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Determination of IAA and ABA in the Same Plant Sample by a Widely Applicable Method Using GC-MS with Selected Ion Monitoring

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Abstract. A method for the purification and subsequent quantification of indole-3-acetic acid (IAA) and abscisic acid (ABA) from the same sample of highly pigmented green tissue has been developed and tested in several species. Solvent partitioning and high-performance liquid chromatography (HPLC) were used for purification. Separate fractions from HPLC-containing IAA and ABA were analyzed by gas chromatography-mass spectrometry (GC-MS) using selected-ion monitoring (SIM). Isotope dilution was used to correct for incomplete recovery. Results are presented for tissue samples from 11 different species and five different plant organs. The method can be completed, for both IAA and ABA, for two samples in 8 h by an experienced technician. IAA and ABA were the dominant peaks in the gas chromatograms from HPLC-purified samples, and amounts of about 1 ng can be detected. The extract was partitioned into an aqueous solution of pH 9.5, a step suspected of ester hydrolysis. By analyzing samples known to contain esters of IAA and ABA and comparing the results with methods which excluded this step, we have shown that this partitioning does not result in erroneously high values due to ester hydrolysis. A direct comparison of the method with one in which HPLC was not employed indicates that our method measures IAA and ABA in samples in which these compounds are not detectable when HPLC is omitted. Thus, HPLC is an essential purification step for samples where contaminating compounds co-purify with IAA and ABA through the solvent-partitioning steps.

In recent years, considerable effort has gone into the development of methods for the purification and quantitative determination of hormones in plant tissue extracts (Chen et al. 1988, Cohen et al. 1986, 1987, Dunlap and Guinn 1989, Funada et al. 1988, Sitbon et al. 1991, Subbaiah and Powell 1987, Vine et al. 1987). In this work, the goal has been to obtain good recovery of hormones and to improve their detection and quantification. The small amounts of hormones, their instability, and the co-purification of interfering compounds have presented major obstacles to the development of reliable routine methods.

During the last several years, we have been attempting to determine plant tissue hormone levels which are involved in the control of function (Li 1989) and developmental phenomena (Cloud 1987, Wear 1986). Our interest has been primarily in indole-3-acetic acid (IAA) and abscisic acid (ABA). In most cases, the plant tissues of interest have been highly pigmented tissues in which the amount of hormone is small compared to the compounds that co-purify with hormones through the standard purification steps. At least two papers have recently appeared in which analysis of both IAA and ABA levels have been reported (Gocal et al. 1991, Vine et al. 1987). In the first (Vine et al. 1987), no high-performance chromatography (HPLC) was used prior to gas chromatography-mass spectrometry (GC-MS), but, as we show here, this has not sufficed for quantifying our samples. We have not been able to get sufficient resolution of IAA and ABA from impurities in our samples during gas chromatography to allow quantitation. In the second (Gocal et al. 1991), separate purification procedures were used for the two hormones in the same sample.

Methods which have been developed for the assessment of IAA and its conjugates (Baldi et al.

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1989, Chen et al. 1988) make minimal use of preliminary partitioning steps prior to HPLC. Our experience is that many contaminating substances are removed by aqueous to organic phase partitioning. These steps result in much better resolution and quantitation. Furthermore, omission of these partitioning steps results in short lifetimes of HPLC columns. We routinely used Sep-Pac C_{18} cartridges prior to HPLC even after extensive solvent partitioning. Sitbon et al. (1991) used solvent partitioning and Sep-Pac but in a different order than we do.

For IAA determinations, we have attempted to modify the procedures used in Bandurski's and Cohen's laboratories (Cohen et al. 1986, Magnus et al. 1980) using the isotope exchange-free ^{13}C-IAA adopted in Cohen's lab. For ABA, the procedure which had evolved in R. Horgan's laboratory (Knox and Wareing 1984, Neill and Horgan 1985) was our starting procedure. It employs the exchange-free methyl-labeled $^{2}H_3$-ABA as internal standard (Neill and Horgan 1987). In general, we have attempted to simplify the procedures so that they require less time and labor and, at the same time, obtain quantitative measurements from a variety of tissues and species with the smallest sample size possible. Previously, 2 days were required to complete the procedure for both hormones.

We describe here our modified method allowing both ABA and IAA to be determined in the same highly pigmented sample at a rate of two samples per day. Because the method involves solvent partitioning at high and low pHs, we report data showing that specific steps in our method do not hydrolyze esters of IAA and ABA. To illustrate the need for purification by HPLC, the method is compared with a more rapid one recently reported by Dunlap and Guinn (1989). Data are reported for samples from several different plant species representing different organs and tissues. Data from corn shoots grown under growth-rate altering environmental conditions are also reported.

Materials and Methods

Plant Materials

The following plant parts were used for our analysis: leaves of soybean (*Glycine max* [L.] Merr.), sorghum (*Sorghum bicolor* L.), sunflower (*Helianthus annuus* L.), tobacco (*Nicotiana tabacum* L.), woolly cupgrass (*Eriochloa villosa* [Thunb.] Kunth) (Oyarzabal 1991), and tomato (*Lycopersicon esculentum* Mill. var Yellow Pear); bulb-scale leaves of onion (*Allium cepa*); shoots of corn (*Zea mays* L.); nodes of Japanese morning glory (*Ipomoea nil* [L.] Roth), strain violet (formerly known as *Pharbitis nil*); axillary buds and stems of green bean (*Phaseolus vulgaris* L. cv Improved Tender Green); and inflorescences of broccoli (*Brassica oleracea* L. cv *botrytis*). Tissues were variously field-, greenhouse-, and growth chamber-grown. When sampled, all tissues were immediately plunged into liquid nitrogen and stored at −77°C or below until extracted.

