Plant Physiol. (1988) 86, 822–825
0032-0889/88/86/0822/04/$01.00/0

A Rapid and Simple Procedure for Purification of Indole-3-Acetic Acid Prior to GC-SIM-MS Analysis[1]

Received for publication August 19, 1987 and in revised form November 2, 1987

KAI-HSIEN CHEN[2], ANITA N. MILLER[3], GLENN W. PATTERSON, AND JERRY D. COHEN*
Department of Botany (K-H.C., G.W.P.) *and Department of Horticulture* (A.N.M.), *University of Maryland, College Park, Maryland 20742; and Plant Hormone Laboratory, Beltsville Agricultural Research Center-West, Beltsville, Maryland 20705* (K-H.C., J.D.C.)

ABSTRACT

A simple and rapid procedure for the purification of indole-3-acetic acid prior to gas chromatography-selected ion monitoring-mass spectrographic analysis was developed using an amino anion exchange minicolumn and a short high resolution C18 column. Since multiple samples can be prepared at one time, the procedure is more rapid and the sample preparation time is reduced to one-third that normally required. In addition, the final recovery was improved by 40 to 50% over that of a solvent partitioning procedure.

Techniques for identification and quantitative analysis of the plant hormone IAA have been recently improved by the utilization of HPLC and GC-MS (12) and by the use of proper internal standards, for example d_4-IAA (3, 4, 10, 14, 16), d_5-IAA (1, 2, 10, 15), and [$^{13}C_6$]IAA (6, 13, 17). Solvent extraction, concentration, and solvent partitioning have been used for IAA purification although these procedures are lengthy, laborious, and result in low recoveries. In addition, problems with solvent purity may lead to a total loss of IAA from the sample (6). The method we present here replaces solvent partitioning and eliminates most of the evaporation steps. This method, based on disposable minicolumns, is a rapid way to isolate IAA from plant material and is especially suitable for further analysis by GC-MS.

MATERIALS AND METHODS

Approximately 0.03 to 3 g of *Avena sativa* pulvini tissue from plants grown as previously described (7) were ground in a Waring Blender[4] or mortar and pestle with 4 ml/g of sample in 65% iso-

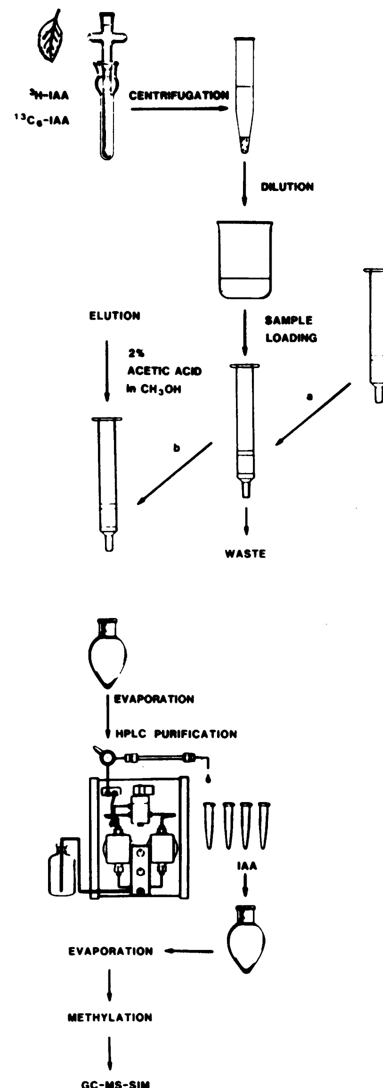

FIG. 1. Diagram of the procedure for analyzing free IAA. The amino column was washed sequentially with hexane, acetonitrile, and double distilled water (2 ml each). It was the then conditioned with 2 ml of 200 mM imidazole buffer to pH 7.0, followed by washing with 10 ml of double distilled water to remove the excess buffer (a). Following application, the sample was purified on the column by sequentially washing with 2 ml each of hexane, ethyl acetate, acetonitrile, and methanol (b). The IAA fraction was then eluted with 2% acetic acid in methanol, evaporated, purified by HPLC, methylated, and analyzed by GC-SIM-MS.

[1] This work was carried out under cooperative agreement No. 56-32U4-7-001 (RSA-7-001-010) of the United States Department of Agriculture/Agricultural Research Service and the University of Maryland and, in addition, was supported by a United States Israel BARD grant U.S.-842-84 and by National Science Foundation Metabolic Biology grant DMB-8617171.

