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Indole-3-acetic Acid Levels of Plant Tissue as Determined by a New High Performance Liquid Chromatographic Method[1]

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ABSTRACT

A method for the analysis of indole-3-acetic acid (IAA) in plant extracts has been developed based on high performance liquid chromatography separation of IAA on a microparticulate strong anion exchange column followed by quantitation with two selective detectors: an electrochemical, carbon paste amperometric detector and/or a fluorescence detector. The detection limit for IAA is less than 1 nanogram with the fluorescence detector and less than 50 picograms with the electrochemical detector.

The IAA levels are reported for various tissues of wheat, pinto beans, soybeans, cotton, and corn.

It has been over 40 years since IAA has been recognized as a plant hormone (16, 34), yet there are still few, if any, convenient, sensitive, reliable assays specific for IAA. This shortcoming has undoubtedly contributed to many controversies in auxin research.

Although there are numerous sensitive biological assays for auxins, most are not specific for IAA, are time-consuming, have poor precision, and are influenced by other components in plant extracts.

Physicochemical methods have been proposed for IAA analysis; however, most have serious shortcomings when applied to plant extracts. For example: colorimetric assays (10, 33) and others are limited because of poor sensitivity. Numerous conditions for paper chromatographic and TLC separations of IAA have been suggested since 1951 (14); however, the poor sensitivity limited their use with plant extracts. The advent of gas chromatography also prompted several proposals for IAA analysis. These included GC[2] coupled with spectrophotofluorimetry (19, 29), GC coupled with ionization detectors (23), and GC coupled with mass spectrometry (2, 11, 24). In most instances the GC methods require extensive purification of the plant extracts, derivative formation for volatility, and selective detectors. Although the absolute sensitivity of the GC and GC-MS methods is very high, the total sample sensitivity is often limited by the size of the sample injectable into the instruments. The high cost of GC-MS instrumentation makes these methods out of range for routine analysis by most workers.

A sensitive spectrofluorometric assay for IAA was proposed (15, 29) in which IAA is converted to indole-α-pyrone by a reaction with acetic anhydride and trifluoroacetic acid. An evaluation of the assay (7) concluded that although the method is technically demanding, it is possible to obtain accurate values for IAA in plant extracts if extreme care is taken to avoid interferences from impurities which cause quenching and scattering. Our evaluation of this assay found the method very laborious and requiring multiple analyses to overcome the poor precision.

Radioimmuno techniques are useful in assays of proteins, drugs and related hormones; however, the IAA molecule may be too small a hapten to impart specificity and sensitivity to the radioimmunoassay (9).

The possible utility of HPLC for the determination of cytokinins in plant extracts was suggested (18) and HPLC was used for the separation and/or determination of plant hormones (3, 5, 6, 20, 32). The HPLC-UV_{254} detector used to determine ABA levels (20, 32) detected ng quantities from a wide variety of plant extracts; however, the determination of IAA is complicated by the low A of IAA at 254 nm and the difficult separation of IAA from other plant components. This report outlines the conditions for the HPLC separation of IAA using several column-mobile phase systems and two sensitive, selective detectors for the quantitation of IAA in plant extracts. The HPLC method has been used to determine the IAA level in various plant tissues and these are compared with published data.

MATERIALS AND METHODS

Plant Material. All plant material was grown in controlled environment growth rooms, in plastic pots containing Jiffy mix (Jiffy Products of America, West Chicago, Ill.), pea gravel mix 1:1, with nutrient supplied daily by a modified Hoagland solution (13). The growth conditions were: soybean (*Glycine max* [L.] min. cv. Wye and Kent), 6,000 ft-c 12-hr photoperiod, 75% relative humidity, 24 C day, 18 C night; pinto bean (*Phaseolus vulgaris* [L.]), 3,000 ft-c, 14-hr photoperiod, 60% relative humidity, 23 C day, 19 C night; cotton (*Gossypium hirsutum* [L.]) cv. Stoneville 213), 2,200 ft-c, 14-hr photoperiod, 50% relative humidity, 27 C day, 18 C night; wheat (*Triticum aestivum* [L.] cv. Selkirk), 3,000 ft-c, 18-hr photoperiod, 75% relative humidity, 22 C day, 19 C night; corn (*Zea mays* [L.] cv. Pioneer brand 3331), 2,200 ft-c, 14-hr photoperiod, 50% relative humidity, 27 C day, 18 C night.

