A Simple Purification of Indole-3-Acetic Acid and Abscisic Acid for GC-SIM-MS Analysis by Microfiltration of Aqueous Samples through Nylon

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ABSTRACT

A simple procedure was developed for the partial purification of plant tissue samples to be analyzed simultaneously for indole-3-acetic acid (IAA) and abscisic acid (ABA). The procedure relies on removal of contaminants by filtration through nylon and partitioning into dichloromethane. This procedure successfully purified both IAA and ABA from muskmelon, cotton, and broccoli tissue. Twenty individual samples can be purified and methylated in 8 h for analysis of free IAA and ABA with gas chromatography-selected ion monitoring-mass spectrometry. The use of microfiltration of aqueous samples through nylon offers new opportunities for improving the efficiency of existing sample purification procedures.

Many procedures have been developed for the isolation and purification of IAA and ABA from plant material (5, 6, 10, 11). Most of these procedures employ an initial extraction of the plant material with an aqueous organic solvent. The nonselective nature of this solvent system yields a highly complex sample containing many polar and nonpolar compounds including most of the plant pigments and polyphenolic compounds. The high concentrations of impurities relative to plant hormones require extensive purification for most analytical techniques (14). Frequently used labor-intensive procedures have required a lengthy sequence of steps including solvent partitioning, open column chromatography, and HPLC (5, 20). Recent procedures have been greatly simplified by using newly available preparative minicolumns and chromatographic cartridges that reduce the time required for sample preparation, particularly in GC-SIM-MS analysis (1, 18).

With the advent of improved microprocessor technology, a new generation of relatively low cost, table-top GC-MS instruments has become available for application to plant hormone analysis (15). GC-MS technology has been used recently in the quantitative analysis of several plant hormones including IAA and ABA (4, 8, 12, 17). The GC-MS analysis is particularly sensitive when only a few selected ions associated with molecular fragments unique to the hormone of interest are monitored (3, 15, 16). The high degree of detector selectivity coupled with the efficiency of capillary gas chromatography requires less sample purity than normally required for analysis of IAA and ABA from plant tissue.

Nylon preparations, including nylon 66, have been used to inactivate and remove polymeric compounds from plant samples (7, 9). Microfiltration through nylon 66 syringe filters was recently reported to be an effective step in the preparation of plant samples for the immunoassay of ABA (13, 21). Consequently, experiments were conducted to assess the potential value of using nylon 66 microfiltration in the preparation of plant samples for the analysis of IAA and ABA by GC-SIM-MS.

MATERIALS AND METHODS

Sample Preparation

Cotton floral buds (squares) and fruiting branches were harvested from field plots prior to anthesis. Muskmeon flowers were harvested from greenhouse-grown plants at anthesis. Broccoli heads including floral buds were harvested at commercial maturity from field plots. Each plant tissue was lyophilized, homogenized with a Polytron PT 10/35 (Brinkman Instruments, Westbury, NY) in 70% acetone (40 mL g⁻¹ dry weight that contained 200 mg of BHT and 100 mg of Na ascorbate L⁻¹) (6). Extraction was continued overnight at 4°C. Standards which included 50 nCi of [¹⁴C]IAA (Amer sham, 59 Ci mmol⁻¹), [¹³C₆]IAA (600 ng sample⁻¹), 100 nCi of [³H]ABA (Amer sham, 69 Ci mmol⁻¹) and [²H₆]ABA (600 ng sample⁻¹) were added at the beginning of each extraction. The acetone extract was suction filtered through Whatman No. 1 paper overlaid with a single layer of Miracloth (Calbi ochem) and evaporated to the aqueous phase by rotary flash evaporation at 40°C. The aqueous residue was removed and the flask rinsed twice with distilled water. The aqueous fraction and rinses were combined and the pH adjusted to 2.8 with 1 M H₃PO₄. The acidified fractions were then filtered through a nylon 66 filter (25 mm diameter, 0.22 μm pore

* Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may be suitable.

† Abbreviations: GC-SIM-MS, gas chromatography-selected ion monitoring-mass spectrometry; BHT, butylated hydroxytoluene; MeABA, methyl ester of ABA; MeI AA, methyl ester of I AA.
size, Micron Separations, Westboro, MA). The aqueous filtrate was partitioned three times against dichloromethane (CH2Cl2), and the combined CH2Cl2 fractions were evaporated to dryness for subsequent methylation.

**GC-SIM-MS Analysis**

Purified samples were methylated with diazomethane (2) and analyzed using a Hewlett Packard 5970 GC-MS. Two-μL splitless injections were chromatographed on a J&W Scientific (Folsom, CA) fused silica capillary column (DB-1701, 10 m × 0.177 mm i.d., 0.4 μm film thickness). After 1 min at 65°C, the column temperature was increased at a rate of 30°C min⁻¹ to 225°C and held for 5 min. The injector was set at 225°C and He carrier flow at 0.8 mL min⁻¹. The IAA levels were determined from peak areas generated by individual traces of ions at m/z 130, 136, 189, and 195 according to procedures described by Cohen et al. (3, 4). The ABA was determined from traces at m/z 162, 166, 190, and 194 using the isotope dilution equation described for IAA (3). Radiolabeled internal standards were used to estimate recoveries of the target compounds (1) and confirmed according to procedures presented by Vine et al. (19). The retention times for IAA and ABA were 7.95 and 9.25 min, respectively.

