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DETERMINATION OF INDOLE-3-ACETIC ACID AND ABSCISIC ACID IN JAPANESE CHESTNUTS BY COLUMN CHROMATOGRAPHY ON SEPHADEX G-10 AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the separation and quantification of indole-3-acetic acid and abscisic acid in Japanese chestnuts has been developed by use of Sephadex G-10 column chromatography and a reversed-phase high-performance liquid chromatography (HPLC). For the first purification step, the plant hormones are extracted with methanol–water (80:20) then eluted as individual hormone fractions from a Sephadex column. The final purification was performed on a reverse phase (LiChrosorb RP-18, 5 μm), eluted with a linear gradient of 10–50% acetonitrile in 1% glacial acetic acid. The column effluent is monitored at 254 nm. This technique has been used effectively to analyze plant hormones in biological samples.

INTRODUCTION

Many research workers have attempted to identify and determine endogenous plant hormones by using various techniques such as chemical reactions, bioassays, gas–liquid chromatography (GLC), GLC–mass spectrometry (MS) and high-performance liquid chromatography (HPLC). The chemical methods[1,2] require large amounts of plant materials and highly pure hormones. The bioassays[3,4] are more sensitive but are limited by interference from excessive quantities of impurities in extracts containing the hormones. Moreover, both these methods are time-consuming. The GLC methods have employed flame ionization[5,6] electron-capture[7,8] and mass spectrometric detection[9,10]. In most cases these methods require highly pure hormones and derivative formation. Although the GLC and GLC–MS methods are highly sensitive, the overall sensitivity is often limited by the sample capacity, and the high cost of GLC–MS instrumentation often precludes its use for routine analysis.

Recently HPLC methods[11–18] were introduced but these also require considerable purification of the hormones prior to analysis. For the purification, various

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stationary phases including Celite[17], charcoal[19], charcoal–Celite[6,10], silica gel[7,10], polyvinylpyrrolidone[14,16,18,20] and Sephadex[12,13] have been employed.

The purpose of this study was to examine the separation and quantification of indole-3-acetic acid (IAA) and abscisic acid (ABA) in the pulps and buds of Japanese chestnuts by using a more simple, rapid and sensitive method using Sephadex G-10 column chromatography and reversed-phase HPLC.

EXPERIMENTAL

Materials

Normal ripe Japanese chestnuts (*Castanea crenata* SIEB et ZUCC., "Ginyose") were purchased from an experimental farm of an Agricultural Experiment Station in Akashi City, Hyogo Pref., Japan.

Reagents

Acetonitrile was a distilled-in-glass chromatographic solvent (Kanto, Tokyo, Japan). Glacial acetic acid, methanol, ethyl acetate and petroleum ether were of a special grade (Wako, Osaka, Japan). Distilled and deionized water was used for all solutions. (±)-*cis*–*trans*-Abscisic acid (ABA) was obtained from Sigma (St. Louis, MO, U.S.A.), IAA was obtained from Wako, and Sephadex G-10 was purchased from Pharmacia (Uppsala, Sweden).

HPLC apparatus

A Hitachi high-performance liquid chromatograph Model-635 equipped with a sampling valve with a 20-μl loop (Rheodyne Model 7120) and Model-635 gradient elution system was employed. Two stainless-steel chromatographic columns (15 cm × 4.0 mm I.D.) connected in series were packed with LiChrosorb RP-18 (particle diameter 5 μm, Merck). The column temperature was maintained at 55°C with a Coolnics Model CTR-120 (Komatsu Electronics, Tokyo, Japan). A flow-rate of 1 ml/min was employed. The UV detector was set at 254 nm, and used at a sensitivity of 0.08 absorbance units full scale (a.u.f.s.) for IAA and 0.16 a.u.f.s. for ABA. The chart speed was 2.5 mm/min.

Extraction of hormones

Samples of 10 g of pulp and 30 buds from Japanese chestnuts were homogenized with cold methanol–water (80:20) in a Waring blender and then stirred overnight at 5°C. The homogenate was filtered and the solid materials were again blended with cold methanol–water (80:20) and filtered. The two filtrates were combined, and the methanol was evaporated *in vacuo*. The residue was dissolved in 100 ml of 0.05 *M* phosphate buffer (pH 8.0), washed three times with half its volume of light petroleum (b.p. 30–60°C) (LP) and the LP phase was discard. The aqueous phase was adjusted to pH 2.8, and extracted (four times) with ethyl acetate. The combined ethyl acetate phases were evaporated *in vacuo*.The residue was dissolved in 4 ml of 0.05 *M* phosphate buffer (pH 8.0).