The plant parts were dissected, weighed, frozen in liquid N_2, and stored at −40 C until extraction for IAA analysis.

Preparation of Plant Extracts. All reagents and solvents were ACS grade. The ether was further purified by passing through an aluminum oxide (Woelm, basic) column just before use. The over-all scheme for the extraction of free IAA, and the clean-up, was similar to that outlined (32). Plant material (0.1–5 g) was removed from the freezer and extracted in a blender with 75 ml of cold 80% methyl alcohol, to which was added a known aliquot of 3-indolyl [1-^{14}C]acetic acid (57 mCi/mmol, Amersham/Searle). After centrifugations and removal of the methyl alcohol with a rotating evaporator, partial clean-up of the extract was made by conventional ether extractions at pH 8 and 2.8. Further clean-up was achieved by either passing the samples through a Sephadex

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[2] Abbreviations: GC: gas chromatography; HPLC: high performance liquid chromatography; E.C.: electrochemical; IPyA: indolepyruvic acid.

G-25 column (32) or a DEAE-Sephadex A-25 column (4.5 cm × 0.70 m Econo-column [Bio-Rad Laboratories]) with 0.1 M NH_4Cl (pH 7.5) as eluate. The IAA eluted in the 10- to 22-ml fraction of the A-25 column and was extracted into ether (at pH 2.8), the ether taken to dryness, and the dry sample stored in the cold until HPLC analysis was performed.

High Performance Chromatographic Separations. Conditions are defined for the separation of IAA from plant extracts with three types of HPLC columns.

1. The main column used for the separation and quantitation of IAA was a strong anion exchange column, Whatman Partisil-10-SAX (25 cm × 4.6 mm) with a mobile phase of 0.01 M NH_2PO_4 and 0.05 M $NaClO_4$, a pressure of 400 to 500 p.s.i., and a flow of 0.6 to 0.8 ml/min. The quantitation of IAA was by peak heights related to a standard sample. The recovery of IAA was determined by collecting the IAA peak (after passing through the electrochemical detector) into a scintillation vial, counting the sample with an Intertechnique SL-30 liquid scintillation spectrometer, and relating the recovered ^{14}C to the total ^{14}C-IAA added to the sample.

2. Two reverse phase columns with a bonded phase of octadecyl silane on totally porous particles were used to confirm the IAA level of the SAX column. These were: (*a*) a Du Pont column of Zorbax-ODS chromatographic packing (25 cm × 4.6 mm) with a mobile phase of 25% acetonitrile, 74.9% H_2O, and 0.1% formic acid, a pressure of 1,200 p.s.i. and a flow of 0.8 ml/min; (*b*) a Whatman Partisil-10-ODS column (25 cm × 4.6 mm) with a mobile phase of 30% methyl alcohol, 70% (0.01 M KH_2PO_4 + 0.05 M $NaClO_4$), a pressure of 1,000 p.s.i., and a flow of 0.8 ml/min.

3. A third HPLC column which could be used to confirm the IAA level from the SAX column was a Du Pont absorption column of Zorbax-SIL chromatographic packing (25 cm × 4.6 mm) with a mobile phase of either 1.5% methyl alcohol, 0.1% formic acid, in CH_2Cl_2 (0.5 H_2O saturated), a pressure of 800 to 1,000 p.s.i., and a flow of 1.4 to 1.8 ml/min; or a mobile phase of 20% ethyl acetate-80% heptane (0.1% in formic acid and 0.005% H_2O), a pressure of 1,000 p.s.i., and a flow of 1.4 to 1.8 ml/min.

