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Note

Semi-automated method for the determination of abscisic acid in crude plant extracts

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Abscisic acid (ABA) is an important plant hormone involved in several processes of plant growth, and its physiological effects have been investigated[1,2]. A suitable method for the extraction and quantification of endogenous ABA levels should be simple and involve the minimum number of steps, but at the same time be efficient in purifying the extracts[3]. All analyses that involve higher plants require rigorous clean-up procedures because of the high concentration of interfering compounds and the low concentration of ABA[4,5].

We have developed a procedure for the quantification of ABA that involves prepurification of plant extracts with Sep-Pak C_{18} cartridges[4,6–9] followed by passage through an RP-18 guard column and RP-8 and SAX analytical columns. Mass spectrometry was used for peak identification and also to measure the purity of the analyte.

EXPERIMENTAL

Instrumentation

A Kontron (Zurich, Switzerland) Model LC 620 liquid chromatograph was used to perform gradients, fitted with a Rheodyne (Berkeley, CA, U.S.A.) Model/125 injector with a 250-μl loop, a Kontron Uvikon 720 spectrophotometric detector, a Kontron Tracer valve-switching module and a Kontron Anacomp 220 microcomputer as a controller and data processor. The tracer switching valves contain four Rheodyne 7010 valves and two low-pressure valves which can be switched from microcomputer by relays.

Three columns were used: a Brownlee RP-18, 10 μm (35 × 4 mm I.D.) stainless-steel guard column (Brownlee, Mississauga, Ont. Canada), a Tracer C-8, 5 μm, 300 Å (150 × 4.6 mm I.D.) stainless-steel column (Teknokroma, Sant Cugat del Vallès, Spain) and a Tracer SAX, 5 μm (250 × 4 mm I.D.) stainless-steel column (Teknokroma). All the columns were operated at room temperature. The detector was set up at 260 nm and 0.1 absorbance V^{-1}.

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The eluents used were: (A) 6.8 m*M* phosphoric acid, (B) methanol and (C) 2 m*M* acetic acid–methanol (60:40, v/v). All of these were degassed by helium flushing. The flow-rate was 1 ml min^{-1}.

A Hewlett-Packard HP 5988 A mass spectrometer at 70 eV was used for ABA peak confirmation. The temperature of the interface was 200°C and the temperature of the ion source was 300°C.

Chemicals and reagents

Methanol of HPLC grade (Probus, Badalona, Spain), phosphoric acid, acetic acid, 2,6-di-*tert.*-butyl-4-methylphenol (BHT), sodium hydrogencarbonate and (±)-abscisic acid (Fluka, Buchs, Switzerland) were used without further purification Milli-Q water (Millipore, Mulhouse, France) was used to prepare solutions.

Plant culture

Plants of *Fatsia japonica* were grown in the experimental fields of the Faculty of Biology (Barcelona University). Maximum photosynthetic photon flux density (PPFD) in the shadehouse was 470 μmol m^{-2} s^{-1} (maximum natural illumination). The minimum and maximum (daily mean) air temperatures were 18 and 25°C, respectively.

Control plants were watered daily to the container capacity and supplied with a nutrient solution. Other plants were subjected to varying degrees of water stress by withholding water supplies for different periods of time, from one to four days.

Leaves were detached, weighed and immediately frozen in liquid nitrogen and lyophilized before storage at −40°C until ABA extraction.

Sample preparation

From 100 to 500 mg (dry weight) of lyophilized and homogenized leaf tissue were shaken in 200 ml of methanol–6.8 m*M* phosphoric acid (80:20, v/v) with 100 mg l^{-1} of 2,6-di-*tert.*-butyl-4-methylphenol as antioxidant at 4°C in the dark for 24 h on a shaker. The homogenate was filtered through an AP Prefilter (Millipore, Bedford, MA, U.S.A.). The remaining fraction was again extracted with 100 ml of methanol–6.8 m*M* phosphoric acid (80:20, v/v) for 4 h under the same conditions until the plant material became colourless. The filtrate was adjusted to pH 8.5 with 0.6 *M* sodium hydrogencarbonate and reduced in a Büchi rotary evaporator at 35°C to an aqueous phase, which phase was frozen at −20°C until the prepurification procedure.