Sephadex G-10 column chromatography

A 4-ml aliquot of the solution obtained as above was applied to a Sephadex

G-10 column (30 × 1.55 cm I.D.) previously equilibrated with 0.05 *M* phosphate buffer (pH 8.0), and the hormones were eluted with the same buffer. The flow-rate was 0.33 ml/min. The eluate was collected in 5-ml fractions and the absorbance of each fraction at 254 nm was measured with a Beckman Model 24 spectrophotometer.

HPLC separation

Each partially purified plant extract of IAA and ABA from the Sephadex column was collected, adjusted to pH 2.8 and then extracted with ethyl acetate (30 ml, five times) and evaporated *in vacuo*. A 60-μl volume of methanol was added to the residue, then 20 μl of this solution were injected into a LiChrosorb RP-18 column and eluted with a linear gradient of 10 to 50% acetonitrile in 1% glacial acetic acid in water.

Identification of IAA and ABA in samples

The identification of IAA and ABA was performed by the following two methods.

(1) The retention times of the peaks in authentic hormones and in the purified plant extracts obtained by HPLC were compared.

(2) The purified extracts were converted into trimethylsilyl ester derivatives using bis(trimethylsilyl)trifluoroacetamide (BSTFA), and then subjected to GLC and GLC–MS. GLC separation was carried out using a Hitachi-063 gas–liquid chromatograph equipped with a flame ionization detector. Samples were injected into a 2 m × 3 mm glass column packed with 3% OV-17 on Chromosorb W AW DMCS (80 mesh). The column oven temperature was initially 150°C, and then programmed linearly (4°C/min) to 260°C. The injection port and detector oven temperatures were 240°C and 270°C, respectively. The carrier gas (helium) flow-rate was 40 ml/min. GLC–MS was carried out on a Hitachi M-80A gas chromatograph–mass spectrometer. A total ion collector was used as the detector. The column packing, size and temperature programming were the same as for GLC. All of the mass spectra were obtained at 20 eV. Other parameters were: injection port temperature, 240°C; interface and ion source temperature, 270°C; accelerating voltage, 3.0 kV; emission current, medium. The range *m/z* 1–700 was scanned. The GLC–MS and mass chromatographic data were recorded, calculated and graphically displayed on-line with a Hitachi M-003 computer.

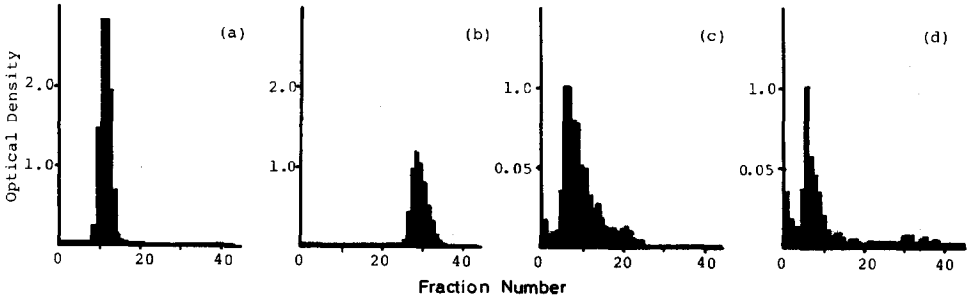

Fig. 1. Elution patterns of authentic ABA (a), IAA (b) and pulps (c) and buds (d) on a Sephadex G-10 column.

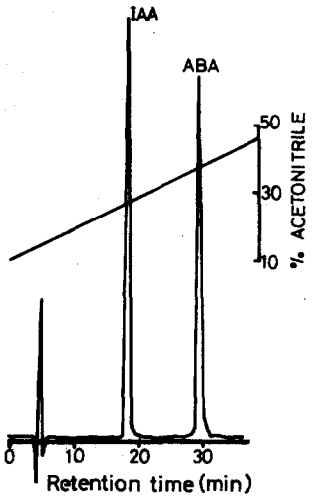

Fig. 2. Chromatogram of a standard mixture of IAA and ABA. Operating conditions: Hitachi Model-635 liquid chromatograph; LiChrosorb RP-18 (5 μm) column; UV detection at 254 nm; flow-rate, 1 ml/min; column temperature, 55°C; mobile phase, linear gradient from 10 to 50% (v/v) acetonitrile in 1% glacial acetic acid; chart speed, 2.5 mm/min.

