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Note

Clean-up and quantification of abscisic acid from citrus leaves by reversed-phase high-performance liquid chromatography

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Abscisic acid (ABA) is an important plant hormone, which is known to control different phases of plant growth processes, and its physiological effects have recently been investigated[1].

The production of ABA by citrus tissues is of interest for its possible role in abscission, senescence and stomatal control[2].

Recently, various methods for the analytical quantification of ABA have been developed. These include gas chromatographic (GC) analysis of the methyl ester of ABA using electron-capture detection (ECD)[3] or flame ionization detection (FID)[4].

More recently, high-performance liquid chromatography (HPLC) has been used for the rapid separation and quantitation of ABA in a wide variety of plant tissue extracts[5–10]. Detection was based on the UV absorbance of underivatized ABA.

Certain plant tissues have been shown to contain, besides free ABA, a polar fraction referred to as "bound ABA" and identified as (+)-abscisyl-β-D-glucopyranoside[11]. This paper describes a simple, sensitive assay for free and bound ABA in citrus leaves by reversed-phase HPLC.

EXPERIMENTAL

Reagents and chemicals

All solvents used in these studies were ACS grade. Standards of cinnamic acid and abscisic acid were purchased from Fluka (Buchs, Switzerland).

Instrumentation

The liquid chromatograph consisted of a Kontron LCS 620 Gradient System (Kontron A.G., Zurich, Switzerland) equipped with a Model 7125 injector with a 20-μl injection loop (Rheodyne, Berkeley, CA, U.S.A.), a Kontron UV Uvikon 722 LC variable-wavelength spectrophotometric detector and a Kontron Anacomp 220 data processor. Chromatography was performed at ambient temperature on a stainless-steel column (25 × 0.4 cm I.D.) packed with Hypersil ODS 5 μm (Policonsult Scientifica, Rome, Italy).

Detection was by UV absorbance at 254 nm and 200 mV full scale.

The flow-rate and chart speed were set at 1 ml/min and 0.5 cm/min, respectively. The mobile phase was 0.2 *N* acetic acid–acetonitrile (6:4, v/v).

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Extraction and purification of free and bound ABA

Fresh tissue (5 g) was homogenized at 5°C in 50 ml of methanol–water (8:2, v/v) containing 200 mg/l of sodium diethyldithiocarbamate. The homogenized tissue was stirred overnight at 5°C and the mixture filtered. The filtrate was concentrated *in vacuo* and filtered, and the solution was divided into two halves.

Free ABA. The first fraction was treated with an equal volume of 0.1 *M* disodium hydrogen phosphate, the pH was adjusted to 10.0 with 0.1 *M* sodium hydroxide, and the solution was washed three times with diethyl ether. The aqueous phase was adjusted to pH 3.0 with 0.1 *M* hydrochloric acid and extracted three times with equal volumes of diethyl ether. The extract was dried over sodium sulphate and evaporated to dryness, the residue was dissolved in 1 ml of methanol–2 m*M* phosphoric acid (75:25, v/v) and introduced on a disposable Sep-Pak C_{18} cartridge (Waters Assoc.) previously washed with 2 ml of 2 m*M* phosphoric acid–methanol (90:10, v/v). Then 2 ml of methanol–2 m*M* phosphoric acid (75:25, v/v) eluted free ABA from the cartridge.

Total ABA. The second fraction was treated with an equal volume of 0.1 *M* sodium hydroxide at 50°C for 30 min; after washing of the solution with diethyl ether, the procedure described above was used.

Calibration curve

Standard solutions containing 0.6, 1, 4, 9, 18 and 25 μg/ml at ABA in HPLC eluent were prepared. The peak height of cinnamic acid (internal standard) is plotted against concentration.

RESULTS

The chromatograms obtained in this study are shown in Figs. 1 and 2. The retention times of ABA and internal standard are 3.7 and 5.0 min, respectively. The calibration curve shows a good correlation coefficient, $r = 0.9997$, between peak heights and ABA concentration in the range 0.6–25 μg/ml. The slope is 0.16 and intercept −0.047.