**HPLC Purification**

One portion of a duplicate sample prepared for GC-SIM-MS analysis was subjected to an additional purification step using reverse phase HPLC. A 100 μL sample was chromatographed on a 15 cm ODS-II column (Whatman, Hillsboro, OR) and detected at 280 nm using a Monitor II detector (Milton Roy/LDC, Riviera Beach, FL). The chromatography was conducted with a Milton Roy/LDC HPLC equipped with dual pumps delivering a gradient of 20 to 60% methanol in water (pH 3) over 20 min at 1 mL min⁻¹. The fractions containing IAA and ABA were collected and prepared for analysis by GC-SIM-MS. The results from HPLC purification were compared to results obtained from the remaining duplicate sample prepared exclusively by microfiltration. In an additional comparative procedure, samples were purified by a simple partitioning technique similar to that described by Vine et al. (19). The aqueous residue left after rotary flash evaporation as described in the section on sample preparation was adjusted to a pH of 8.5 with a saturated solution of NaHCO3 and partitioned 3 times against equal volumes of petroleum ether followed by 3 equal volumes of diethyl ether. The aqueous fraction was then adjusted to a pH of 2.8 with 1 N HCl and partitioned 3 times against equal volumes of ether. The ethereal fraction was evaporated to dryness and methylated for GC-SIM-MS analysis as previously described.

**RESULTS AND DISCUSSION**

Tissue samples from muskmelon floral buds, cotton floral buds, cotton fruiting branches and broccoli floral buds were purified according to the procedure described in Figure 1. Although the appearance of the extract differed among species and organs, microfiltration produced samples of free IAA and ABA that were of sufficient purity for direct GC-SIM-MS analysis. A plot of ions at m/z 130 and 190 representing MeIAA and MeABA, respectively, were resolved to baseline without interferences from other compounds (Fig. 2). The retention times for authentic standards were the same as target peaks in the sample extracts identified as MeIAA and MeABA (Fig. 3). A mass spectral scan of the target peak tentatively identified as MeIAA was identical to the scan of authentic MeIAA (Fig. 4). Ions in the scan at m/z 130 and 189 represented the base peak and parent ion, respectively, of MeIAA. A scan of the target peak representing MeABA was also identical to a scan of authentic MeABA (Fig. 5). The base ion at m/z 190 and the ion m/z 162 were used in the GC-SIM-MS analysis of ABA from sample extracts. Ions including the parent ion of MeABA occurring at m/z greater than 200 were not produced in sufficient quantities by electron impact ionization at 70 eV to make any detectable contribution to the spectral scan (12). The ratio of base peak to molecular ion for MelAA (130/189) and the ratio of m/z 162/190 for MeABA were the same as ratios determined for the respective authentic standards. Using cotton floral buds as a typical example, additional purification with reverse phase HPLC did not change the quantities of IAA and ABA purified by microfiltration and analyzed on GC-SIM-MS (Table I). Concentrations of the hormones in different plant tissues ranged from undetectable to 980 and 170 to 1360 ng g⁻¹ dry weight for IAA and ABA, respectively (Table II). Recoveries ranged from 50 to 60% for IAA and 60 to 70% for ABA.

The analytical procedure successfully quantified both ABA and IAA at several concentrations and from different tissue sources. Unmethylated samples or methylated samples of

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**Figure 1.** Procedure used in preparation of tissue samples for analysis of free IAA and ABA.
SIMPLE PURIFICATION OF IAA AND ABA BY MICROFILTRATION

Figure 2. Selected ion chromatograms of m/z 130 (MeIAA) and m/z 190 (MeABA) from muskmelon floral buds (A), cotton floral buds (B), cotton fruiting branches (C), and broccoli floral tissue (D).

Figure 3. Selected ion chromatogram at m/z 130 (MelAA) and m/z 190 (MeABA) from methylated extract of cotton floral buds (Sample) and authentic standards (Standard) of IAA and ABA.

Figure 4. Mass spectra of target peak identified as MelAA from methylated extract of cotton floral buds (Sample) and authentic standards (IAA).

Blank extracts without tissue gave no peak response at the retention times associated with MeIAA or MeABA. Therefore, the purification procedure was not considered to be a source of extraneous ions contributing to the MelAA or MeABA peaks. Using the GC-SIM-MS analysis, as little as 200 and 500 pg of IAA and ABA, respectively, can be detected and reliably quantified. Less than 500 mg dry weight of any tissue is required for the analysis. The success of this procedure relies on the unique ability of GC-SIM-MS to analyze relatively impure samples. Our experience with GC-SIM-MS indicates that even these crudely purified samples have not fully exploited the limits of this technology.

