Purification and Measurement of Abscisic Acid and Indoleacetic Acid by High Performance Liquid Chromatography

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ABSTRACT

A procedure was selected for the simultaneous extraction and purification of abscisic acid (ABA) and indoleacetic acid (IAA). Unnecessary steps were eliminated and an accumulation of aqueous phase was avoided. The superior performance of diethyl ether (compared to ethyl acetate) for bulk purification and the superior resolution provided by 250 millimeter columns packed with 5-micrometer spherical particles of strong anion exchanger and octadecylsilane (C18) greatly facilitated the purification of samples. A fixed-wavelength (254 nanometer) ultraviolet detector and a fluorescence detector connected in series on a high performance liquid chromatograph permitted nondestructive monitoring and measurement of ABA and IAA. Derivatization was not necessary for chromatography or for detection. Isocratic elution with simple mobile phases gave sharp peaks. A few simple precautions minimized losses. Recoveries through the entire procedure averaged about 75% for ABA and about 50% for IAA. Purified ABA and IAA fractions were usually free of interfering contaminants. Identities were confirmed by gas chromatography-mass spectrometry.

Poor recovery is a frequent problem with IAA determination because IAA is easily oxidized and is subject to photodecomposition (11, 14). ABA is also somewhat labile, although not as labile as IAA. Because of variable losses during extraction and purification, the use of internal standards is essential for accurate quantification of ABA and IAA (4). trans-ABA has been used as an internal standard for the estimation of ABA (9, 19), but the acceptability of its use depends upon its absence from the tissue being analyzed and upon lack of interconversion of ABA and trans-ABA during the procedure. The use of radioactive ABA as an internal standard circumvents these potential problems. Indolebutyric acid has been used as an internal standard for IAA (5, 19), but its value is questionable because of differences in partitioning and chromatographic behavior and possible differences in stability. Labeled IAA is preferable as an internal standard.

HPLC and GLC are frequently used in the final stages of purification and for quantification of ABA and IAA. HPLC has advantages over GLC in quantification because UV and fluorescence detectors, used with HPLC, are nondestructive, and the entire sample can readily be collected for measurement of radioactive internal standards. Also, it is not necessary to form volatile derivatives (e.g. by methylation or silylation) for HPLC as it is for GLC. The UV detector, however, is not very specific and impurities are likely to interfere with quantification unless the sample is almost pure.

The objectives of the work reported here were to select procedures that would (a) permit simultaneous extraction and purification of ABA and IAA, (b) provide purification sufficient that ABA could be quantified by UV absorbance and IAA could be quantified by natural fluorescence with the detectors connected in series on an HPLC, (c) give a high percentage recovery so that small amounts of tissue could be analyzed, and (d) be as simple as possible.

MATERIALS AND METHODS

Plant Material. Young fruits of cotton (Gossypium hirsutum L.) and leaves of grapefruit (Citrus paradisi Macf. 'Ruby Red'), mulberry (Morus alba L.), and ash (Fraxinus uhdeli Wenzig) were quickly frozen at −85°C, freeze dried, and ground to pass a 40-mesh screen. The tree leaves were included because we wanted to test our procedure with material likely to contain a large amount of contaminants such as polyphenolics. The ground samples were stored at −85 or at −20°C until they were analyzed.

Chemicals and Solutions. Methanol (OmniSolv) was used for extraction and in the HPLC solvents. Na ascorbate (100 mg) was dissolved in water, the pH adjusted to 7, and the volume made to 200 ml. BHT (200 mg) was dissolved in 800 ml of methanol. The methanolic and aqueous solutions were then mixed to give 80% methanol-BHT-ascorbate extracting solvent. Ethyl acetate and diethyl ether (separately) were extracted twice and three times, respectively, with one-half volume of water and filtered through a plug of cotton fiber (11). BHT was added to give 10 mg L−1. Water was HPLC quality.

