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TRACE ENRICHMENT OF INDOLE-3-ACETIC ACID FROM LEAF MATRIX

KYOUNG R. KIM
College of Pharmacy, Sungkyunkwan University, Suweon 170 (Korea)
ALBERT ZLATKIS* and SHARY WEISNER
Department of Chemistry, University of Houston, Houston, TX 77004 (U.S.A.)
and
JUNG H. KIM and KYOUNG S. CHOI
Department of Food Engineering, Yonsei University, Seoul 120 (Korea)
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SUMMARY

Eight different sorbents were evaluated for their adsorptivity and desorptivity of indole-3-acetic acid (IAA), a plant hormone. Among the sorbents studied, Thermosorb was found to be most efficient in enriching the unionized IAA from dilute aqueous acidic solution. Interfering components in plant leaf matrix could be washed off with water-immiscible organic solvents from a Chromosorb P column, while the ionized carboxylic acids were retained in the adsorbed water on the surface of Chromosorb P.

The method of simple and efficient purification-enrichment of IAA using Thermosorb in conjunction with Chromosorb P, followed by the quantitative analysis employing C_{18} reversed-phase high-performance liquid chromatography–fluorescence detector, has been applied to the determination of IAA in soybean plant leaves.

INTRODUCTION

Indole-3-acetic acid (IAA) is a naturally occurring plant hormone and its involvement in a variety of vegetative functions has been well established. Quantitative analysis of IAA from plant materials is required to investigate metabolic changes and other biochemical processes during plant growth and development.

Highly sensitive gas chromatographic (GC) and combined GC–mass spectrometric techniques[1–3] have been developed to replace the biological assays originally used for the determination of IAA. More conveniently, high-performance liquid chromatography (HPLC) has recently been used for the analysis of IAA. It has been analyzed by various normal phase[4], reversed-phase[5–7], ion pair[2] and silica gel adsorption[8] methods, but the reversed-phase mode with a very sensitive fluorescence detector system[9] is the most commonly employed procedure.

We can now analyze traces of organic compounds down to ng/g concentrations

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using various sensitive chromatographic systems. The primary problem associated with trace analysis is that the trace amounts of the substances of interest are sometimes far too low to be detectable due to the limit of sensitivity of the present detectors. Such problems can be solved by preliminary isolation of the desired substances from the remaining material, followed by the concentration of the isolated substances to achieve detectable levels.

Indole-3-acetic acid is present in most plant tissues in low concentrations, ranging 20–250 ng/g fresh weight. Most of the purification–enrichment procedures reported in the literature employed lengthy liquid–liquid partitions with several pH adjustments, and/or liquid–solid adsorption methods, usually requiring large amounts of solvents and time.

Durley *et al.*[9] reported an extraction-purification procedure for IAA from sorghum bicolor leaves. The procedure included three solvent extractions with three pH adjustments, one polyvinylpyrrolidone (Porapak N) column fractionation, and one preparative C_{18} reversed-phase HPLC cleanup, with 64% recovery of IAA.

Sandberg *et al.*[2] extracted and purified IAA from the leaves of the Scotch pine tree for the quantitative HPLC analysis. The method involved three solvent extractions, five pH adjustments, and one Celite-PVP-Sephadex LH-20 column fractionation, followed by an additional silica gel thin-layer chromatographic purification.

The solvent partitioning step is not only time consuming and laborious, but evaporation of a large volume of solvent leads to loss of the photo-oxidatively labile IAA, and to contamination which comes from large amounts of added solvents.

In an attempt to develop a more efficient sampling method without the solvent partitioning step, we tried adsorption chromatography. A crude sample is first adsorbed on an adsorbent and then either the compound of interest (IAA) is eluted selectively from the adsorbent, or the interfering compounds are removed from the adsorbent first, followed by the desorption of the compound of interest. Solid supports or adsorbents reported in the literature for the purification-enrichment of IAA included Porapak N[1–3,9–13], XAD-7[3,17], C_{18} Sep-Pak[1,12], octadecyl bonded silica (C_{18})[3], μSpherogel[14], Sephadex G-10[16], Sephadex G-15[15], and Sephadex LH-20[2,3,14].

