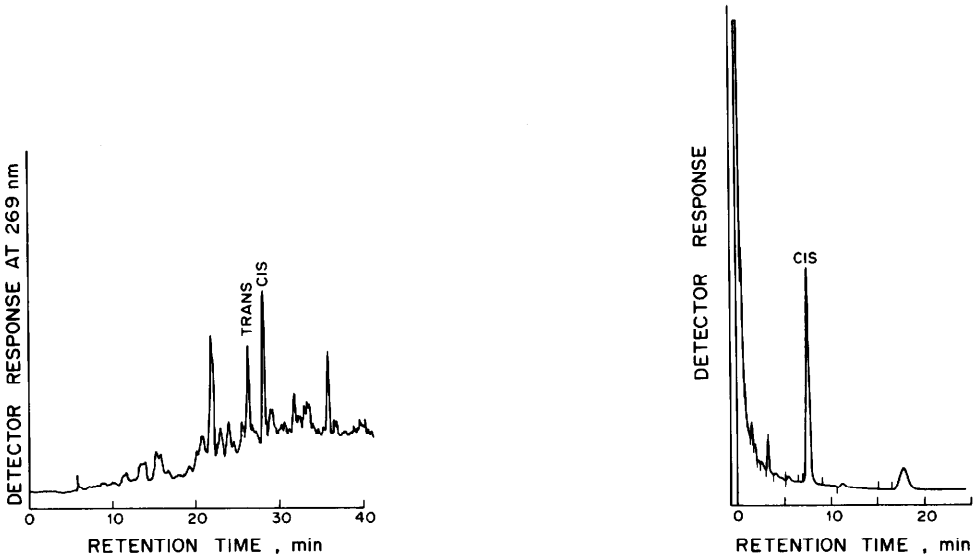

Fig. 6. Separation of the ABA fraction from a partially purified extract of salt-stressed tomato leaves (1.5 g) by semi-preparative HPLC. Sample dilution, 1 ml; injection volumes, 100 μl. Chromatographic conditions as in Fig. 5.

Fig. 7. Electron-capture analysis following methylation of the *cis*-ABA fraction collected by semi-preparative HPLC (Fig. 6). Chromatographic conditions as in Fig. 2.

time and damage to fittings that may be incurred with the frequent change of columns usually required with methods employing both preparative and analytical HPLC[16,18,20].

A setting of 269 nm on the variable-wavelength UV detector proved optimal for ABA. Background peaks evident at 254 nm were reduced, tentative identification and quantitative evaluation were improved and cleaner more precise fractions could be collected for further analyses by GLC with detection limits in the picogram range.

Methylation of the *cis*-ABA fraction and subsequent separation on a column prepared according to Aue *et al.*[14] using electron-capture detection gave a peak equivalent to *ca.* 800 ng/g dry weight of ABA (Fig. 7). The peak is highly symmetrical and equivalent to scans obtained with ABA standards (Figs. 2 and 4a) and, of greater importance, the high resolution contributes to consistency of results among replicates and smaller standard errors. Although faster analysis times and better separations make procedure I the method of choice, a comparison of Figs. 3 and 7 indicates that both procedures produce well defined ABA peaks. Further, when [^{14}C]ABA is added at the extraction stage, both procedures yield recoveries that consistently exceed 85%.

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