For corn shoots from growth-rate-altering temperatures, seeds were germinated and seedlings grown in vermiculite in a darkened incubator at 30°C or 14°C (Stewart et al. 1990). For growth-rate-altering water supply, seeds were imbibed then planted either in water-saturated vermiculite (560 g water/100 g vermiculite) or 16% water-saturated vermiculite (100 g water/100 g vermiculite) (Rayapati and Stewart 1991). Growth was at 30°C in a darkened incubator.

Chemicals

[$^{13}C_6$]-[benzene ring]IAA (Cohen et al. 1986) and racemic trideuterated ABA (RS-[3-methyl-$^{2}H_3$] ABA) were used as internal standards. The deuterated ABA was repurified by HPLC to remove *trans*-ABA before using it. Organic solvents were HPLC grade. Deionized water was filtered through a 0.45-μm filter.

Extraction and Purification

The standard, dual-purpose purification procedure is summarized in the flow diagram of Fig. 1. Frozen (−77°C) plant tissue samples of 0.5–5 g (fresh weight) were added to a mortar containing about 100 ml of liquid nitrogen. Approximately 3 g of sea sand were added and the tissue ground to a fine powder. Amounts of [$^{13}C_6$]IAA and [$^{2}H_3$]ABA internal standards equal to those expected in the tissue sample were added along with butylated hydroxytoluene (10 mg/g fresh wt) as an antioxidant.

After grinding, the sample was extracted three times for 20 min at 0°C in 8.3 ml of 80:20 (vol/vol)::acetone:water/g fresh weight of tissue. After filtering through two layers of Whatman no. 1 paper, the extract was reduced to the aqueous phase by flash evaporation at 35°C. All manipulations were done in a dimly lit room (<2 μmol m^{-2} s^{-1}) to prevent photoisomerization to *trans*-ABA. The pH of the extract was made basic with NaOH (0.1 or 0.5 N depending on the amount of tissue extracted) while being rapidly stirred. Equivalent results were obtained when the pH was adjusted to either 7.0 or 9.5, and the 9.5 pH did not result in the hydrolysis of ABA esters (see Table 3). The basic extract was partitioned three times against 5 ml of hexane/g fresh weight, and the pigment-rich hexane phase was discarded. The aqueous phase was acidified to pH 2.5 with 1 N HCl and partitioned three times against 30 ml of ethylacetate. The aqueous phase was discarded. The pooled ethylacetate phase (~90 ml) was flash evaporated at 35°C to near-dryness. Residual pigment was removed by dissolving the sample in 5 ml of 70:30 (vol/vol)::methanol:pH 8.5 water and passing it through a C_{18} Sep-Pak cartridge (Waters Assoc., Watertown, MA, USA) prepared by passing 5 ml of 100% methanol and 5 ml of the alkaline methanol solution through it. After the sample, two additional 5-ml volumes of the alkaline methanol were passed through the cartridge. The pooled eluates were dried in vacuo at 35°C. The residue was dissolved in three 0.4-ml portions of 20:80 (vol/vol::methanol:0.1 M acetic acid.

The sample was filtered through a nylon 66 filter (25 mm diameter, 0.45 μm pore size; Alltech Associates, Inc., Deerfield, IL, USA) prior to injection into HPLC. A total volume of 1.2 ml was injected into a 10 × 250 mm Phenomenex semi-preparative reverse-phase C_{18} column of 5 μm particle size. IAA and ABA were eluted from the column by a two-slope linear gradient using