Recovery test

The recovery test was carried out as follows. Volumes of 100-μl of authentic IAA (20.8 mg per 10 ml of methanol) and ABA (19.0 mg per 25 ml) were placed on the Sephadex G-10 column and eluted with 0.05 *M* phosphate buffer (pH 8.0). A

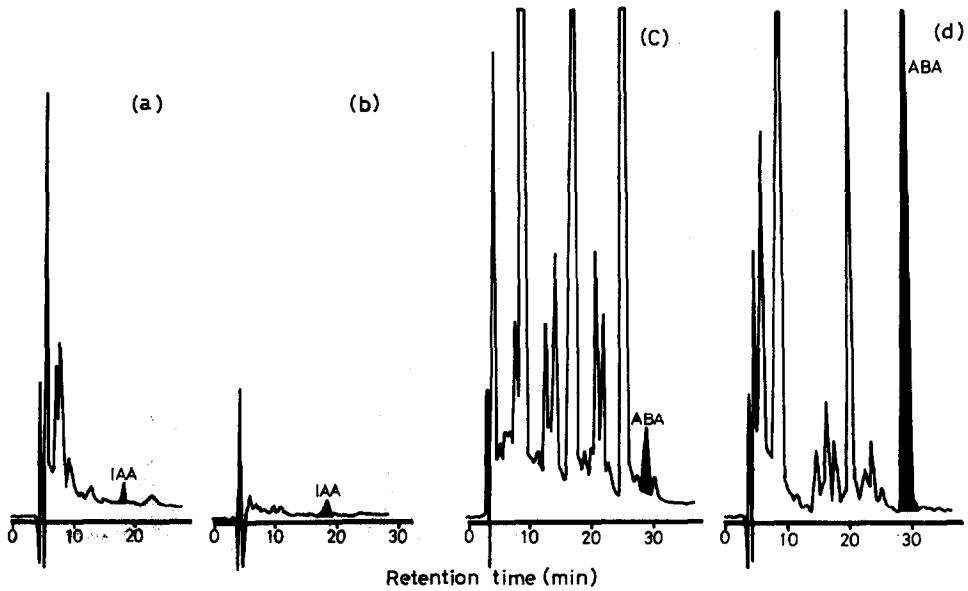

Fig. 3. Chromatograms of IAA (a, b) and ABA (c, d) from pulps (a, c) and buds (b, d) obtained on a Sephadex column. Operating conditions as in Fig. 2.

TABLE I

OVERALL RECOVERY (%) OF AUTHENTIC IAA AND ABA

	Sephadex G-10	*HPLC*
IAA	115.7	76.7
ABA	99.4	97.1

5-ml fraction was collected and the UV absorbance at 254 nm was measured. Fractionated IAA and ABA zones were collected, acidified at pH 2.8 and extracted with ethyl acetate. The ethyl acetate phase was evaporated *in vacuo*, transferred to a small vial, taken up in 100 μl of methanol and 10 μl of this solution were directly subjected to HPLC