\( \text{d}_{5-\text{cis}, \text{trans}}^{[2-14\text{C}]} \text{ABA} \) (947 MBq mmol−1) and 3-indolyl-[2-14C]acetic acid (2.04 GBq mmol−1) were obtained from Amer sham and used as internal standards. All glassware was silylated before use to minimize the possibility of adsorption of ABA and IAA to glass surfaces (3, 14).

Extraction and Bulk Purification. Portions of ground tissue, usually 200 to 1,000 mg dry weight, were placed in 125-ml Erlenmeyer flasks with 30 ml of extracting solvent. Twenty-five μl portions of [14C]ABA and [14C]IAA (about 6,000 and 10,000 dpm, respectively) were added as internal standards. The samples were stirred gently overnight at about 4°C on a magnetic stirrer. They were filtered with suction through Whatman No. 1 paper. The residues in the flask and on the filter were rinsed four times with 1.00 ml of methanol and extracted as above.

Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by United States Department of Agriculture.

1 Abbreviations: BHT, butylated hydroxytoluene or 2,6-di-tert-butyl-p-cresol; RFE, rotary flash evaporation; SAX, strong anion exchange.
with 10-ml portions of extracting solvent. Methanol was removed by RFE at about 35°C and the aqueous residue (about 10 ml) was transferred to 50-ml polypropylene centrifuge tubes. The flask, used for RFE, was rinsed with 10 ml of hexane and the hexane was then used to extract Chl and lipids from the aqueous residue (6). This step was repeated with another 10 ml of hexane. The pH of the aqueous phase was then adjusted to 8 with K2HPO4 and the samples were centrifuged 10 min at 12,100g. The supernatant fraction was partitioned twice against 10-ml portions of washed ethyl acetate-BHT to remove phenolics.

Some samples were subjected to alkaline hydrolysis for estimation of ester IAA and conjugated ABA. Instead of being adjusted to pH 8 with K2HPO4, they were made 1 N with respect to KOH and kept 1 h at about 25°C (2). The samples were centrifuged to remove a precipitate and the supernatant fractions were decanted into clean centrifuge tubes. Each sample was then partitioned three times against 10-ml portions of ethyl acetate-BHT to remove phenolics and other impurities. The ethyl acetate fraction was discarded.

Residual ethyl acetate was removed with 10 ml of hexane. The pH of the aqueous fraction was adjusted to 2.8 with H3PO4 and the acidified solution passed through a C18 cartridge (e.g., Waters Sep-Pak preconditioned with methanol followed by 1 mm HCl) to trap ABA and IAA. After the sample passed through, the C18 cartridge was rinsed with 10 ml of 1 mm HCl. ABA and IAA were then eluted with 5 ml of NH4OH and the pH quickly adjusted to 2.8 with H3PO4, ABA and IAA were extracted into three 10-ml portions of washed diethyl ether-BHT. Each 10-ml portion of ether was partitioned, in turn, against a 10-ml portion of 1 mm HCl to remove residual polar contaminants. (The same 10 ml of 1 mm HCl was used for all three 10-ml portions of ether.) The ether was evaporated by RFE (hood). The flask was cooled with a sponge dipped in ice water before releasing the vacuum. The residue was immediately dissolved in acetonitrile and transferred to a small centrifugal filter. The filtered samples were stored overnight (or longer when necessary) in a freezer.

HPLC. A 250 × 4.6-mm column of strong anion exchanger (5-μm spherical particles of Adsorbosphere SAX, Alltech Associates) was used for the next step of purification. Acetonitrile was evaporated with a stream of N2. The samples were dissolved in 50 μl of methanol and loaded into a Waters U6K injector with a 250-μl Hamilton gas-tight syringe (model 1825 with Teflon plunger). The syringe was partially filled with 200 μl of 0.02 N NH4OH before the sample was injected up so that all of the 0.02 N NH4OH passed through the syringe and needle. Development was isocratic with 80% methanol-0.02 N acetic acid at 1.5 ml min−1. A Waters model 440 fixed-wavelength UV absorbance detector (254 nm) and a Waters model 420-AC fluorescence detector (254 nm excitation, 360 nm emission) were connected in series and used to detect ABA and IAA, respectively (14).