Instrumentation. The HPLC instrumentation used for the separation and quantitation of IAA is diagrammed in Figure 1. The chromatographic separation of IAA in a SAX column was made with a Du Pont model 840 liquid chromatograph (operated with a high pressure nitrogen cylinder). The two selective detectors were connected in series. The fluorescence detector was a Du Pont model 836 detector operating with a medium pressure mercury lamp, a Corion 281 nm interference filter (maximum band pass *A* of 0.52 at 281 nm), and a 0 to 54 cut-off emission filter (50% transmittance at 310 and 85% at 340 nm). The electrochemical detector was similar to that described by Swartzfager (31), operating in the amperometric mode with a modified cell (Fig. 2). The carbon paste electrode was made from a mixture of 10 g of spectroscopic graphite (Union Carbide, Grade SP-1) and 6 ml of mineral oil (Nujol). The reference electrode was a 6.35 cm Calomel electrode (No. 4092-K-30, A. H. Thomas). The carbon paste electrode was operated at +0.83 to 0.90 v with a Princeton Applied Research model 170 polarographic analyzer.

The HPLC separations on the ODS-reverse phase column and the SIL-absorption column were made with a Du Pont model 841 high performance liquid chromatograph, which utilized a miniature high pressure pneumatic amplifier pump and either the standard 254 nm UV photometric detector and/or the Du Pont model 836 fluorescent detector. Both HPLC instruments were fitted with Valco CU-6-UHP injection valves with syringe injection adaptors which allowed variable volume injection with minimal sample loss.

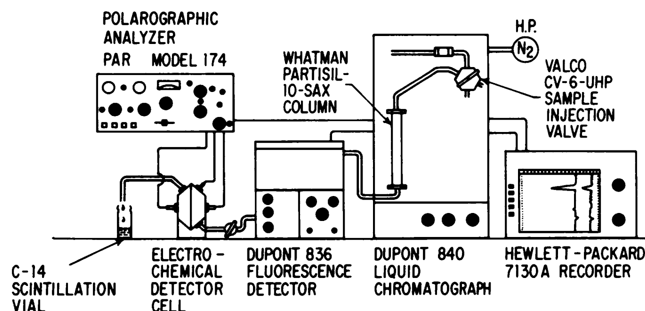

FIG. 1. HPLC instrumentation used for the separation and quantitation of IAA from plant extracts. A fluorescence detector, in series with an E.C. detector, was used for selective detection of IAA.

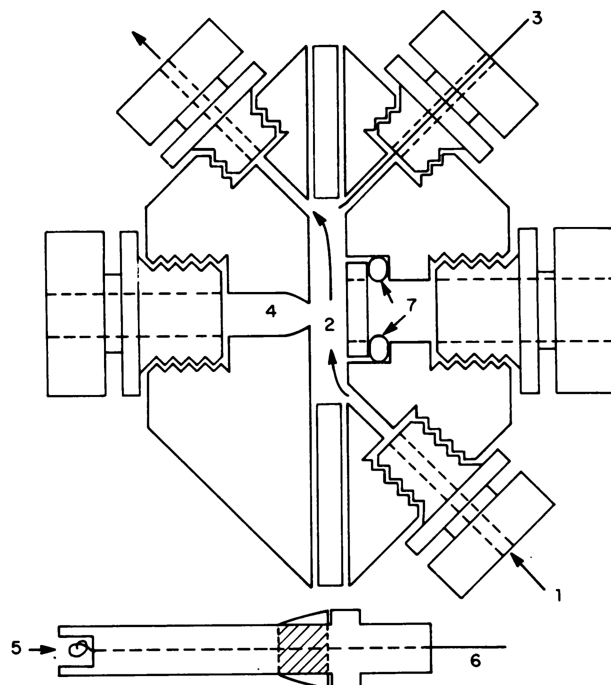

FIG. 2. Diagram of the carbon paste, E.C. detector. 1: Cell inlet; 2: flow cavity; 3: platinum wire-auxiliary electrode; 4: position of reference electrode; 5: carbon paste electrode; 6: platinum wire contact; 7: O-ring seal.