To choose the appropriate adsorbents for our purpose, we tested the inorganic adsorbents Chromosorb P, Chromosorb W, Thermosorb (TS), and Carbopak B; we also tested the organic adsorbents Sep-Pak C_{18}, Porapak O, Chromosorb 102, and Chromosorb 107.

Finally, to prove the effectiveness of the present sampling method, IAA in soybean plant leaves was quantitatively determined by C_{18} reversed-phase HPLC–fluorescence detector.

EXPERIMENTAL

Plant material

Young leaves and fully grown leaves from a soybean plant (*Glycin Max.* var. Hwang-gum) were used. Freshly harvested leaves were freeze-dried and were stored at −40°C until analyzed.

Chemicals

IAA (Aldrich, Milwaukee, WI, U.S.A.), L-ascorbic acid (Kanto, Tokyo, Japan), methanol (Rots, Belgium), and diethyl ether (Sohwa, Japan).

Adsorbents

Chromosorb P (acid-washed, 80–100 mesh), Chromosorb 102 and 107 (both 60–80 mesh), and Carbopak B (80–100 mesh) were purchased from Supelco, Bellefonte, PA, U.S.A. Chromosorb W (acid washed, 100–120 mesh; Johns-Manville, Denver, CO, U.S.A.), Thermosorb (80–100 mesh; Chrompack, Bridgewater, NJ, U.S.A.), Sep-Pak C_{18} (200–400 mesh; Waters Assoc., Milford, MA, U.S.A.) and Porapak Q (50–80 mesh; Alltech, Deerfield, IL, U.S.A.).

IAA standard solution

10 mg of IAA was dissolved in 10 ml of pH 7.0 sodium phosphate buffer (0.2 *M*) containing 500 mg/l of L-ascorbic acid. A volume of 0.1 ml of the resulting solution was taken and diluted to 10 ml with pH 7.0 sodium phosphate buffer to make pH 7.0 standard, or with 0.1 *N* hydrochloric acid solution to make pH 2.5 standard.

Instrumentation

Centrifuge (Model H-251; Kokusan, Japan), freeze-dryer (corrosion resistant; FTS Systems, Stone Ridge, NY, U.S.A.), and Waters Assoc. high-performance liquid chromatograph (Model 244) equipped with data module 730, variable wavelength UV detector 450, fluorescent detector 420, and solvent delivery system 45.

HPLC analysis

The HPLC column employed was 30 cm × 3.9 mm I.D., 10-μm μBondapak C_{18}. A sample of 15 μl was eluted isocratically with methanol–water (40:60) containing 5% acetic acid at a flow-rate of 1 ml/min. The UV detector was operated at 254 nm and the fluorescence detector was operated with excitation at 254 nm and emission at 360 nm.

IAA adsorptivity tests for various adsorbents

An amount of 0.2 g of each adsorbent was packed into a polypropylene column (Econo™ column; BioRad, Richmond, CA, U.S.A.) secured with a glass wool plug. Sep-Pak C_{18} was used as supplied (0.35 g/pack) without modification. The adsorbents were then thoroughly washed with methanol, with the exception of Porapak Q which was washed with acetone followed by methanol. Each adsorbent was allowed to equilibrate before use with pH 7.0 buffer or 0.1 *N* hydrochloric acid solution (pH 2.5). IAA standard solution (1 ml) was passed through the column, collecting the eluate; the column was further eluted with an aqueous solution of the same pH as the standard, and the first 2-ml fraction of the eluate was collected. After the remaining water on the column was removed under vacuum, the column was eluted with mathanol, collecting the first 2-ml portion of the eluate. Each eluate was monitored for IAA by HPLC.