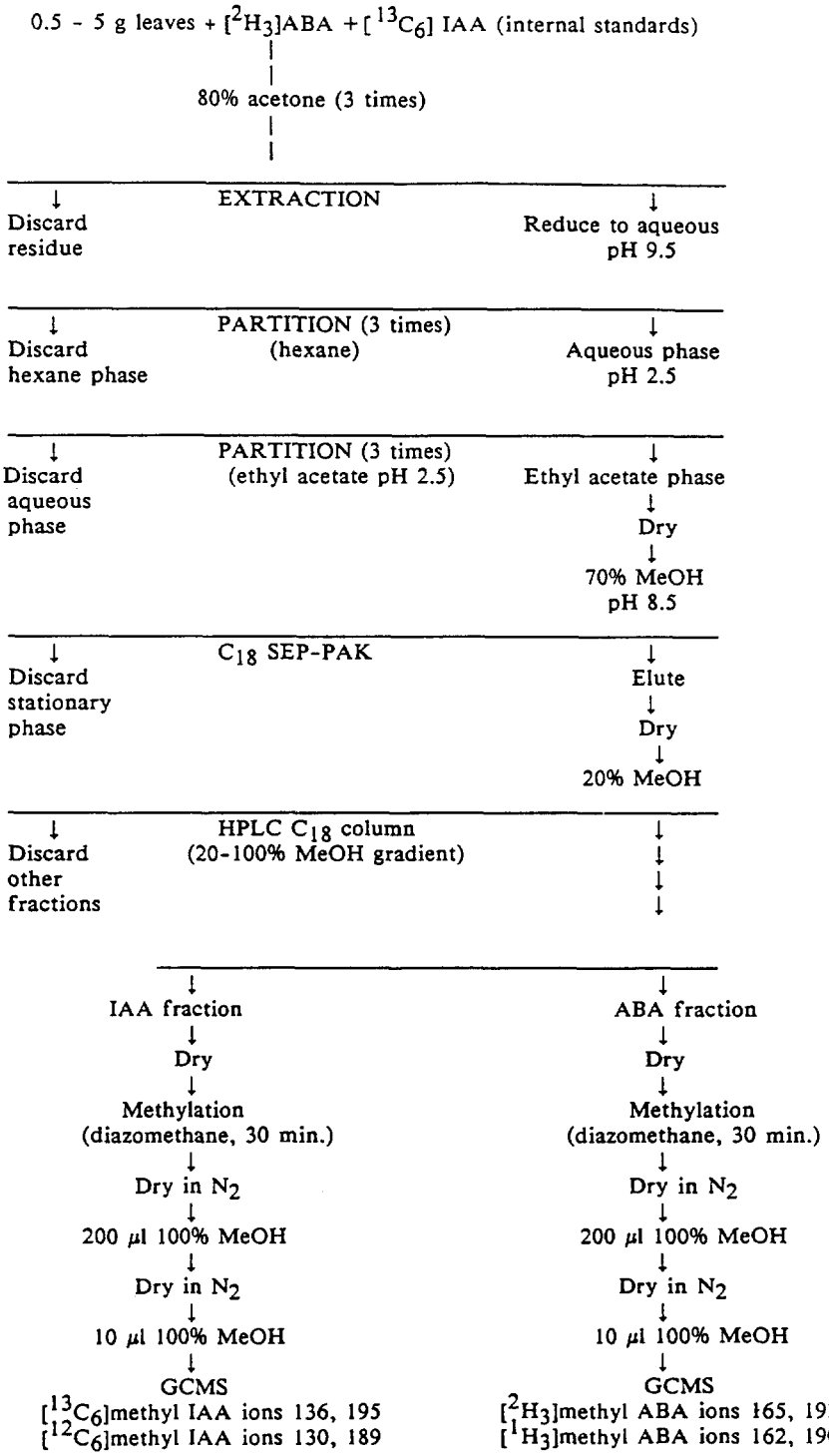

Fig. 1. Flow diagram for the extraction, purification, and determination of IAA and ABA from plant tissues.

a binary solvent system consisting of methanol and water containing 0.1 M acetic acid with a flow rate of 2.5 ml min^{-1}. The first-stage linear gradient was 20:80 to 45:55 (vol/vol)::methanol:0.1 M acetic acid for 11.6 min; the second-stage linear gradient was 45:55 to 80:20 (vol/vol)::methanol:0.1 M acetic acid for 20.2 min. The fractions from HPLC corresponding to IAA elution (23.3–26.6 min) and ABA elution (27.5–31.0 min) were collected separately and pooled (~10 ml) in 25-ml pear-shaped flasks and dried in vacuo at 35°C. The dry samples were methylated in 1 ml of a diazomethane solution (Fieser and Fieser 1967) for 30 min, dried in a stream of N_2, taken up in three rinses, of 100% methanol totaling 200 µl, transferred, to a 0.3-ml reac-

tion vial ("mini-vial" Reliance Glass Works, Inc., Bensenville, IL, USA), again dried under N_2, and finally dissolved in 10 μl of 100% methanol.

GC-MS of Methyl-IAA and Methyl-ABA

A gas chromatograph (Hewlett-Packard model 5890) linked to a quadrupole mass spectrometer (HP model 5970) was used in the selected-ion monitoring (SIM) mode. The MS ion source was operated at 70 eV and 200°C. The GC-MS interface was at 280°C. A capillary column (Econo-Cap SE-54, Alltech Associates, Inc., Deerfield, IL, USA) 30 m × 0.25 mm ID with a fused silica stationary phase coated with a 0.25-μm thick polydiphenyldimethylsiloxane was used. A 1.5 m × 0.25 mm ID deactivated fused silica "guard" column (Alltech Associates, Inc, cat. no. 602930) was attached to the chromatographic column to protect it. We routinely break off the first 15 cm of the guard column after every 10 or fewer samples (depending on observed "tailing" of the methyl IAA peak). The guard column is replaced when it becomes less than 75 cm in length. Using this routine, we have been able to run at least 200 samples on a chromatographic column and still obtain acceptable peaks. The guard column was necessary to protect the front end of the column from activation by plant-derived impurities.

Carrier gas was helium at a flow rate of 50 ml min^{-1}. Column temperature was programmed to change linearly from 100°–250°C at a rate of 10°C min^{-1} during 15 min, then stay at 250°C for 5 min. Duplicate 2-μl subsamples of each sample were injected using the splitless injection mode.

Methyl-IAA and methyl-ABA were analyzed separately by electron impact (EI) GC-MS. With exception of the corn samples, the four most abundant ions for both IAA and ABA were monitored. They were 130 and 189 m/z for methyl-IAA and 136 and 195 for methyl-[$^{13}C_6$]IAA, but the calculations of concentration presented here are based on the 130/136 ion currents except in one case noted in Table 1. In all samples with the exception of those from corn seedlings, ions of m/z 162 and 190 for methyl-ABA and 165 and 193 for methyl-[$^2H_3$]ABA were monitored, but the calculations of concentration presented here are based on the 190/193 ion currents only. In corn, a contaminating fragment ion at m/z 162 precluded its use in ABA determination. Instead, results with the 190 and 193 ion pair were checked against results from the 134 and 137 ion pair. Corrections for recovery were made using the isotope dilution equation of Magnus et al. (1980). For both IAA and ABA two kinds of calibrating GC-MS determinations were made. One was done routinely with each batch of samples by methylating a mixture consisting of the isotope-labeled hormone and a known quantity of unlabeled hormone. The second was done once on each batch of labeled hormone to derive a standard curve (see Neill and Horgan 1987). To do this, variable quantities of unlabeled hormone were mixed with a constant quantity of labeled hormone, and the mixtures were methylated and injected into the GC-MS. A regression equation, derived for each standard curve, was used in calculating IAA and ABA concentrations.