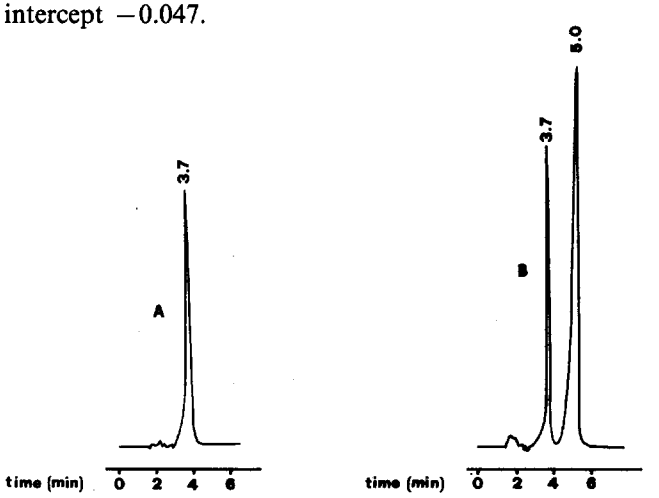

Fig. 1. Chromatography of standards: (A) ABA; (B) ABA and internal standard.

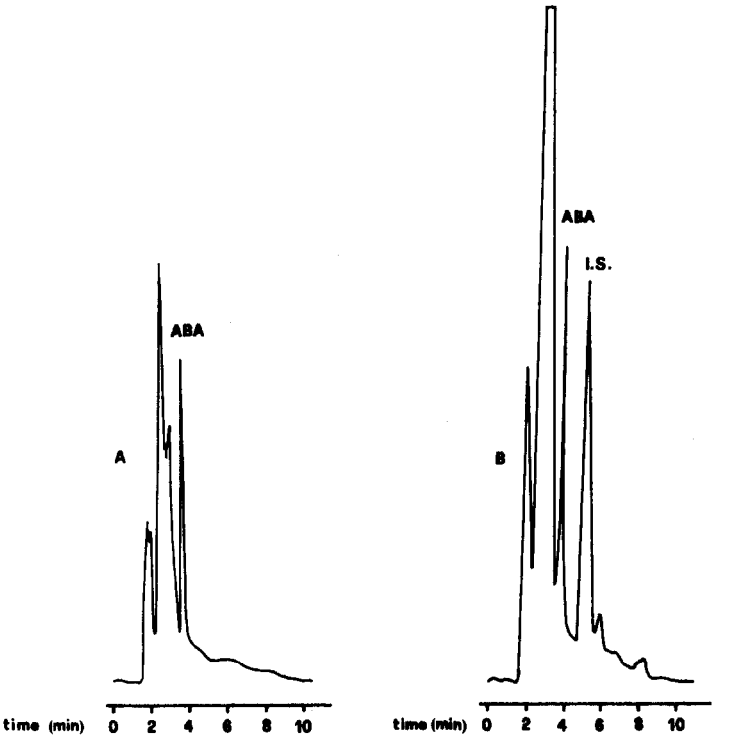

Fig. 2. Chromatography of citrus leaves extract: (A) total ABA (35 nmol/g fresh tissue); (B) total ABA (47 nmol/g fresh tissue) and cinnamic acid (I.S.).

Recovery and sensitivity

We have estimated the recovery by adding the solution of ABA standard in leaves extract containing the same amount of ABA. The extraction percentage is *ca.* 80%. Results are shown in Table I. The limit of sensitivity was 1–2 nmol/ml.

DISCUSSION

To demonstrate the biological applicability of the method, the values of free ABA were determined in twenty samples of citrus leaves, all from different trees. The

TABLE I

RECOVERY OF ABA FROM TISSUE

Number of samples is 5.

Amount added (μg/ml)	*Amount found (μg/ml)*	*Coefficient of variation (%)*	*Recovery (%)*
4	3.2 ± 0.21	6.5	80
9	7.24 ± 0.18	2.5	80.4
18	14.34 ± 0.30	2.1	79.6

TABLE II

LEVELS OF ABA FROM CITRUS LEAVES

Free ABA ± S.D. *(μg/g wet wt. of tissue)*	*Bound ABA ± S.D.* *(μg/g wet wt. of tissue)*
0.46 ± 0.31	15.14 ± 2.99

peaks of ABA were compared with the peak of the internal standard which had been added in the sample before extraction. The obtained values are shown in Table II.

The clean-up method based on using a C_{18} cartridge is simple and efficient; it allows rapid analysis of various biological samples containing ABA. The determination of ABA is made easier by the introduction of cinnamic acid as internal standard, which presents similar recovery of ABA.

The reversed-phase cartridge used in this HPLC method also facilitates the determination of ABA in complex biological matrices.

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