The ABA fraction was collected in a small conical tube that contained 50 μg of BHT in 50 μl of methanol. The collected sample was concentrated to the aqueous phase, but not to dryness, under a stream of N2 at about 35°C. The IAA fraction was collected in a 100-ml spherical flask that contained 50 μg of BHT. Methanol was removed by RFE at 35°C; the sample was concentrated to the aqueous phase but not to dryness. The flask was lifted from the water bath and cooled with a sponge dipped in ice water before the vacuum was released. In some cases the IAA fraction was further purified on a new SAX column developed with 80% methanol-0.1 N acetic acid. Rheodyne switching valves were used for selecting solvents and columns.

A 250 × 4.6-mm column of octadecylsilane (5-μm spherical particles of Adsorbosphere or Econosphere C18, Alltech Associates) was used for final purification and quantification. The ABA and IAA fractions were injected and chromatographed separately. The samples were loaded with a bracketing technique that improved sample recovery and resolution (10). Development was isocratic with 50% methanol-0.02 N acetic acid at 1 ml min−1. The ABA and IAA fractions were collected in scintillation vials. Scintillation cocktail (Beckman ReadySolv MP) was added with mixing and the samples were counted for 4 min each in a Beckman LS 7500 liquid scintillation spectrometer.

Measurement of Radioactivity in Various Fractions. We used insoluble PVP in some of our earlier procedures. Radioactivity remaining in the insoluble PVP was measured by carefully extruding it from the glass column and slicing it into bands. The C18 cartriges were also cut into sections. These materials were combusted in a biological oxidizer (R. J. Harvey Instrument Corp., model OX400). The CO2 was trapped in phenylethyamine (18) and the radioactivity was measured in the scintillation counter.

To estimate losses in the various liquid fractions, the liquids were evaporated to dryness, the residue was combusted in the presence of a small amount of powdered sugar, and radioactivity of the CO2 determined. Fractions collected from the C18 column (HPLC) were counted without combustion.

GC-MS. For confirmation of identity, some samples without internal standards were subjected to GC-MS analysis. The putative ABA and IAA fractions, collected from the C18 column, were extracted into ether that had been washed with water, filtered through cotton, and dried by passage through dry Na2SO4 (11). The samples were methylated with diazomethane (15). The ether and methanol were evaporated with a stream of N2 in glass vials with screw-top caps fitted with Teflon-lined septa. As soon as the ether and methanol were evaporated the cap was tightened while N2 was flowing through a needle inserted through the septum. The vials were wrapped in Al foil and shipped to College Station, Texas for GC-MS analysis.

A Varian 6000 capillary GC was directly coupled to a VG 70-250 mass spectrometer. The column was an SPB-1 (Supelco, Inc.) fused silica capillary column, 30 m long by 0.25 mm i.d. The GC injector temperature was 250°C. The oven was programmed from 5 min at 80°C to 240°C at 15°C min−1 and then held at 240°C for 30 min. The mass spectrometer was run in the EI+ mode at 70 eV with the source temperature at 190°C and GC interface line heated at 260°C. Helium at 0.07 MPa was used as the carrier gas. Samples were dissolved in methanol for injection.

RESULTS AND DISCUSSION

Extraction and Bulk Purification. Aqueous solutions of methanol, ethanol, or acetone are most commonly used as extracting solvents (4, 14). We compared 80% methanol with 80% acetone. The acetone extract contained material which caused a darker amber color and somewhat larger contaminant peaks than 80% methanol. Otherwise, the results were similar (not shown).