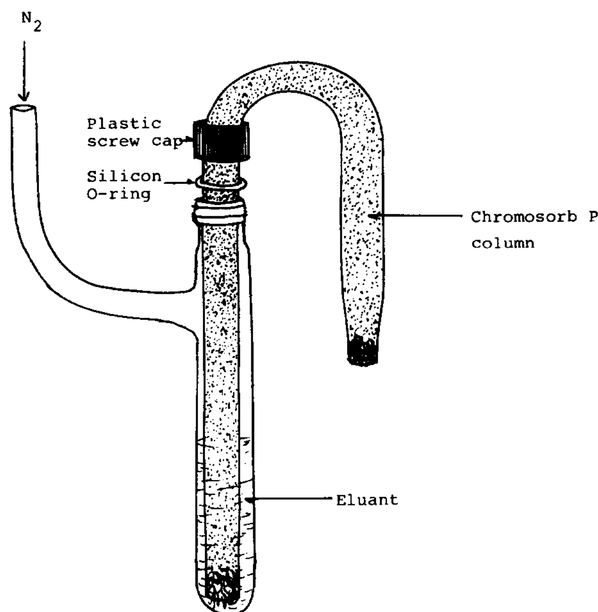

Fig. 1. Sampling apparatus.

Plant pigments cleanup tests with various adsorbents

Fresh soybean plant leaves (10 g) were homogenized in 20 ml of either pH 7.0 buffer or 0.1 *N* hydrochloric acid solution to make plant samples of pH 7.0 or pH 2.5, respectively. A volume of 1.0 ml of each homogenate was passed through the column and the color of the filtrate was visually compared with that of the leaf homogenate.

Test for the optimal elution volume for Chromosorb P column

An amount of 2.3 g of Chromosorb P was packed into a U-shaped glass column (0.6 cm I.D., 0.8 cm O.D.) which was fitted into a modified Kimax culture tube with a side arm (Fig. 1). The screw cap can be tightened via a silicon O-ring to make a gastight connection between the column and the tube. A volume of 2 ml of pH 7.0 standard IAA solution was placed in the tube and loaded onto the column by applying a positive nitrogen stream through the side arm. The column was wetted all the way up to 80% of the packing and the remaining 20% was dry. Then pH 7.0 buffer solution was introduced into the tube and the column was eluted by applying nitrogen pressure. Eluates of 1 ml were collected for the first five consecutive fractions and each was analyzed by HPLC to evaluate the percent recovery of IAA.

Determination of the optimal amount of Thermosorb for enriching IAA in aqueous solution

A constant amount of IAA (10 μg) was dissolved in 1 ml, 5 ml, and 10 ml of pH 2.5 hydrochloric acid solution. TS columns containing 0.2 g or 0.5 g of TS were prepared using polypropylene columns. The IAA samples of various concentrations were passed through each TS column and the remaining water in the column was

removed by suction. The column was eluted with methanol and 1 ml of eluate was collected to be monitored for IAA by HPLC.

Tests for the efficiency of combined Chromosorb P–TS sampling procedure

Five different IAA samples were prepared by dissolving 1.0, 2.5, 5.0, 7.5, and 10.0 μg of IAA in 2 ml each of pH 7.0 buffer. Each sample was loaded onto the U-shaped Chromosorb P column (see Fig. 1) containing 2.3 g of the adsorbent. The column was washed with 5 ml of diethyl ether, then eluted with 5 ml of pH 7.0 buffer under the stream of nitrogen. The collected buffer eluate was treated with 6 N hydrochloric acid to adjust the pH to 2.5. The resulting solution (about 4 ml) was passed through a polypropylene column packed with 0.5 g of TS. The remaining water on the TS was removed and the column was eluted with methanol. The methanol eluate (1 ml) was analyzed by HPLC for IAA to evaluate the percent recovery of each IAA sample.

The quantitative analysis of IAA from soybean leaves

A mixture of 10 g of the freeze-dried soybean leaves and 0.1 g of glass beads was homogenized in 20 ml of pH 7.0 buffer containing 500 mg/l of ascorbic acid. The leaf homogenate was centrifuged for 30 min at 4000 g. The supernatant was taken and diluted with pH 7.0 buffer to 20 ml. Two ml of the crude sample were loaded onto the U-shaped Chromosorb P column to be processed in a similar manner as the sampling procedure described above. The first 1.0 ml of methanol eluate was analyzed for IAA content.