In the prepurification step, the aqueous phase was defrozen, poured into a beaker and the flask was washed with 2 ml of Milli-Q water. The remaining solid suspension was filtered off. The pH was adjusted to 2.6 with concentrated phosphoric acid (8.6 *M*) and loaded with a syringe onto a Sep-Pack C_{18} cartridge, prewetted with 5 ml of methanol and then with 5 ml of water. The cartridge was washed with 5 ml of methanol–6.8 m*M* phosphoric acid (30:70, v/v) to elute the most polar compounds. The cartridge was then washed with 3 ml of methanol–6.8 m*M* phosphoric acid (60:40, v/v) and the eluted fraction was evaporated to dryness in a lyophilizer.

Determination of abscisic acid

The dried sample was dissolved in 250 μl of methanol–6.8 m*M* phosphoric acid (50:50, v/v) and 200 μl were injected into the high-performance liquid chromato-

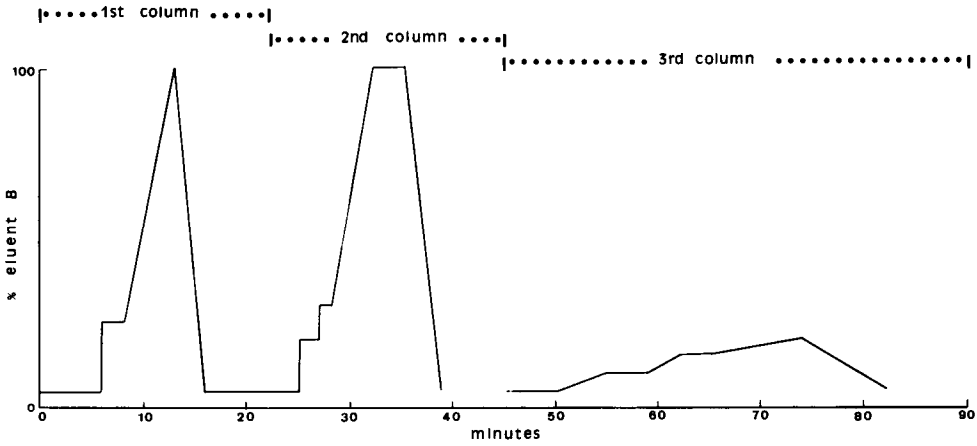

Fig. 1. Gradient scheme relative to eluent B (% methanol) during 90 min of the chromatographic separation that included three different columns: first RP-18, second RP-8 and third SAX.

graphic (HPLC) system for ABA separation and further quantification. The gradient programme included the necessary changes in the valves to turn each column system on or off (Fig. 1).

The eluents for the first (Brownlee RP-18) column were A and B. From time zero (injection) to 6 min, the relative proportion of B was 5% and from 6 to 8 min it was 25%; from 8 to 13 min there was a linear gradient from 25 to 100% B. The eluate from 9 to 10.50 min was introduced automatically at the head of the second (Tracer C-8) column. The eluents for the second colum were also A and B as follows: from 0 to 4 min, 100% A; from 4 to 6 min, 20% B; from 6 to 7 min, 30% B; from 7 to 11 min there was a linear gradient from 30 to 100% B. The eluate from 11 to 13 min was introduced into the head of the third Tracer SAX column. The eluents for this column were C and B as follows: from 0 to 5 min, 5% B; from 5 to 10 min there was a linear gradient from 5 to 10% B; from 10 to 14 min, 10% B; from 14 to 17 min a linear gradient was run from 10 to 15% B; from 17 to 20 min, 15% B; and from 20 to 29 min, 20% B. The ABA fraction was eluted at 5.7 min. The time involved in the whole process was 90 min.