Onion scale-leaf ABA determinations made by EI-GC-MS were validated by negative chemical ionization GC-MS using the method of Rivier and Saugy (1986). A Finnegan model 4000 MS with a model 4500 ion source and a model 9610 GC with a DB-1 fused glass capillary column, 30 m × 0.25 mm ID, having a 0.25-μm polydimethyl siloxane film were used. Electron voltage was 70 eV; ammonia in the ion volume was at approximately 0.9 torr.

Table 1. IAA and ABA concentrations in various plant parts as determined in sets of the same purified plant samples.

Plant part	IAA conc. (ng g^{-1} fresh wt)	ABA conc. (ng g^{-1} fresh wt)
Japanese morning glory nodes	30	4.7
Bean primary-leaf nodes	32	14
Corn shoots	22	2.3
Bean axillary buds	194	83
Soybean leaves	14	203
Sorghum leaves	47	5.7
Sunflower leaves	30	7.3
Tobacco leaves	15[a]	13
Woolly cupgrass leaves	47	45
Onion bulb-scale leaves		
Adaxial epidermis	47	11
	—	12[b]
Parenchyma and abaxial epidermis	25	4.5
	—	4.7[b]
Broccoli heads	202	68

[a] Using ion-pair m/z 189/195 instead of 130/136 because of a contaminant at the leading edge of the m/z 130 peak (see Fig. 4).
[b] Determined by the identical method except that negative chemical ionization (ammonia) GC-MS (Rivier and Saugy 1986) replaced electron impact GC-MS.

Results

Qualitative and Quantitative Results Using the Standard Dual-Purpose Method

Quantitative measurements of IAA and ABA in samples from a variety of tissues and organs from various species are presented in Table 1, including highly pigmented leaf and bud tissues. All of the samples represented in Table 1 contained numerous contaminants in the fractions injected into the GC. Four ion chromatograms, each from an HPLC fraction, along with their respective single-ion traces are presented in Figs. 2 and 4 for the methyl-IAA fraction and Figs. 3 and 5 for the methyl-ABA fraction. Numerous peaks are present in the chromatograms of both IAA and ABA fractions, especially the ABA fraction from tobacco. Chromatograms from samples other than tobacco represented by the data in Table 1 indicated similar or smaller numbers and quantities of contaminants than those shown for tobacco (Figs. 4 and 5). We observed as many as five peaks, which were higher than the ABA peak, and as many as 10 peaks that were greater than half-height of the ABA peak. In all cases, however, the IAA and ABA peaks were reasonably well re-

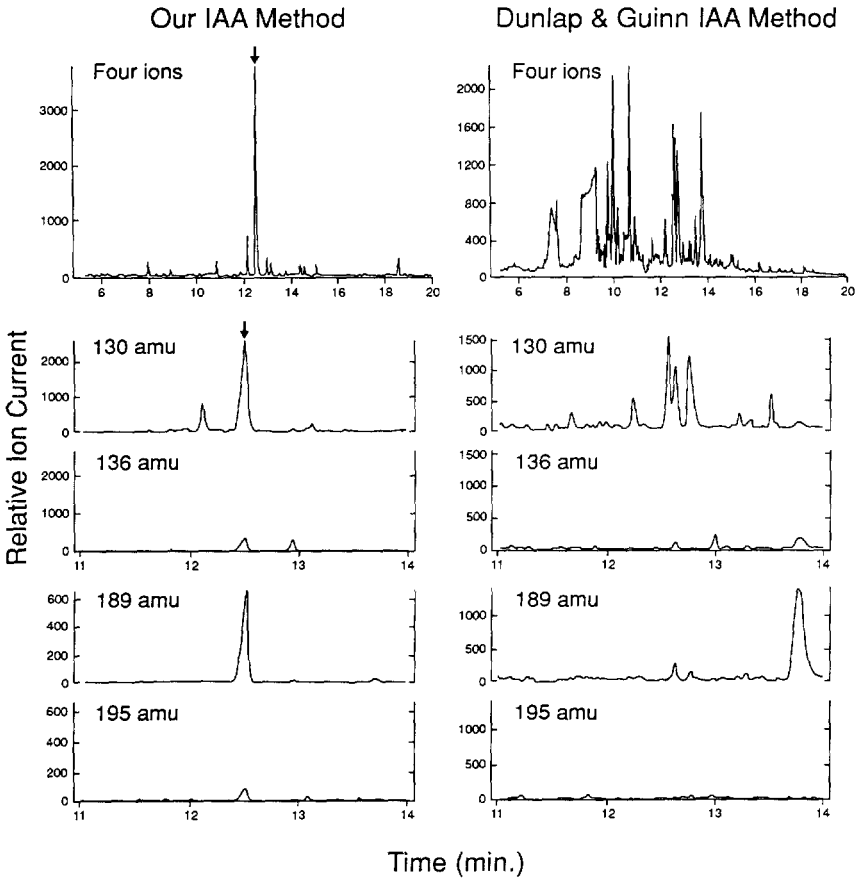

Fig. 2. GC retention time profiles showing ion currents for selected ions in the IAA-containing fraction of samples prepared from broccoli heads by our procedure and by that of Dunlap and Guinn (1989). The arrows designate the methyl-IAA peak.

solved from contaminants based on their retention times. This resolution can be seen in the single-ion current traces in Figs. 2–5. In general there were fewer contaminants in the IAA fraction than in the ABA fraction.