[2] Present address: Department of Pharmacology and Molecular Science, Johns Hopkins University, Baltimore, MD 21205.

[3] Present address: Horticulture Department, Oregon State University, Corvallis, OR 97331.

[4] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

propanol with 0.2 M imidazole (Kodak, recrystallized from methanol) buffer pH 7.0. [$^{13}C_6$]IAA as an internal standard (0.1–1 μg/g sample) and approximately 50,000 dpm of ^{3}H-IAA (22.5 Ci/mmol, Amersham) as a radiotracer were added. After the isotopes equilibrated in the extract for 1 h at 4°C, the extract was centrifuged at 10,000*g* for 5 min. The supernatant fluid was then divided into three equal parts, the first for analysis of free IAA, the second was hydrolyzed for 1 h at 25°C with 1 N NaOH to measure free plus ester IAA, and the third was hydrolyzed for 3 h at 100°C with 7 N NaOH in order to measure total IAA (free + esters + amides). All assays were performed in duplicate.

For free IAA analysis, a 4 ml aliquot was diluted to 25 ml with double distilled water to reduce the isopropanol concentration to about 10%. The diluted extract was then applied to a conditioned amino anion exchange column (BAKER-10 SPE 3 ml, Baker; PrepSep 0.3 g, Fisher; or Extra-Sep 2.5 g, Lida; see Fig. 1). Flow was adjusted to about 5 ml/min using a Baker Extraction System. After the diluted extract passed through the column, aspiration was continued for 30 s to remove the excess water, and the column was washed sequentially with hexane, ethyl acetate, acetonitrile, and methanol (2.0 ml each). The IAA was eluted from the amino column using 3.0 ml of methanol containing 2% acetic acid. The acidic methanol eluent was evaporated to near dryness using a rotary evaporator then to dryness using a microrotary evaporator with a two-stage vacuum pump. The residue was resuspended in 100 μl of 50% methanol for HPLC purification. The HPLC procedure was similar to that used in our prior studies (6, 13, 17) except that a rapid analytical column (12.5 cm × 4.6 mm) was eluted with 20% acetonitrile/water and 1% acetic acid. The C18 column was packed with 1.2 g of 5 μ Whatman ODS-3 resin using a slurry of 1:1 ethylene glycol:methanol at 8500 psi. The radioactive fractions collected from HPLC (Fig. 2) were pooled, reduced to dryness, resuspended in 100 μl of methanol, methylated using ethereal diazomethane (5), and then analyzed by GC-SIM-MS as previously reported (6).

For determination of the IAA in free plus ester form, 2-propanol was removed *in vacuo* and equal amounts of 2 N NaOH added. After hydrolyzing the ester conjugates for 1 h at room temperature, the hydrolysate was diluted, titrated to pH 2.5 with 1 N HCl and passed through a conditioned (washed with hexane, methanol, water, and 1% acetic acid successively, each 5 ml) Baker C18 column to desalt. The C18 column was rinsed with 5 ml of distilled water, and the IAA eluted with 2 ml of acetonitrile. Imidazole buffer (20 mM, pH 7.0) was added to the eluent to dilute the organic phase to 10%, and then the sample was applied to the conditioned amino column and prepared following the separation procedure described above for free IAA.

For determination of total IAA, appropriate amounts of NaOH pellets were added to the extract after evaporation of the 2-propanol. The hydrolysis was done in a capped PTFE vial purged with water saturated nitrogen gas for 3 h at 100°C. The hydrolysate was diluted, and titrated with 2 N HCl on ice to pH 2.5. It was then passed through the conditioned C18 column and prepared following the separation procedure described above for free and ester IAA.

Identical procedures to those described above were used for the analysis of IAA in field grown peach fruit pericarp (13), greenhouse grown tobacco leaves (*Nicotiana glauca* and *N. langsdorffii*), and caryopses obtained from imbibed wheat seeds (*Triticum aestivum* cv Itana).