RESULTS AND DISCUSSION

IAA is an acetic acid derivative (pK_a = 4.75) with a relatively lipophilic indolyl

TABLE I

EFFECT OF pH OF INDOLE-3-ACETIC ACID (10 μg) AQUEOUS SOLUTION (1 ml) ON ADSORPTIVITY AND DESORPTIVITY OF ADSORBENTS (0.2 g)

Adsorbent	*Percent recovery of IAA*			
	pH 2.5		*pH 7.0*	
	pH 2.5 eluate	*Methanol eluate*	*pH 7.0 eluate*	*Methanol eluate*
Chromosorb P	100.0	0.0	100.0	0.0
Chromosorb W	100.0	0.0	100.0	0.0
Thermosorb	9.8	90.5	70.6	28.4
Carbopak B	0.0	6.6*	0.0	0.0*
Sep-Pak C_{18}**	0.0	65.9	0.0	78.3
Porapak Q	11.4	78.2	31.4	68.6
Chromosorb 102	6.0	66.6	13.5	80.2
Chromosorb 107	Trace	76.9	3.2	64.3

* Chemical alteration.
** 0.35 g.

TABLE II

EFFECT OF pH OF LEAF HOMOGENATE AQUEOUS SOLUTION (1 ml) ON ADSORPTIVITY OF ADSORBENTS (0.2 g) FOR PLANT PIGMENTS

Adsorbent	*Adsorptivity**	
	pH 2.5	*pH 7.0*
Thermosorb	0.4	0.2
Carbopak B	0	0
Sep-Pak C_{18}**	0.6	0.4
Porapak Q	0.8	0.2
Chromosorb 102	0.4	0.2
Chromosorb 107	0.6	0.4

* Total retention = 1.0, determined by visual detection.
** 0.35 g.

moiety. Therefore, the solubility of IAA in water and organic solvents will depend very much on the pH of the medium. For instance, at pH 2.5, IAA is mostly in unionized form thus dissolving in organic solvents, but at pH 7.0 it exists mostly in ionized form, dissolving readily in water.

It was our intention to achieve a comparison among some commercially available adsorbents, thus the adsorptivity and desorptivity of IAA for eight different adsorbents were determined at pH 7.0 and at pH 2.5, respectively (see Table I).

As we expected, because of their polar nature, both Chromosorb P and Chromosorb W as filtering media did not retain IAA in either ionized or unionized form, while TS, a nonpolar hyrophobic adsorbent, could hold unionized IAA relatively well and give it up readily upon methanol elution. On the other hand, Carbopak B, a graphitized carbon which is also a non-polar hydrophobic sorbent, caused alteration of chemically labile IAA. Sep-Pak C_{18}, a non-polar organic sorbent, adsorbed IAA almost completely in both charged and uncharged forms, but desorption with methanol was not easily achieved. Chromosorb 107, a moderately polar polymeric adsorbent, retained IAA fairly well in either form, but desorption with a small quantity of methanol was incomplete. Non-polar Porapak Q and Chromosorb 102 retained IAA somewhat non-selectively in both forms.

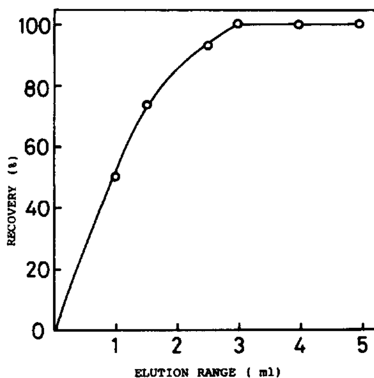

Fig. 2. Recovery of IAA (10 μg) from Chromosorb P column with phosphate buffer (pH 7.0) elution.

An ideal sorbent for our purpose should selectively retain plant pigments, phenolic compounds and all the other UV absorbing or fluorescent interfering substances and pass the IAA through the column or *vice versa*.

We tested some of the non-polar sorbents as filtering media to remove plant pigments from crude leaf homogenate (see Table II). At pH 2.5, Sep-Pak C_{18}, Porapak Q and Chromosorb 107 could retain plant pigments but IAA was also retained and at pH 7.0, most of the pigments passed through adsorbents together with IAA. Thus we concluded that non-polar sorbents cannot be used as a filtering medium to remove pigments selectively.