The identity of the methyl-IAA and methyl-ABA fractions was confirmed by comparing the EI mass spectrum of the methylated material from broccoli head having a GC retention time identical to methylated synthetic IAA and ABA (Sigma Chemical Co., St. Louis, MO, USA). These spectra were compared with a truncated National Bureau of Standards database which references amplitudes of the 10 most abundant ions for methylated IAA and ABA (see also Neill and Horgan 1987). Our purified materials from broccoli head showed a 95% spectral match for both IAA and ABA. Spectra from most of the samples we analyzed showed >98% spectral match.

Even though the observed spectra match well with the spectra in the truncated database, it is important to evaluate the purity of each sample for quantitative calculations. We have used the two ion pairs having the largest ion current for quantitation except in analyzing corn samples. Corn samples were monitored for the methyl-ABA fragment giving the first and third largest current. One test of whether or not these fragments derived from the hormone molecule is to compare the ratio of the two fragments from a sample with the same ratio of fragments from a standard (either an authentic methylated compound or the internal standard in each sample). For all data reported in this paper, the ratio was similar to that obtained for the standard.

Method Precision and Accuracy

From the corn shoot data of Table 3 methodological error can be estimated. To do so, IAA and ABA in triplicate aliquots from each extract were analyzed and the 95% confidence interval of each mean (a *t*-based statistic) was calculated. In both cases the 95% confidence interval was approximately ±10% of the mean.

To insure accuracy of the method described here, it was validated by determining onion tissue ABA concentrations obtained using it with those ob-

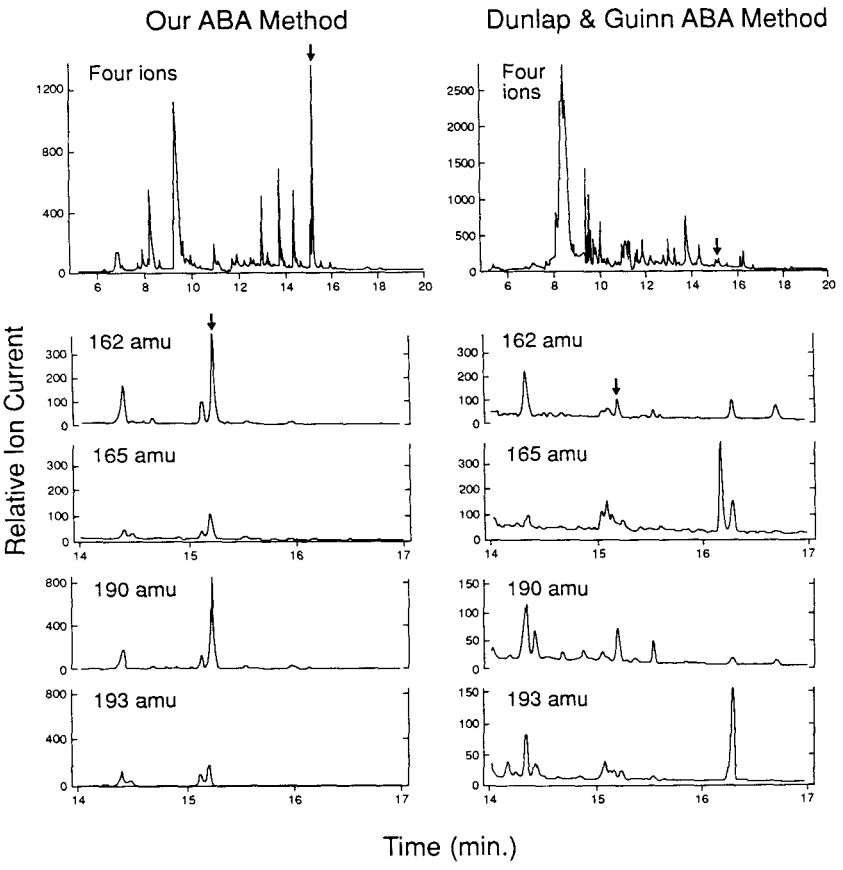

Fig. 3. GC retention time profiles showing ion currents for selected ions in the ABA-containing fraction in samples prepared from broccoli heads by our procedure and by that of Dunlap and Guinn (1989). The arrows designate the methyl-ABA peak.

tained using negative chemical ionization GC-MS for methyl-ABA with ammonia as the reagent gas. This latter method is at least 200-fold more sensitive than the EI-GC-MS used routinely by us. Both methods gave similar results (Table 1).

No Ester Hydrolysis During Partitioning at pH 9.5

Our method includes a step in which the pH of an aqueous extract is adjusted to 9.5, then partitioned against hexane. This partitioning is the most effective method for removing large amounts of contaminating substances, particularly pigments, which are abundant in many of the samples of interest to us. This step is not included in the procedure of Neill and Horgan (1985), and we had previously used a lower pH in a longer IAA purification procedure (Cloud 1987). Since this step is apparently advantageous in purification, we wanted to be sure that esters of IAA and ABA were not hydrolyzed so as to erroneously affect the determined levels of free IAA and ABA. To examine this question, we chose tomato leaves which are known to contain ABA glucose esters (Loveys and Milborrow 1981) and corn shoots which are known to contain IAA esters (Bandurski and Schulze 1974).

A comparison of the amounts of IAA and ABA obtained with our method with that of Neill and Horgan (1985) in which the extracts are maintained acidic throughout the initial purification steps is shown in Table 2. Even though these leaves are known to contain large quantities of ABA glucose ester relative to ABA (Loveys and Milborrow 1981), the amount of ABA was determined to be the same by both methods. The pH 9.5 partitioning step therefore did not hydrolyze ABA glucose ester and cause erroneously high values for ABA. The Neill and Horgan method was not developed for the purpose of determining IAA. However, IAA results comparable to ours were obtained using their method but the results with their method were more variable (Table 2).