RESULTS AND DISCUSSION

The technique described above (Fig. 1) has been applied to measure the amount of IAA in oat pulvini, as well as in a variety of plant samples including *Arabidopsis*, corn (leaves and seeds), peach (pericarp, mesocarp and ovule), cherry (stems and buds), tobacco (leaves, stems, and genetic tumors), *Kalanchoe* crown gall tumors, and wheat aleurone layers (data not shown). In these studies, the recovery of IAA typically ranged from 30 to 70% based on recovery of radioisotope internal standards. Recovery

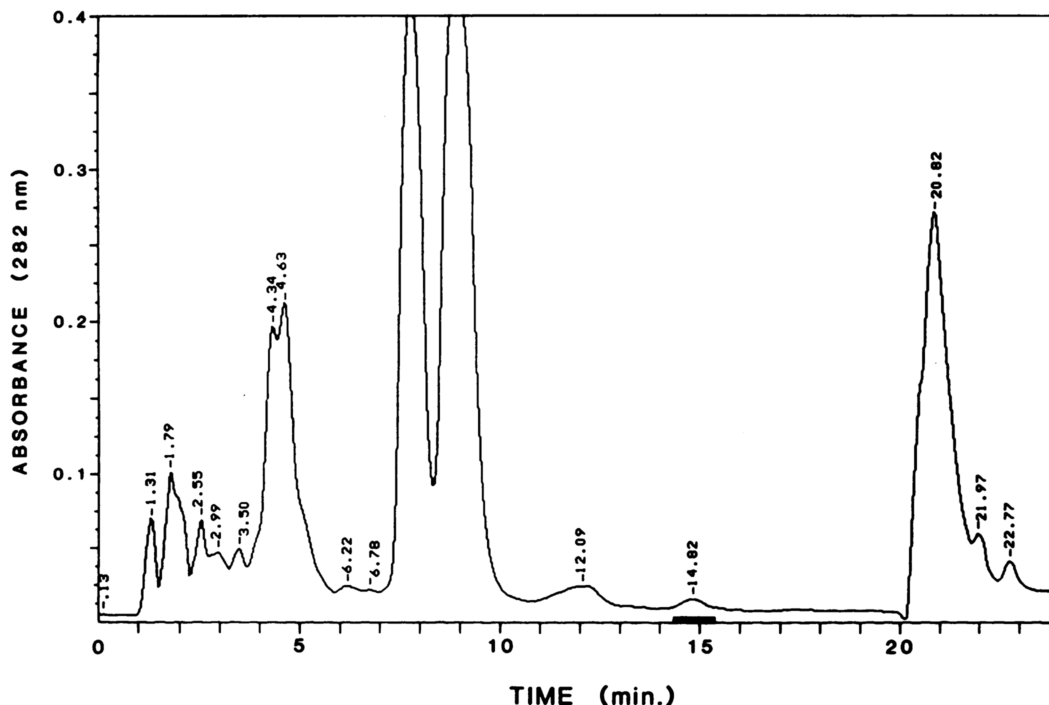

FIG. 2. HPLC chromatogram of amino column purified oat pulvini tissue. The column was 12.5 cm × 4.6 mm packed with Whatman ODS-3 5 μm, the mobile phase was 20:79:1 acetonitrile:water:acetic acid, flow rate was 1 ml/min. Fractions between 14.3 min and 15.3 min contained the radioactivity from [^{3}H]-IAA.

Table I. *Recoveries of IAA following Purification Prior to Analysis by GC-SIM-MS*

Plant Material	Sample Size	Recovery	IAA Level
	g	*%*	*ng/g fresh wt*
Experiment series 1			
Prunus persica cv. Redhaven (pericarp of developing fruit, free IAA)			
a) Purification using an amino minicolumn[a]	1.0	62 ± 4	7
b) Purification as in Refs. 6 and 13.	3.0	25 ± 10	6
Experiment series 2			
a) Purified using an amino minicolumn[a]			
Nicotiana sp. (leaves, free IAA)	0.05–1.0	45 ± 10	5–15
Triticum aestivum (caryopses, free + ester IAA)	0.05–0.1	64 ± 3	1600–2400

[a] See "Materials and Methods."