IAA is readily destroyed by oxidation (11, 14). Therefore, precautions are required during extraction and purification to prevent excessive losses. Lino et al. (11) systematically studied this problem and recommended steps to minimize such losses. Their precautions included the washing of organic solvents with water, filtering them through cotton fiber, and the addition of the antioxidant BHT. In addition, we used ascorbate in our extracting solvent, as did Morgan and Durham (14), because BHT is apolar and unlikely to remain in the aqueous phase when it is partitioned against an organic solvent such as hexane or ethyl acetate.

Some workers have used insoluble PVP for partial purification of plant extracts (5, 7–9, 11, 12, 19). Phenolics are more effectively retained by PVP at low than at a higher pH (8), but IAA is also strongly retained at low pH (7). Pure IAA readily passes through insoluble PVP at pH 8, but we found that some of the
[14C]IAA was retained on PVP in the presence of plant extract, possibly because IAA combined with other organic components in the extract that were bound. Radioactivity remaining in the PVP column was associated with dark-colored material. Losses were variable and unpredictable. Furthermore, the use of insoluble PVP considerably increased the volume of aqueous material and increased the time required per sample, but did not greatly increase the purity of the extract at pH 8. Therefore, we discontinued its use.

An accumulation of aqueous phase was avoided. Rather than extract ABA and IAA into an organic solvent at low pH and then into aqueous solution at high pH, we adjusted the pH of the aqueous solution to 8 with K2HPO4, or higher with KOH (to hydrolyze ester IAA and conjugated ABA), and then extracted some of the impurities with ethyl acetate. Some ABA and IAA partitioned into ethyl acetate at pH 8 (Table 1), but these losses were considered acceptably small. A little IAA partitioned into hexane used to remove lipids before pH adjustment, but almost none went into hexane after the pH was adjusted to 8 (Table 1). Very little ABA partitioned into hexane either before or after pH adjustment, in agreement with results of Cjha et al. (6).

Centrifugation to remove a precipitate at high pH facilitated sample cleanup. A large amount of material precipitated from cotton boll extract, but little precipitated in the extracts of tree leaves. Alkaline hydrolysis caused these extracts to become very dark. Extraction with ethyl acetate at high pH after hydrolysis removed some of the UV-absorbing material.

We used Sep-Pak C18 cartridges to adsorb ABA and IAA from aqueous solution at low pH (5, 9, 12), but became concerned about possible losses due to overloading and to incomplete elution. Measurement of radioactivity indicated some loss due to incomplete retention when relatively crude extracts were used. Such losses would probably vary with the kind and amount of plant tissue used, with the volume of liquid, and with the stage of purification. Other apolar substances in the extract could overload the cartridge and cause incomplete retention of ABA and IAA. We also found some radioactivity remaining in the cartridges after elution of ABA and IAA, possibly because of ion pairing with organic cations to give less polar complexes. Recoveries were somewhat higher when C18 cartridges were not used, but the life of the SAX column was considerably shortened. Furthermore, the SAX column sometimes failed to resolve the ABA and IAA fractions from contaminants when the C18 cartridges were not used for preliminary cleanup. If large amounts of plant tissue are used, or if the tissue contains high concentrations of contaminants, it may be preferable to extract ABA and IAA into ether before loading onto the C18 cartridges. In that case, evaporate the ether by RFE (hood) and dissolve the residue in 0.5 ml of methanol. Add, with mixing, 5 ml of 1 mM HCl before loading the sample on the C18 cartridge. Rinse the cartridge with water or 1 mM HCl and discard the effluent. ABA and IAA can be eluted with methanol or dilute NH4OH. Pure methanol eluted more impurities than 50% methanol or 0.02 N NH4OH. Aqueous K2HPO4 buffer did not satisfactorily elute ABA and IAA from the C18 cartridges and should not be used.