A comparison of the amounts of IAA and ABA in corn shoots, a tissue with large amounts of IAA ester, using two different pH values at the same water-hexane partitioning step is presented in Table

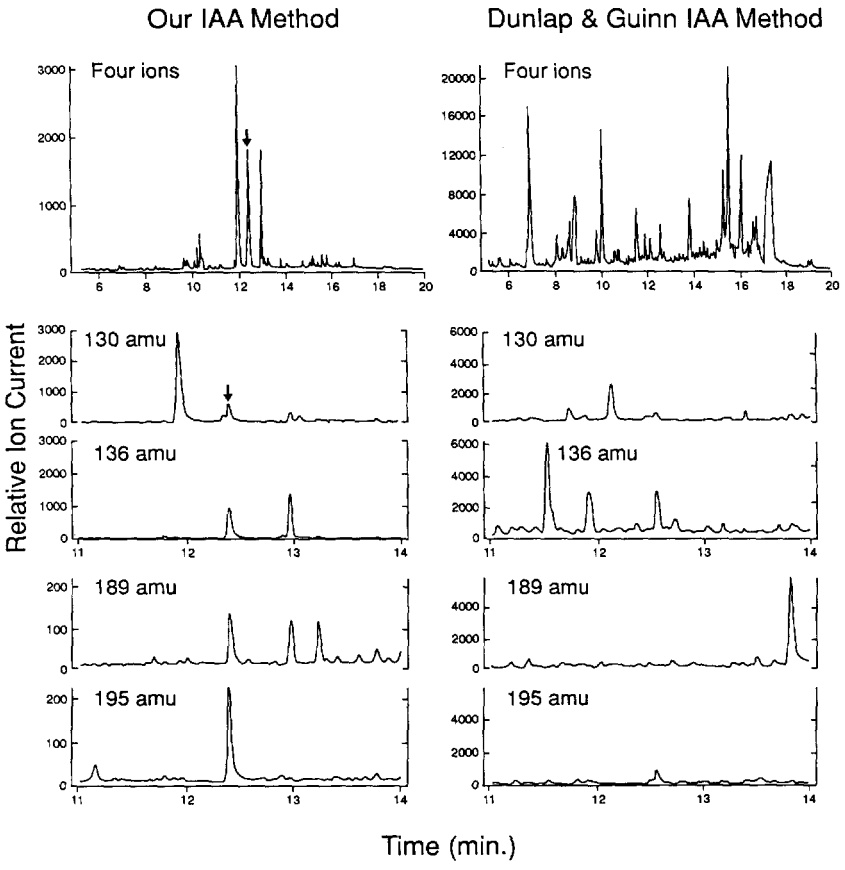

Fig. 4. GC retention time profiles showing ion currents for selected ions in the IAA-containing fraction in samples prepared from tobacco leaves by our procedure and by that of Dunlap and Guinn (1989). The arrows designate the methyl-IAA peak.

3. The results clearly indicate that pH 7.0 and pH 9.5 gave the same quantities of IAA and ABA. Thus, there was no hydrolysis of IAA ester at the pH 9.5 partitioning step. The partitioning was equally effective in the removal of large quantities of impurities, particularly pigments, at both pH values.

HPLC Requirement

We compared our method with the recently published method of Dunlap and Guinn (1989), which omits HPLC, processes several samples simultaneously, and, thus, decreases sample purification time and labor. Chromatograms from the ABA- and IAA-containing samples from broccoli heads and tobacco leaves prepared by the two methods are presented in Figs. 2–5. Samples prepared from these tissues by our method were suitable for IAA and ABA analysis by GC-MS. When HPLC was omitted, only the ABA-containing fraction from broccoli was suitable for analysis by GC-MS (Fig. 3). The ion current from methyl-ABA prepared with HPLC was 11.6 times higher (m/z 190 = 18,926 "counts") than that of the sample prepared without HPLC (m/z 190 = 1636 "counts"). Much better resolution of the methyl-IAA and methyl-ABA peaks was observed when HPLC was employed. Furthermore, a much more frequent replacement of the GC guard column was required when the samples had not been HPLC purified. Even with the careful use of the guard column, the life of a chromatography column would be much shorter when injecting the less pure samples. Based on our observations of methyl-IAA peak tailing when using HPLC-purified samples, methyl-IAA is highly susceptible to column activation caused by impurities. Sample purification without HPLC could, therefore, result in very high GC column replacement costs.

Our quantitative results using the method of Dunlap and Guinn (1989) agree with their report according to which the IAA from broccoli heads was below the threshold level for detection (Fig. 2). However, ABA could be determined by them and was found to be 362 ng g^{-1} dry weight, which is similar