RESULTS AND DISCUSSION

The analysis of IAA by HPLC techniques presents several problems which were not encountered in the earlier work with ABA (20, 32). First, the column selection is more critical since, with most column-mobile phase systems, IAA tends to elute quite rapidly and is thus poorly separated from some components of the plant extracts. This problem was overcome with the Partisil-SAX column. Separation of IAA, t-ABA, and ABA by the SAX column are shown in Figure 3. Under these conditions IAA has a K′[3] of 5 to 5.6. This large K′ facilitates the separation of IAA from most other components. The second major problem was detection. The IAA levels in plant tissues tend to be as low or lower than ABA levels, yet the UV sensitivity of IAA is considerably less than for ABA. This is illustrated in Figure 3 where the ABA and IAA concentrations are the same, but the IAA peak height is less than 10% of the ABA peak.

To increase the sensitivity and extend the selectivity of IAA detection, we used two sensitive, selective HPLC detectors: a

[3] $K' = [(t_r - t_0)/t_0]$ where t_r and t_0 are the elution time of the retained and unretained solutes, respectively.

fluorescent detector and an electrochemical detector. The HPLC separations and dual detection of IAA are shown in Figure 4. In this run, a 50-μl standard sample of IAA (4 ng) was injected at zero time and then was followed by a second standard sample of IAA (0.4 ng) after 8 min. The response of the E.C. was linear from the sub-ng level to the μg level (detection limit about 0.05 ng). Large impurity peaks from the tissue extracts caused some loss of E.C. detector response; however, this could be greatly reduced by diverting the column effluent from the E.C. detector during the early part of the HPLC run. When this procedure was used, the change in the E.C. response was generally less than 20%/day. To increase the precision of the E.C. detector, a standard IAA solution was injected 8 min after the sample injection; this standard IAA peak was used to calculate the response for both the E.C. and fluorescence detectors. The life of the carbon electrode was 1 to 3 days; however, the electrode could be easily repacked with carbon paste and the detector again operable within 15 to 30 min. The fluorescence detector gave a linear response for IAA from 0.5 ng to over 400 ng (detection limit 0.5–1 ng). This detector was at least 13 times more sensitive than the 254 nm detector and gave considerably more selectivity. Although the fluorescent detector was less sensitive than the E.C. detector, it was more convenient and had good long term stability.

Typical chromatograms from the HPLC analysis of IAA in plant extracts are shown in Figures 5, 6, and 7. The SAX column gave good separations of IAA. One of the advantages of the HPLC method is that only mg amounts of plant tissues are required. This point is illustrated in Figure 6 in which the IAA level is determined on a single abscission zone region of a cotton petiole. In some analyses it is possible to have interference with one detector and not the other (Fig. 7). The IAA level of pinto

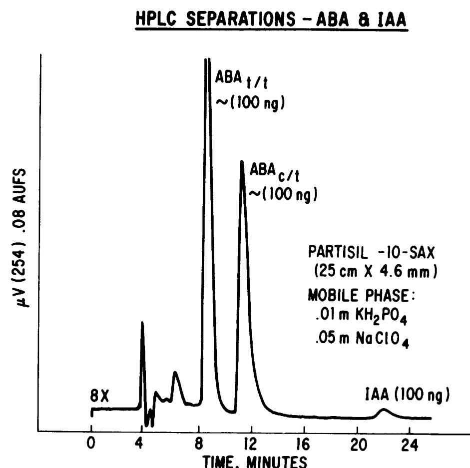

FIG. 3. HPLC separation of IAA, t-ABA, and ABA. Chromatographic conditions: Partisil-SAX (25 cm × 4.6 mm) column, mobile phase, 0.01 M KH_2PO_4 + 0.05 M $NaClO_4$, 500 p.s.i., 0.8 ml/min flow, and 254 nm detector.

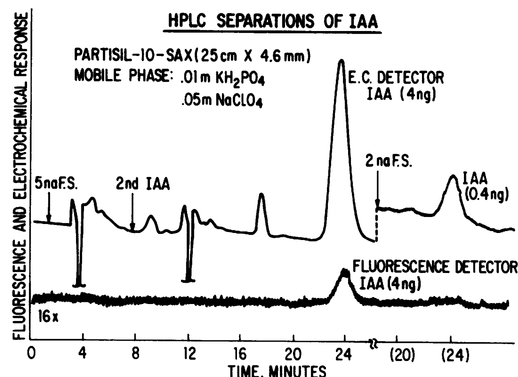

FIG. 4. HPLC separation of IAA and detection with dual detectors. Chromatographic conditions the same as Figure 3 but with fluorescence and E.C. detectors. na F.S. = nanoamps full scale.