Ethyl acetate and diethyl ether are the two organic solvents most commonly used for extracting ABA and IAA from aqueous solution at low pH. Ether is extremely volatile and tends to form peroxides that must be removed before use (11). Therefore, we preferred ethyl acetate. Attempts at measuring ABA and IAA in leaves of mulberry and ash, however, demonstrated the superiority of ether (Figs. 1 and 2). Ether extracted much less of the contaminants from the acidified aqueous phase than did ethyl acetate. ABA in the ether extract was cleanly separated from most of the UV-absorbing contaminants when chromatographed on the SAX column (Fig. 1), but ABA in the ethyl acetate extract was almost completely obscured by contaminants (Fig. 2).

**Chromatography.** ABA and IAA are both anions except at low

![Chromatograph of mulberry leaf extract on the SAX column after extraction from the acidified aqueous phase with diethyl ether.](image-url)
ABA is phosphate (a) it concentrated to C18 column. It will column of well works Guinn (9) and DEAE, respectively. ABA, from each of phase pH. Therefore, chromatography in an anion-exchange column works well for both. Several workers have used DEAE-cellulose or DEAE-Sephadex for partial purification of IAA (1, 12, 16). HPLC is capable of greater resolution than older methods that employ DEAE, TLC, or paper chromatography (4), and has the further advantage of sensitive on-line detectors that permit highly selective collection of fractions. Sweetser and Swartzfager (16) and Guinn (9) used microparticulate SAX columns for IAA and ABA, respectively. Both used 10-µm particles. Five-µm spherical particles give much better resolution and, therefore, better separation from contaminants (e.g. Figs. 1 and 3).

Sweetser and Swartzfager (16) used an aqueous mobile phase of 10 mM NaH₂PO₄ and 50 mM NaClO₃. We selected a mobile phase of 80% methanol-0.02 N acetic acid (800 ml methanol, 1.2 ml acetic acid, plus water to 1 L) for the SAX column because (a) it gave good separation of ABA and IAA from impurities and from each other and (b) the collected fractions were readily concentrated to a relatively small volume for loading onto the C18 column. (Note: If phosphate has been used in the SAX column it will not give the indicated performance until all phosphate is displaced by acetate.)

The retention time for IAA was much longer than that for ABA on the SAX column (Fig. 3). The retention times decreased over a period of months, but retention times for contaminants did not decrease as much as for IAA. Retention times were strongly influenced by pH; they were decreased by increasing the concentration of acetic acid or by substituting HCOOH.

Especially difficult samples were further purified by passing the IAA fraction from the first SAX column through a second (newer) SAX column. The different retention characteristics of the older (more used) and the newer SAX columns facilitated separation of IAA from impurities. We used 80% methanol-0.02 N acetic acid for the first SAX column and 80% methanol-0.1 N acetic acid for the second. The SAX columns were cleaned with 80% methanol-1.0 N acetic acid followed by pure methanol.

Alkaline hydrolysis of conjugates with KOH caused an increase in a UV-absorbing component that eluted slowly from the SAX column and sometimes contaminated the IAA fraction. This contaminant eluted quickly from the C18 column, however, and was well separated from IAA (Fig. 4).

Because their modes of separation differ, the SAX column and the C18 column are highly effective when used in sequence. In most cases, after cleanup on the SAX column, ABA eluted from the C18 column as a single dominant peak when monitored at 254 nm. Likewise, IAA eluted as a single major peak when monitored by fluorescence (Fig. 4). Extract from ash leaf tissue

![Fig. 2. Chromatograph of mulberry leaf extract on the SAX column after extraction from the acidified aqueous phase with ethyl acetate.](image-url)

![Fig. 3. Chromatograph of hydrolyzed cotton fruit extract on the SAX column showing the relative retention times of ABA and IAA.](image-url)
was cleaned with 8 when the ice samples contained strongly decomposed IAA when dried and evaporated. Jaworski quickly elutes this ABA in cotton. Despite that, interfering contaminants are still present. These results indicate that any procedure selected must be tested with the plant tissue of interest.