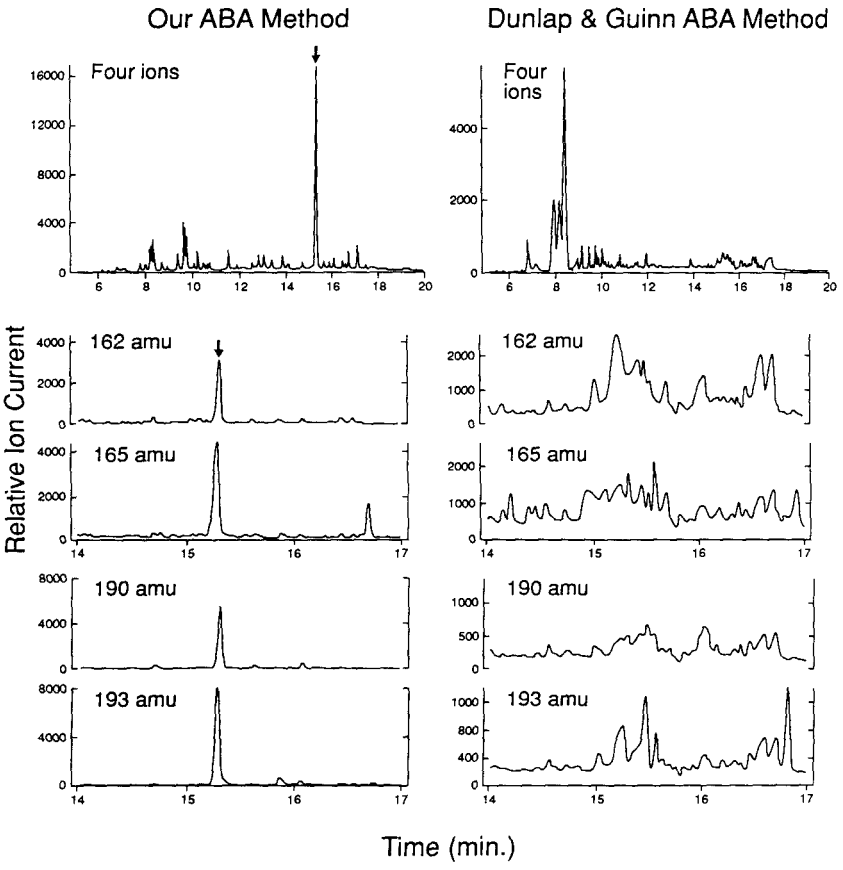

Fig. 5. GC retention time profiles showing ion currents for selected ions in the ABA-containing fraction in samples prepared from tobacco leaves by our procedure and by that of Dunlap and Guinn (1989) The arrows designate the methyl-ABA peak.

to our estimate of 417 ng g^{-1} dry weight (43 ng g^{-1} fresh weight) using their method. Inclusion of HPLC in the purification procedure allowed us to measure IAA, as well as ABA, in broccoli heads (Table 1, Figs. 2 and 3). Including HPLC yielded reliable estimates of IAA and ABA in tobacco leaves (Table 1), whereas we were unable to detect either without its inclusion (Figs. 4 and 5).

Table 2. IAA and ABA concentrations in tomato leaves measured by the method described herein and by the method of Neill and Horgan (1985) which differ in the pH of the early purification steps.

Method	IAA conc. (ng g^{-1} fresh wt)[a]	ABA conc. (ng g^{-1} fresh wt)[a]
Present study (≤ pH 9.5)	20.7 (0.4)	77.2 (2.6)
Neill and Horgan (≤ pH 4)	26.6 (3.6)	74.7 (2.4)

Samples were ground, and methanol and internal standards were added. The extract was then divided into six subsamples. Three subsamples were processed identically using each method as described in Materials and Methods.

[a] Mean and SE (in parentheses) of triplicate subsamples.

Hormone Concentrations in Plants Grown Under Growth-Altering Conditions

IAA and ABA concentrations in corn seedlings grown under different temperature and moisture conditions are shown in Table 4. Low temperatures retards growth and has been shown to alter the lev-

Table 3. IAA and ABA concentrations in corn shoots measured in samples purified as described in Materials and Methods using two different pH values in the water-hexane partitioning step.

pH of aqueous phase during hexane partitioning	IAA conc. (ng g^{-1} fresh wt)	ABA conc. (ng g^{-1} fresh wt)
7.0	29.8 (0.3)[a]	2.3 (0.1)[a]
9.5	30.7 (0.5)[b]	2.2 (0.1)[b]

Samples were ground, and methanol and internal standards were added. The extract was then divided into subsamples. During the water-hexane partitioning step, subsamples were partitioned after raising the pH to 7.0 or 9.5. All other steps were identical.

[a] Mean and SE (in parentheses) of duplicate subsamples.

[b] Mean and SE (in parentheses) of triplicate subsamples.

Table 4. IAA and ABA concentrations in samples from shoots of corn seedlings grown under growth-rate altering conditions.

Growth conditions	Age[a] (h)	IAA conc. (ng g^{-1} fresh wt)[b]	ABA conc. (ng g^{-1} fresh wt)[b]
Well-watered, 30°C	56	22.4 (0.04)	2.3 (0.4)
Well-watered, 14°C	288	15.2 (0.1)	10.1 (1.4)
Well-watered, 30°C	96	43.2 (3.8)	2.1 (0.8)
Drought-stressed, 30°C	140	19.0 (1.5)	5.1 (0.005)
Drought-stressed and rewatered, 30°C	156	32.5 (0.1)	0.9 (0.1)

[a] The well-watered 56-h seedlings grown at 30°C were at the same developmental stage as the 288-h seedlings grown at 14°C. The well-watered 96-h seedlings grown at 30°C were at the same developmental stage as the 140-h drought-stressed seedlings. The rewatered seedlings were only slightly larger than the drought-stressed seedlings.

[b] Mean and SE of determinations on duplicate samples.

els of the alternative respiratory pathway (Stewart et al. 1990). Drought stress reduces growth rate in corn seedlings (Sharp et al. 1988) and alters the level of mitochondrial proline dehydrogenase (Rayapati and Stewart 1991). The effect of temperature is seen by comparing the hormone concentrations from seedlings grown 288 h at 14°C with those grown 72 h at 30°C. In the slower growing seedlings (low temperature), the levels of IAA were lower and of ABA higher.

The effect of drought on ABA was less than expected for a shoot water potential of −0.7 MPa. Rewatering decreased the ABA level to the well-watered level (Table 4). As mentioned above, a large ion current was observed at 162 m/z in the samples from corn seedlings compared to that expected from examining ion currents at 165, 190, and 193. This contaminant was particularly prominent in the samples from drought-stressed seedlings. Drought decreased the IAA level to about one-half of the well-watered level. This drought-induced low level was increased upon rewatering. A longer time after rewatering might have allowed the IAA levels to return to that in the well-watered shoots. Rapid growth had not resumed in the rewatered shoots 16 h after rewatering.