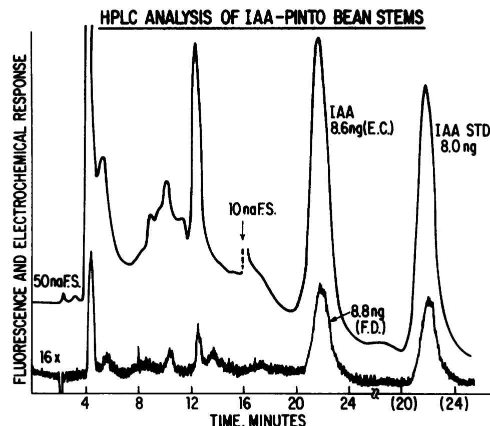

FIG. 5. HPLC analysis of IAA from pinto bean stem extracts with fluorescence (F.D.) and E.C. detectors. Chromatographic conditions similar to Figure 4. The stem tissue was taken from between third and fourth trifoliate of 17-day old pinto beans. Forty-μl sample injected, representing 0.5 g (fresh wt) of tissue.

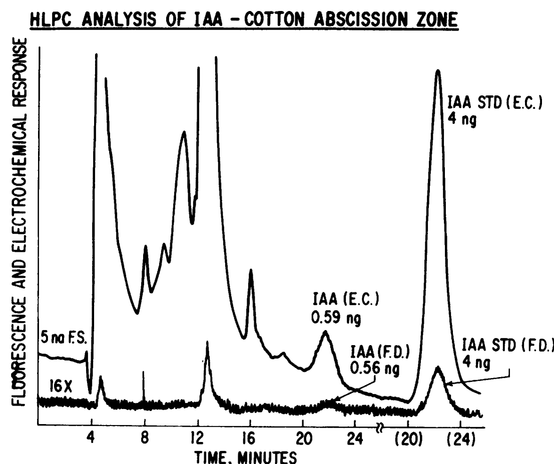

FIG. 6. HPLC analysis of IAA from a single cotton abscission zone. Chromatographic conditions similar to Figure 4. Abscission zone was from third leaf of 23-day-old cotton plants. Forty-μl sample injection, representing 285 mg (fresh wt) of tissue.

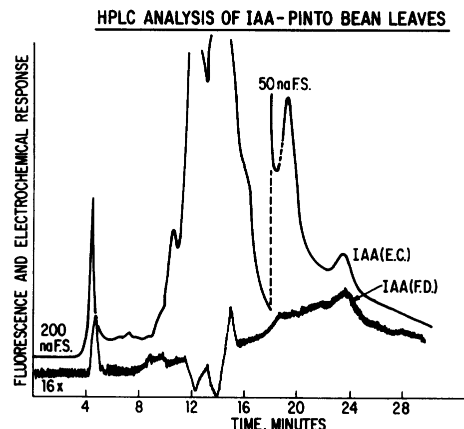

FIG. 7. HPLC analysis of IAA from pinto bean leaves. Chromatographic conditions similar to Figure 4. Trifoliate leaves (fully expanded) from 22-day-old pinto bean plant, 40-μl sample injected, representing 3.7 g (fresh wt) of tissue.

bean leaves is low, and the leaf extracts contain peaks which tend to overlap the fluorescent detector peak for IAA; the E.C. detector, however, gave an IAA peak free of interferences. In most instances either or both detectors could be used and there was good agreement between the IAA values calculated. A study on the precision of this HPLC method with the E.C. and/or fluorescence detector showed a standard deviation of 1.7 to 2.5% (data not shown). This precision is considerably better than the variation in IAA levels within individual plants where such factors as age, time of day, light conditions, water stress, etc., can easily cause 10 to 50% variations.

The IAA level of various plant tissues has been determined by the HPLC method (Table I). The IAA values are reported as ng of IAA/g fresh wt of tissue. The values are corrected for IAA recovery (the ^{14}C-IAA recovery was 30–50% for most samples).