We focused our attention on the possibility that by employing Chromosorb P in conjunction with TS, IAA could be purified and enriched from dilute aqueous solution. For example, when crude IAA solution at pH 7.0 is loaded onto a Chromosorb P column, the adsorbent will hold the ionized IAA dissolved in water. However, by eluting the column first with a water-immiscible organic solvent such as diethyl ether or dichloromethane, the major interfering substances such as chlorophyll, carotenoids and phenolic compounds could be selectively removed from the column, while the ionized IAA dissolved in water, could be retained by the adsorbent until it is eluted with water. The IAA retained by the Chromosorb P could be recovered by eluting with pH 7.0 buffer. After the pH of the IAA sample was adjusted to 2.5, it was passed through a TS column, which adsorbed unionized IAA selectively. The IAA retained by TS column can be quantitatively desorbed by eluting with a small amount of methanol, thereby further purifying and enriching of IAA from dilute aqueous solution.

To explore the use of Chromosorb P for initial purification, the optimal elution volume to effect 100% recovery of IAA from the adsorbent was determined (see Fig. 2). The test results show that 3 ml of pH 7.0 buffer is sufficient to elute IAA quantitatilvely from 2.3 g Chromosorb P column which was loaded with 10 μg of IAA dissolved in 2 ml of pH 7.0 buffer.

Since the adsorbing capacity of an adsorbent for IAA is likely to depend on its concentration, a series of experiments were conducted to determine the optimal amount of TS for enriching a constant amount of IAA (10 μg) in different dilutions at pH 2.5 (see Table III). As was expected, the test results showed that for a given amount of the adsorbent, the percent recovery rate of IAA became lower for a more dilute solution. It was found that when a sample of 10 μg IAA in 5 ml of pH 2.5 hydrochloric acid solution was passed through a 0.5-g TS column, followed by a methanol elution, 92.8% of the original IAA could be recovered.

TABLE III

EFFECT OF DILUTION OF IAA (10 μg) WITH ACIDIC SOLUTION (pH 2.5) ON TS ADSORPTIVITY

Amount of TS (g)	*Recovery* of IAA (%)*		
	1 ml	*5 ml*	*10 ml*
0.2	86.0	62.4	48.1
0.5	98.7	92.8	80.2

* Single elution of IAA with 1 ml methanol after adsorption.

TABLE IV

EFFICIENCY OF SAMPLING PROCEDURE

*Added** (μg)	*Found* (μg)	*Recovery* (%)
1.0	0.96	95.6
2.5	2.11	84.4
5.0	4.10 ± 0.1**	82.0 ± 2.0
7.5	6.00	80.0
10.0	7.85	78.5

* Added to 2 ml of pH 7.0 buffer solution.
** Average of four measurements.

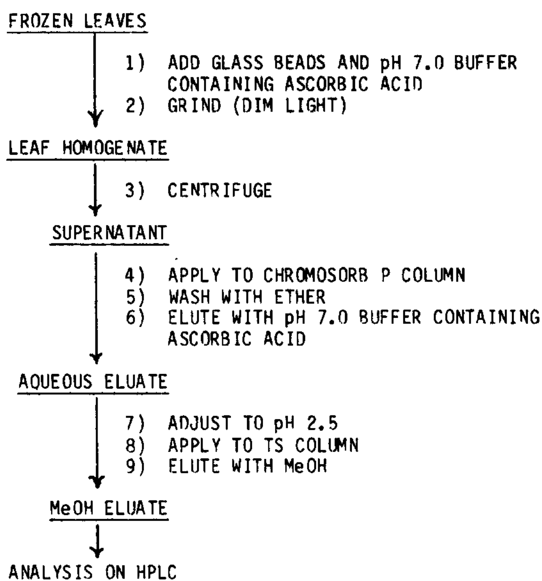

Fig. 3. New sampling procedure.