Precautions. BHT should be added to the tubes and flasks used for collecting ABA and IAA from the SAX column because BHT elutes quickly and thus becomes separated from ABA and IAA. Despite this precaution, we occasionally observed large losses of both ABA and IAA after purification on the SAX column. Mann and Jaworski (13) reported that IAA can be lost by sublimation when dried in vacuo. Iino et al. (11) thought it more likely that decomposition products were volatile. We suspect that ABA and IAA are very susceptible to oxidation if dried to a thin film and exposed to air while warm. Losses were minimal when the samples contained BHT, samples were evaporated only to the aqueous phase, and the flasks were cooled with a sponge (dipped in ice water) before air was admitted. Even though BHT absorbs strongly at 254 nm it does not interfere with quantification during chromatography on C18 because BHT is strongly retained by C18 when the mobile phase contains water. The C18 column was cleaned with 100% methanol.

Recovery through the entire procedure averaged about 75% for [14C]ABA and about 50% for [14C]IAA when the indicated precautions were taken.

Confirmation of Identity. Material tentatively identified as ABA co-chromatographed with authentic ABA in both the SAX and the C18 columns. Methylation and GLC on a glass column of OV-1 also yielded a peak that co-chromatographed with ABA when monitored with an electron-capture detector (not shown). Likewise, putative IAA co-chromatographed with authentic IAA on the SAX and C18 columns. Furthermore, the ratio of A at 254 nm to fluorescence was the same (±10%) as for authentic IAA. Confirmation of identities was obtained by GC-MS. The mass fragmentation patterns were very similar for authentic ABA and plant extract ABA (Fig. 5), and were virtually identical for IAA and plant extract IAA (Fig. 6).

Because contaminants vary with different tissues, freedom from interfering contaminants should be determined with specific tissue extracts by comparing results obtained with different detection methods. For example, GLC with electron-capture detection is frequently used for quantification of the methyl ester of ABA. A N-specific detector can be used for the detection of IAA separated by GLC, or an amperometric detector can be used with HPLC (16). The indolo-a-pyrone fluorescence method (11) is very sensitive and almost specific for IAA. Immunoassays are available for both ABA and IAA (17). The most definitive method may be GC-MS, for those fortunate enough to have

![Fig. 4. Chromatographs of IAA and ABA from cotton fruit extract on the C18 column after partial purification on SAX columns. The IAA sample was purified through two SAX columns before loading onto the C18 column.](image)

![Fig. 5. Mass fragmentation patterns of authentic ABA (upper) and ABA in hydrolyzed cotton fruit extract (lower).](image)
Chl and lipids before pH adjustment with minimal losses of ABA and IAA. Formation of emulsions was never a problem. Ethyl acetate removed some of the UV-absorbing contaminants from the aqueous phase, after it was adjusted to pH 8 or higher, with only small losses of ABA and IAA into the ethyl acetate. Aqueous phase did not accumulate because we did not extract from an organic into an aqueous phase. C18 cartridges were used to concentrate ABA and IAA from aqueous solution, and their use provided additional cleanup and protection of the HPLC columns. Elution of ABA and IAA from C18 cartridges with dilute NH$_2$OH or 50% aqueous methanol was more effective than elution with 100% methanol as a purification step. Diethyl ether was much superior to ethyl acetate for removing ABA and IAA from contaminants in the acidified aqueous phase.

The resolving power of 250-mm columns packed with 5-μm spherical particles of SAX and C18 facilitated the final stages of purification. Simple methanolic solvent systems for isocratic elution gave good separation of ABA and IAA from contaminants in both columns. Derivative formation was not necessary for chromatography nor was detection. Entire samples were collected for determination of radioactivity of internal standards, thereby increasing the precision of estimates of recovery.

The entire procedure is relatively simple, gives good recovery of both ABA and IAA, and, with most tissues tested, gives fractions sufficiently pure that ABA can be measured by A at 254 nm and IAA can be measured by natural fluorescence with on-line HPLC detectors.

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Fig. 6. Mass fragmentation patterns of authentic IAA (upper) and IAA in hydrolyzed cotton fruit extract (lower).