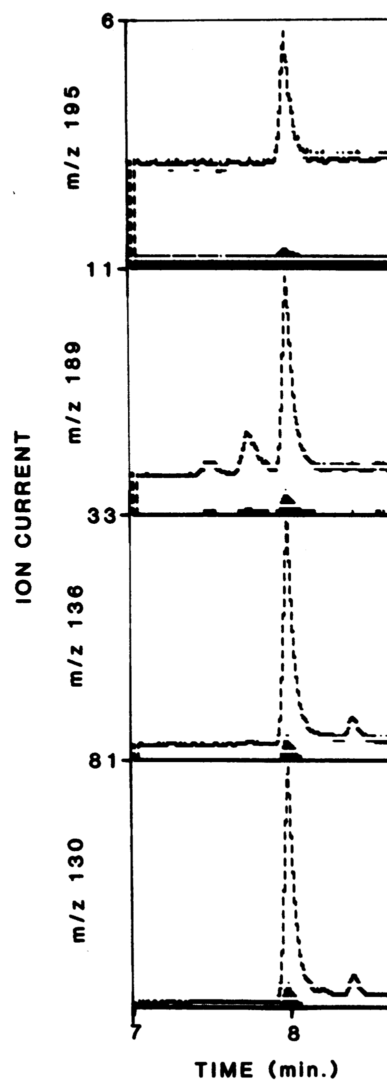

FIG. 3. Selected ion chromatogram of methylated, HPLC purified, sample of free IAA from *Avena sativa* pulvini. The ions at m/z 130 and 189 are the quinolinium ion and molecular ion, respectively, of the methyl ester of the endogenous IAA. Ions at m/z 136 and 195 are the corresponding ions from the [$^{13}C_6$]IAA internal standard. The chromatogram was obtained on an 11 meter 0.32 mm i.d. fused silica column CP Sil 19 CB (Chrompack) using helium as carrier gas at a flow rate of 1 ml/min. Mass spectrometry was carried out on a Hewlett Packard 5992 GC-MS with a dwell time of 50 msec for each ion.

was highly dependent upon the plant material. For example, in the case of tobacco, a species high in phenolics, the typical recovery was within the range of 30 to 50% (Table I), while in the case of wheat aleurone layers, samples that are relatively clean, the typical recovery was usually 60 to 70%. In comparison to our prior experience with the solvent partitioning methods (6, 13, 17), the final recovery was improved by 40 to 50% (Tables I and II). The low and variable recoveries of IAA during isolation and purification (8, 9) have been reported to be due to the ease of indole oxidation, adherence to glassware, and problems with solvent purity (6, 11). It is clear that procedures requiring less handling time, less transfer steps, and less glassware offer important advantages in terms of sample recovery. It should be noted, however, that knowledge of sample recovery is not necessary for quantitation by isotope dilution analysis (6). Higher recoveries, nevertheless, allow smaller samples to be analyzed effectively by these methods.

In addition to recovery improvement, multiple samples can be prepared at the same time using this procedure and this, together

Table II. *Comparisons of Ion Exchange Minicolumn Technique and Solvent Partitioning Method*

	Solvent Partitioning	Ion Exchange
Total time required (single preparation)	6–8 *h*	3–4 *h*
Optimal number of samples processed together	1–2 samples	6–8 samples
Approximate analysis time per sample (multiple samples)	5 h	1.5 h
Total recovery	20–50%	30–70%
Sample size	1–100 g	0.05–2 g
Sources of difficulties	a) Solvent impurities (see Ref. 6) b) HPLC recovery relative low c) Emulsion formed during partitioning	a) Overloading ion exchange column. b) pH control during extraction and on the ion exchange column

Table III. *Loading Capacity of Different Amino Columns used for Purifying Tobacco Leaf Tissue*

Loading capacity was determined by an increase in the amount of ^{3}H-IAA eluted from the column during the hexane, ethyl acetate, acetonitrile, methanol washing steps.

Column	Amount of Tobacco Leaf Tissue
	g fresh wt
Prep-Sep 0.3 g (Fisher)	<0.2
Baker-10 SPE 3 ml (Baker)	~1.0
Extra-Sep 2.5 g (Lida)	>2.5

with the more rapid fractionation on the short column (Fig. 2), improved the speed of preparation without decreasing the final purity of the samples (Figs. 2 and 3). Six or more samples can be processed in the time necessary to prepare two samples by the prior method (Table II). A critical point of this technique is matching the capacity of the amino column with sample size and plant specimen (Table III). For organic acid-rich tissue such as plant tumors, a larger amino column is needed to avoid overloading. This should be determined in a pilot experiment prior to analysis of a new plant material. Some pigments bind to the amino column in an apparent nonionic fashion and reduce the capacity of the amino column. For highly pigmented plant material, a simple hexane extraction of the diluted extract prior to loading on to the amino column will help prevent overloading problems (data not presented). Preconditioning the amino column to pH 7.0 was necessary to obtain reproducible results. Water washing of amino columns from various manufacturers results in a different pH of the eluent. Thus, it is advisable to select a volume of imidazole buffer sufficient to neutralize the column used. In order to avoid contamination, a high purity grade of imidazole or recrystallized reagent material should be used.