Now, to evaluate the overall efficiency of the new Chromosorb P–Thermosorb purification–enrichment procedure for IAA, the percent recovery of IAA for samples containing different amounts of IAA dissolved in a fixed volume (2 ml) of pH 7.0 buffer, were determined (Table IV). For a fixed amount of adsorbent (2.3 g of Chromosorb P and 0.5 g of TS the overall percent recovery of IAA dropped as the IAA

TABLE V

LEVELS OF IAA IN SOYBEAN LEAVES OF *GLYCIN MAX*. VAR. HWANG-GUM

Age	*ng/g fresh weight*
Fully grown	44.6
Young	26.8

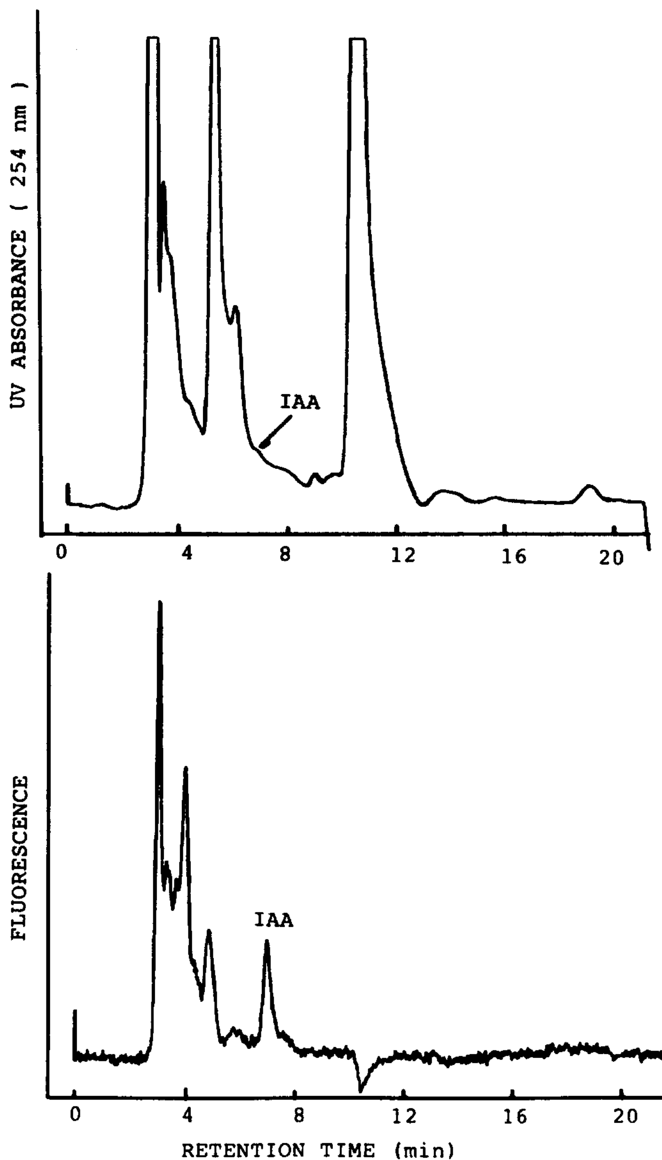

Fig. 4. HPLC of soybean leaf sample spiked with IAA (10 ng). Column, μBondapak C_{18} (10 μm, 30 cm × 3.9 mm I.D.); mobile phase, methanol–water (40:60) containing 5% acetic acid; flow-rate, 1 ml/min; sample size, 15 μl; UV detector, 254 nm; fluorescence detector, excitation at 254 nm, emission at 360 nm.

concentration of the sample became higher, from 95.5% for a sample of 1 μg IAA/2 ml to 78.5% for 10 μg/2 ml. For a sample of 5 μg/2 ml buffer, the recovery rate for an average of four measurements turned out to be a fairly reliable 82% ± 2.0%.

Finally, to examine the applicability of the new sampling procedure (see Fig. 3) to plant material, the IAA in soybean plant leaves was quantitatively analyzed

(see Table V). The HPLC chromatogram monitored by UV absorbance (see Fig. 4) shows very clean UV background compared with chromatograms of published results reported elsewhere[2,11,16]. Also the HPLC chromatogram in Fig. 4 monitored by fluorescence detector (detection limit, 0.1 mg) reveals a well resolved IAA peak.

In conclusion, the pesent work demonstrated that Chromosorb P in conjunction with TS is readily adaptable for the purification and concentration of IAA with high percent recovery from plant leaf matrix without laborious liquid–liquid extractions. The entire sampling procedure usually takes less than 45 min and elutions for the purification step are done under a nitrogen stream, thus minimizing the oxidation of the sensitive IAA. The usefulness of the present method might be further extended to other acidic phytohormones in plants.

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