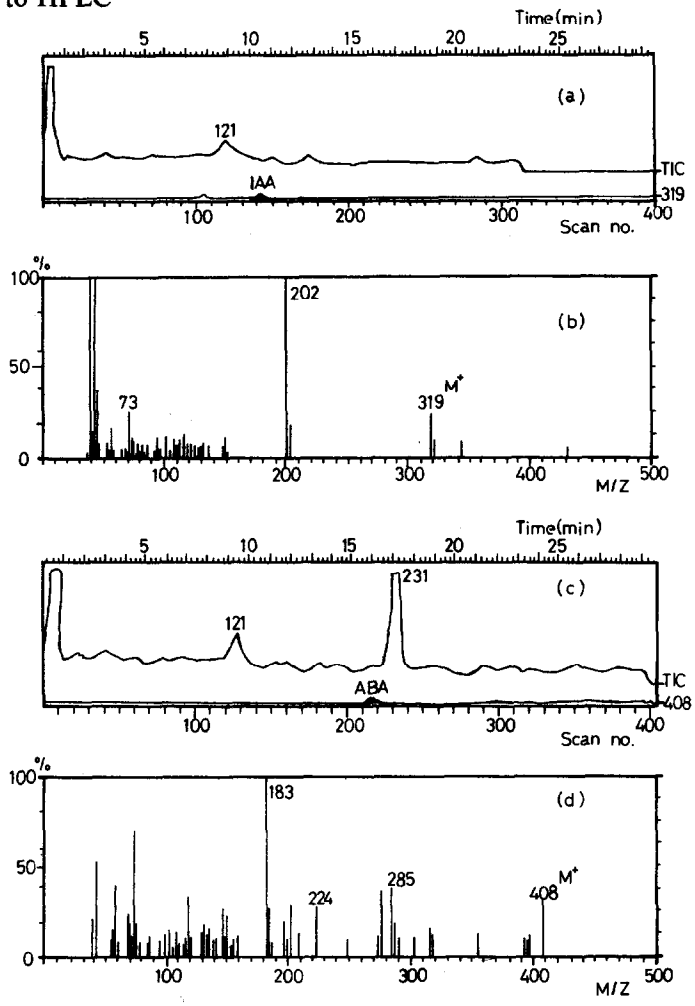

Fig. 4. Mass chromatograms (a, c) and mass spectra (b, d) of trimethylsilyl derivatives of IAA (a, b) and ABA (c, d) in samples purified by HPLC. In all cases, the mass chromatograms and mass spectra were identical to those of authentic standards.

TABLE II

LEVELS (ng PER g FRESH WEIGHT) OF GROWTH REGULATORS EXTRACTED FROM PULPS AND BUDS OF JAPANESE CHESTNUTS

Means from duplicate determinations.

	Pulps	*Buds*
IAA	0.59	60.59
ABA	62.00	1197.59

Quantification

Quantification of IAA and ABA was accomplished by comparing their peak areas in samples to the peak area of the standard by use of an electronic integrator (SiC Electric Company, Tokyo, Japan).

RESULTS AND DISCUSSION

Fig. 1 shows the elution pattern on Sephadex G-10 of authentic ABA (a), IAA (b), extracts obtained from pulps (c) and from buds (d). ABA was eluted in fractions 5–15, IAA in fractions 23–35 and brown impurities were retained on the top of the column.

The chromatographic behaviour of authentic ABA and IAA eluted from the Sephadex column was investigated on LiChrosorb RP-18 with a linear gradient from 10 to 50% acetonitrile.

Fig. 2 shows a typical chromatogram obtained from the Sephadex column. The peaks of IAA and ABA were well separated with retention times of 17.4 and 29.8 min respectively. The detection limit for IAA was about 3 ng and for ABA about 1 ng.

Table I shows the overall recovery of authentic IAA and ABA. Approximately 77% IAA and 97% ABA were recovered upon HPLC. The efficiency of recovery was fairly good in comparison with that reported previously[5,12,13].

Final purification and separation of IAA zones in both pulps and buds (Fig. 3a and b) fractionated on a Sephadex column were accomplished by reversed-phase HPLC. Several impurity peaks, especially in pulps, were detected, but the peak corresponding to standard IAA was free from interference. Although the chromatogram of the ABA zone (Fig. 3c and d) showed many more impurity peaks than those of the IAA zone, the peak corresponding to standard ABA was separated without interference.

For final identification the individual fractions containing IAA and ABA were collected and converted into the trimethylsilyl derivatives using BSTFA. The trimethylsilyl esters were subject to temperature-programmed GLC and were analysed by GLC–MS. The mass chromatograms gave peaks with retention times and mass spectra identical to those of standard IAA and ABA (Fig. 4a, b and c, d).

In conduction, two hormones, IAA and ABA, in the samples were isolated by using Sephadex G-10 column chromatography and reversed-phase HPLC. The levels of each of the hormones extracted from the chestnuts are shown in Table II, expressed as ng per g fresh weight. It was found that in both pulps and buds, the levels of ABA

were much higher than those of IAA; moreover, the levels of both IAA and ABA in buds were considerably higher than those in pulps.

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