Discussion

Prior to this research, our analytical procedures for IAA and ABA analysis required two full days. Different samples and separate procedures were used for the analysis of the two hormones, thus introducing both methodological and biological variation. Furthermore, IAA measurements in the literature were primarily from tissues with a relatively high ratio of IAA to contaminating, co-purifying substances. With the method described here, one person familiar with the procedures can complete the extraction, purification, and analysis of two samples for both IAA and ABA in the same day. The results presented in this paper show that the method is suitable for a variety of plant samples that have interfering substances, such as those in highly pigmented leaf tissues. Quantitative recovery of IAA through the whole protocol is ≥30% (Cloud 1987) as determined by ^{14}C-IAA dilution. The method should be particularly useful for establishing hormone identity and for validating and calibrating the antibody methods, generally less selective but more rapid and sensitive than GC-MS.

The method described here is widely applicable. In addition to the 11 species of angiosperms whose analysis is reported here, we have also successfully applied this method to the bryophyte, *Marchantia polymorpha* (data not shown).

Some of the partitioning steps we have used prior to HPLC have been considered unnecessary for hormone determinations in some plant tissues. The susceptibility to alkaline hydrolysis of IAA and ABA esters has lead to the omission by others of the high pH (>7.0) aqueous-hexane partitioning step. The effectiveness of this partitioning step in removing contaminants, particularly pigments, led us to include it. Our results comparing the procedures with and without this step by using them on tissues relatively rich in esters of IAA and ABA allows us to conclude that no hydrolysis occurs under the conditions specified for the partitioning. Furthermore, the partitioning is equally effective at pH 7.0. The findings of Table 3 extend the findings made in maize kernel extracts by Baldi et al. (1989) to green leaves of maize. Baldi et al. (1989) made an extensive study of the effect of pH and time of exposure on hydrolysis of IAA esters, but did not examine de-esterification of ABA esters. Thus, we are confident that this step does not result in erroneous data due to ester hydrolysis and that it is worth including in the procedure for samples with large amounts of co-purifying contaminants.

It is apparent that an HPLC purification step is required for hormone analysis by GC-MS in order for the method to be applicable for a large range of plant samples. Any samples other than floral buds (Dunlap and Guinn 1989) and other rapidly developing parts containing relatively high hormone levels prepared without this purification step simply contain too many interfering substances to permit reliable quantitative determination of IAA and ABA. In the case of IAA, it is not detectable in some samples without HPLC purification even though the level, determined using HPLC (see broc-

coli in Table 1), was higher than any other tissue IAA level measured in this study. Even with floral buds, frequent replacement of columns will be necessary to avoid IAA "tailing."

The tissue IAA concentrations reported here match rather closely those reported in the literature for corn (Bandurski and Schulze 1974) and tobacco leaves (Chen et al. 1988, Thornburg and Li 1991) using similar methodology. In the axillary buds of bean, the average concentration of IAA measured by us (194 ng g^{-1}) falls into the low end of the range (106–600 ng g^{-1} fresh weight) calculated by us from the data provided by Gocal et al. (1991) and below the range (330–1080 ng g^{-1}) found by Cohen et al. (1987) using a GC-MS-validated ELISA for IAA.

Tissue ABA concentrations reported here for leaves of corn are within the range (2–21 ng g^{-1} fresh weight) reported earlier by Neill et al. (1986) for unstressed, wild-type seedlings. For leaves of unstressed tomato plants, we found a level of 76 ng g^{-1}, whereas Neill and Horgan (1985) have reported levels somewhat higher (111–423 ng g^{-1} fresh weight) in another variety. In broccoli inflorescences, we found ABA concentrations 1.8 times higher than that calculated by us (38 ng g^{-1} fresh weight) using the data of Dunlap and Guinn (1989) and our own dry and fresh weight measurements (dry weight is 10.4% of fresh weight). Such differences can, of course, be attributed to sampling differences as well as to methodological differences. In bean stems, our average ABA estimate is one-sixth of the 80 ng g^{-1} fresh weight reported by Knox and Wareing (1984). Likewise, in measurements made on bean axillary buds, our ABA estimate of 83 ng g^{-1} was well below the range (460–2000 ng g^{-1} fresh weight) calculated by us from data reported by Gocal et al. (1991).

We believe our method is reliable for the determination of IAA and ABA in plant samples and provides physiologically interpretable data. Environmental conditions which slowed the growth of corn shoots lowered the level of IAA and increased the level of ABA. These results are as expected, based on the general knowledge that IAA promotes and ABA inhibits shoot growth. The relatively small effect of drought on ABA levels in corn seedlings could be a result of the mild stress (-0.7 MPa) and its long duration which may have allowed degradation of ABA accumulated earlier. The disproportionately large ion currents at 162 m/z, an ABA fragment, in both stressed and rewatered samples, suggest that at least one contaminant was present in the samples that fragments to 162 m/z in the mass spectrometer. The data suggest that this contaminant is more abundant in the stressed than the unstressed shoots.

We are now using this method routinely in several physiological studies that require IAA and ABA analysis. These include samples from (1) transgenic plants, (2) plant tissues representing developmental sequences, (3) tissues known to control the development of other plant parts, (4) plants grown under varying environmental conditions, (5) field-grown plants that are component parts of agronomic studies, and (6) plants other than Tracheophytes. Thus, the method is suitable to a broad range of experimental applications.

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