The highest concentration of IAA in the tissues examined was in immature seeds. The range of IAA concentrations reported in Table I for leaf, petiole, and stem tissue is mainly due to the age of the tissue; the younger tissue generally shows higher IAA levels. In most species, the stem and petioles have an IAA concentration higher than even the young leaves. This could indicate that the stems are either a significant storage site for IAA or an important source of IAA synthesis (26).

Differences in extraction and assay methods and in growth conditions make it difficult to compare values of IAA reported in Table I with those reported by others. Nevertheless, a summary is given in Table II of the reported IAA levels in a number of plant tissues. No individual comparison can be made because of the obvious age and varietal differences; however, the range in IAA levels of most related plant tissues is similar.

All IAA values reported in Table I were obtained with the Partisil-SAX column coupled to the electrochemical detector and/or fluorescent detector. However, with the diversity of components in plant tissues, it is probably too much to expect that a single column will separate IAA from all interfering compounds in every plant tissue even with selective detectors. Therefore, other column-mobile phase systems have been developed for the possible confirmation of IAA found by the SAX column. Both the reverse phase ODS columns (column 1) and the absorption column (column 2) of Zorbax-SIL chromatographic packing could be used to collect the IAA fraction. It can then be concentrated and injected into the SAX column for quantitation; or used directly, in some instances, for the quantitation of IAA with the fluorescence detector. Typical separations of IAA and ABA on the ODS and SIL columns are given in Figures 8 and 9.

Although we used an 80% methanol extraction of IAA from plant tissue, other extraction procedures should be suitable. Atsumi *et al.* (1) have developed a new extraction procedure for senescent cultured tobacco cells which reportedly avoids possible interferences from indolepyruvic acid which can spontaneously oxidize to IAA under some conditions. A check on the interference from added IPyA with the HPLC method indicates that 5 to 20% of IPyA could be converted to IAA during work-up. A comparison

Table I

IAA Levels of Plant Tissue as Determined by the HPLC Method

Species	Plant Part	IAA ng/g (fresh wt) (1)
Pinto Bean		
(Phaseolus vulgaris)	Seeds-immature	200-336
	trifoliates (older)	4-5
	trifoliates (younger)	14-16
	stem (lower)	22-36
	stem (upper)	50-54
	petioles (younger)	25
Soybean		
(Glycine max cv Wye)	seeds-immature	50-200
	primary leaves	6-10
	trifoliates	6-10
	stems	25-48
	cotyledon	18
	roots	14
Soybean		
(Glycine max cv Kent)	primary leaves	2-3
	trifoliates	4
	stems	12-45
	cotyledon	69
	petioles	45
	roots	14-22
Cotton	apex	17-40
(Gossypium hirsutum	leaves (younger)	18-30
cv Stoneville 213)	leaves (older)	3-10
	petiole (younger)	40-50
	petiole (older)	5-24
	stem	32-59
Wheat	Seed-immature	150-800
(Tritricum aestivum		
cv Selkirk)		
Corn	Stem (seedling)	10-12
(Zea mays cv Pioneer brand 3331)	Leaf (seedling)	5-15