Using the microfiltration procedure, 20 individual tissue samples were prepared for GC-SIM-MS analysis within 8 h (Table III). The sample preparation time with this procedure was less than 50% of time required by solvent partitioning or HPLC. The microfiltration procedure also circumvented the need for prepacked cartridges, minicolumns, large volumes of
highly purified solvents or HPLC equipment. Effective filtration of the initial tissue extract must precede rotary flash evaporation and microfiltration to minimize obstruction of the micropores with precipitate and loss of filtration capacity. The microfilters can be cleaned by backflushing with methanol and can be recycled. The microfiltration procedure alone may not be adequate to purify samples treated with NaOH to hydrolyze the conjugated forms of IAA (1). The hydrolyzed samples contain large amounts of impurities that cannot be completely removed by microfiltration and interfere with the methylation step prior to GC-SIM-MS analysis. Consequently, samples prepared for the analysis of conjugated IAA require additional purification. Similar difficulties may be encountered in tissue samples containing high levels of interfering pigments and phenolic compounds relative to very low concentrations of free IAA (Suttle, USDA-ARS, Fargo, ND, personal communication).

Microfiltration of acidified aqueous samples through nylon 66 is a quick, simple, and effective step in the purification of IAA and ABA prior to GC-SIM-MS analysis. Additional purification using HPLC provided no improvement to the GC-SIM-MS analysis and was considered unnecessary. Extraction, purification, and analysis that previously required up to a week was to be conducted within 24 h. We have shown that the procedure is broadly applicable as demonstrated by the successful purification of tissue samples from several different plant sources. The retention times and mass spectra of target peaks confirmed the identity of IAA and ABA in the sample extracts. Although a relatively crude preparation, several hundred samples purified by microfiltration have been analyzed with no adverse effect on the operation or maintenance of the GC-MS. The application of this procedure to investigations with other tissues and additional plant hormones offers an opportunity to greatly accelerate physiological studies dependent on plant hormone analyses.

ACKNOWLEDGMENTS

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LITERATURE CITED


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Table I. Comparison of IAA and ABA Levels in Cotton Floral Buds Prepared by Microfiltration and Subjected to Additional Purification Using HPLC

<table>
<thead>
<tr>
<th>Purification Method</th>
<th>IAA (ng g⁻¹ dry wt)</th>
<th>ABA (ng g⁻¹ dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfiltration</td>
<td>980 ± 153</td>
<td>1268 ± 40</td>
</tr>
<tr>
<td>Microfiltration + HPLC</td>
<td>879 ± 135</td>
<td>1404 ± 138</td>
</tr>
</tbody>
</table>

* Values represent mean ± 1 SE.

Table II. Simultaneous Quantitation of Free IAA and ABA from Muskmelon Floral Buds, Cotton Floral Buds, Cotton Fruiting Branches, and Broccoli Floral Tissue Purified Using Microfiltration and Analyzed by GC-SIM-MS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>IAA (ng g⁻¹ dry wt)</th>
<th>ABA (ng g⁻¹ dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muskmelon floral buds</td>
<td>130 ± 20</td>
<td>401 ± 35</td>
</tr>
<tr>
<td>Cotton floral buds</td>
<td>984 ± 153</td>
<td>1360 ± 196</td>
</tr>
<tr>
<td>Cotton fruiting branches</td>
<td>80 ± 8</td>
<td>170 ± 9</td>
</tr>
<tr>
<td>Broccoli floral tissue</td>
<td>ND *</td>
<td>362 ± 50</td>
</tr>
</tbody>
</table>

* Values represent mean ± 1 SE.  
* Below the threshold level for detection.

Table III. Comparison of Microfiltration, Solvent Partitioning, and HPLC Techniques Used in the Purification of IAA and ABA for Plant Tissue Samples Prior to GC-SIM-MS Analysis

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Samples/Run</th>
<th>Time/sample (h)</th>
<th>Sample capacity (g)</th>
<th>Recovery (%)</th>
<th>Disadvantage</th>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfiltration</td>
<td>20</td>
<td>0.4</td>
<td>0.05–1.0</td>
<td>50–70</td>
<td>Precipitate obstruction of filter</td>
<td>6</td>
</tr>
<tr>
<td>Solvent Partitioning</td>
<td>8–10</td>
<td>0.9</td>
<td>0.05–1.0</td>
<td>40–60</td>
<td>Solvent waste, Separate fractions of sample impurities</td>
<td>1.3</td>
</tr>
<tr>
<td>HPLC</td>
<td>6</td>
<td>1.3</td>
<td>0.05–1.0</td>
<td>30–60</td>
<td>ABA</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* One support person in an 8-h day.  
* Combined range for IAA and ABA.