The final GC-SIM-MS[5] analysis showed good agreement between the ratios of the molecular ion and base peak (Fig. 3). In this way the GC-MS technique internally corroborates the analysis of IAA (6). We have found that the method is highly reproducible and the data is consistent with the previous solvent partitioning method (6). This method has increased the speed and extended the versatility of the GC-SIM-MS technique using $^{13}C_6$-IAA as an internal standard for plant IAA measurement (6), without altering the precision and chemical certainty inherent in stable isotope dilution quantitative mass spectrometry.

[5] Abbreviation: SIM, selected ion monitoring.

LITERATURE CITED

1. Allen JRF, J-E Rebeaud, L Rivier, P-E Pilet 1982 Quantification of indole-3-acetic acid by GC/MS using deuterium labelled internal standards. *In* H-L Schmidt, H Forstel, K Heinzinger, eds, Stable Isotopes, Elsvier, Amsterdam, pp 529–534
2. Allen JRF, L Rivier, P-E Pilet 1982 Quantification of indole-3-acetic acid in pea and maize seedlings by gas chromatography-mass spectrometry. Phytochemistry 21: 525–530
3. Bandurski RS, A Schulze 1983 Gravitational effects on plant growth hormone concentration. Adv Space Res 3: 229–235
4. Cohen JD, A Schulze 1981 Double-standard isotope dilution assay I. Quantitative assay of indole-3-acetic acid. Anal Biochem 112: 249–257
5. Cohen JD 1984 Convenient apparatus for generation of small amounts of diazomethane. J Chromatogr 303: 193–196
6. Cohen JD, BG Baldi, JP Slovin 1986 $^{13}C_6$-[benzene ring]-indole-3-acetic acid: a new internal standard for quantitative mass spectral analysis of indole-3-acetic acid in plants. Plant Physiol 80: 14–19
7. Dayanandan P, FV Hebard, PB Kaufman 1976 Cell elongation in grass pulvini in response in geotropic stimulation and application. Planta 131: 245–252
8. Hamilton RH, RS Bandurski, BH Grigsby 1961 Isolation of indole-3-acetic acid from corn kernels and etiolated corn seedlings. Plant Physiol 36: 354–361
9. Little CHA, JK Heald, G Browning 1978 Identification and measurement of indoleacetic acid and abscisic acids in the cambial region of *Picea sitchensis* (Bon.) Carr. by combined gas chromatography-mass spectrometry. Planta 139: 133–140
10. Magnus V, RS Bandurski, A Schulze 1980 Synthesis of 4,5,6,7 and 2,4,5,6,7 deuterium-labeled indole-3-acetic acid for use in mass spectrometric assays. Plant Physiol 66: 775–781
11. Mann JD, EG Jaworski 1970 Minimizing loss of indoleacetic acid during purification of plant extracts. Planta 92: 285–291
12. McDougall J, JR Hillman 1978 Analysis of indole-3-acetic acid using GC-MS technique. *In* JR Hillman, ed, Isolation of Plant Growth Substances. Cambridge University Press, Cambridge, pp 1–25
13. Miller AN, CS Walsh, JD Cohen 1987 Measurement of indole-3-acetic acid in peach fruits (*Prunus persica* L. Batsch cv Redhaven) during development. Plant Physiol 84: 491–494
14. Momonoki YS, A Schulze, RS Bandurski 1983 Effect of deseeding on the indole-3-acetic acid content of shoots and roots of *Zea mays* seedlings. Plant Physiol 72: 526–529
15. Noma M, N Koike, M Sano, N Kawashima 1984 Endogenous indole-3-acetic acid in the stem of tobacco in relation to flower neoformation as measured by mass spectroscopic assays. Plant Physiol 75: 257–260
16. Pengelly WL, PJ Hall, A Schulze, RS Bandurski 1982 Distribution of free and ester indole-3-acetic acid in cortex and stele of the *Zea mays* mesocotyl. Plant Physiol 69: 1304–1307
17. Wodzicki TJ, H Abe, AB Wodzicki, RP Pharis, JD Cohen 1987 Investigations on the nature of the auxin-wave in the cambial region of pine stems. Validation of IAA as the auxin component by *Avena* coleoptile curvature assay and by gas chromatography-mass spectrometry-selected ion monitoring. Plant Physiol 84: 135–143