(1) IAA values corrected for recoveries

Table II

IAA Levels of Plant Tissue Cited in the Literature

Species	Plant Part	IAA ng/g (fr wt)	Assay Method	Ref.
Bean	Cotyledon (3-5 days)	14-24	PC + WC	36
(Phaseolus vulgaris)	Primary leaves (7-19)	9-15	PC + WC	36
	Mature trifoliates	~3	PC + AC	27
	Shoot tips	7-12	PC + AC	37
	Shoot tips	2-29	GLC + MS	37
Soybean	Biloxi			
(Glycine max)	apex + leaves (short days)	~400	PC + Col	35
	apex + leaves (long days)	<.3	PC + Col	35
	Lincoln			
	apex + leaves (short days)	.3-.7	PC + Col	35
	apex + leaves (long days)	.3	PC + Col	35
Cotton				
(Gossypium hirsutum)	Ovules	9.5	GLC	28
Tomato	Old leaves	17	PC + WC	17
(Lycopersicon	Young leaves	77	PC + WC	17
esculentum)	Stem (base)	14	PC + WC	17
	Stem (tip)	53	PC + WC	17
	Stem (mid)	~50	PC + WC	22
Sunflower	Hypocotyl	44-68	Spec Fluo	4
(Helianthus annuus)	Cotyledon	22	Spec Fluo	4
Coleus blumei	Apex + leaves	3.1-12.1	TLC + AC	8
Castor Bean	Phloem sap	4.0 (ng/ml)	GLC-MS	12
(Ricinus communis)	Xylem sap	3.3 (ng/ml)	GLC-MS	12
Corn	Seedling shoots (dark grown)	16-36	GLC + Id	8
(Zea mays)	Root (apical sections)	76-360	GLC + MS	21
Oats	Seedling shoots (dark grown)	11-20	GLC + Id	8
(Avena sativa)	Primary young leaves (tips)	1-8	PC + AM	25
Mung bean	Seedling roots	17	PC + ACu	1
(Phaseolus mungo)	Seedling hypocotyl	5.4	PC + ACu	1

(1) Abbreviations
AC = Avena coleoptile; ACu = Avena curvature; AM = Avena mesocotyl; Col = Colorimetric; Id = Isotope dilution; PC = Paper chromatography; Spec Fluo = Spectrofluorometric; WC = Wheat Coleoptile

HPLC SEPARATIONS

IAA & ABA

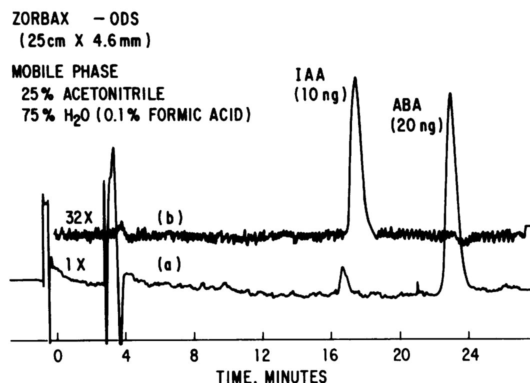

FIG. 8. HPLC separation of IAA and ABA. Chromatographic conditions: Zorbax-ODS (25 cm × 4.6 mm), mobile phase 25% acetonitrile + 75% H_2O (0.1% formic acid), 1,200 p.s.i., 0.8 ml/min flow. (a): 254 nm detector; (b): fluorescence detector.

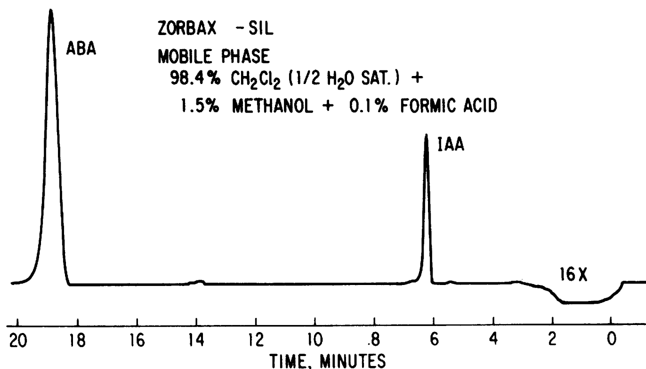

FIG. 9. HPLC separation of IAA and ABA. Chromatographic conditions: Zorbax-SIL (25 cm × 4.6 mm), mobile phase, 98.4% CH_2Cl_2 (0.5 H_2O-saturated) + 1.5% methanol + 0.1% formic acid, 800 p.s.i., 1.7 ml/min flow.

of the IAA values obtained by the usual 80% methanol extraction procedure with those obtained by Atsumi's $(NH_4)_2SO_2Cl_2$ extraction indicates that the IPyA levels of cotton and soybeans are low since there is good agreement with the IAA values found by both extraction methods. However, the possible interference of IPyA in any IAA assay method should be kept in mind.

Our results indicate that HPLC, when combined with selective detectors, is a very valuable technique for IAA analysis. The ease and precision of this IAA analysis should greatly facilitate a better understanding of the role of auxins in plant growth and development.

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