The fraction containing the ABA peak was collected after detection. It was lyophilized and stored at -20°C until mass spectrometric analysis for peak confirmation.

Calibration graph

ABA was identified by comparison with the retention time of standards. For the calibration graph, amounts from 42 to 529 ng were used. Standards solutions containing 0.21, 0.24, 0.26, 0.53, 0.80, 1.32, 1.85 and 2.64 ng μl^{-1} of ABA in methanol–6.8 m*M* phosphoric acid (1:1, v/v) were prepared and three replicates of each were injected into the HPLC system.

The calibration graph of peak area *vs.* ABA concentration was linear from 42 to 529 ng of ABA standard (correlation coefficient $r = 0.9976$). A calibration run was made daily.

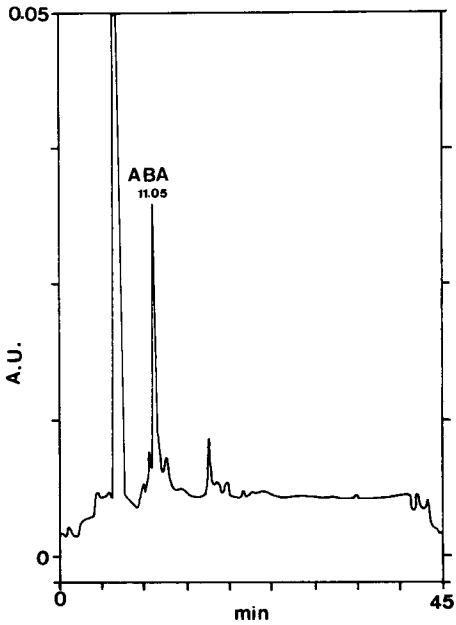

Fig. 2. Chromatography of 0.26 g dry weight of *Fatsia japonica* leaf extract, passed through a Sep-Pak C_{18} cartridge and redissolved in 250 μl of methanol–6.8 m*M* phosphoric acid (1:1, v/v). Injection volume 200 μl. The ABA peak was eluted from the SAX column at 5.7 min, which is equivalent to 11.05 min in the second part of the chromatography.

RESULTS

Clean-up procedure

The method for the prepurification of the crude leaf extract on Sep-Pak C_{18} proved to be a simple and efficient means of removing substances that interfere with subsequent chromatographic steps. The sample and eluent for the clean-up in reversed-phase chromatography had to be acidic for maximum recovery and to reduce tailing. Of the acids tested, phosphoric acid was the most satisfactory in controlling pH. The RP-18 cartridge and automated cut-off are nevertheless necessary in order to avoid damaging the analytical columns.

Chromatographic analyses were performed better by using a sequence of two differents mechanisms. In the first, the RP-8 column separates the components in order of their hydrophobicity, and in the second, an automatic cut-off fraction from the RP-8 eluate is separated by using a strong anion-exchange column.

Quantification

For ABA quantification, an SAX column provides satisfactory resolution and a good separation from interfering substances from the plants. We selected a methanol–2 m*M* acetic acid (60:40, v/v) mobile phase for the SAX column because it gave a good separation of ABA from impurities (Fig. 2). If phosphate is used in the SAX column it does not give a satisfactory performance.

TABLE I

QUANTIFICATION OF ABA LEVELS IN *FATSIA JAPONICA* LEAVES

Different levels of ABA [ng g^{-1} dry weight (D.W.)] in *Fatsia japonica* leaves subjected to varing degrees of water stress from 2 to 4 days. (A) control plants; (B) plants with 2 days of water deficit; (C) plants with 3 days of water deficit; (D) plants with 4 days of water deficit. All plants were sampled at 12 p.m. solar time. Samples of the same plant (A-1, A-2, A-3; B-1, B-2, B-3; C-1, C-2, C-3; D-1, D-2, D-3) showed very similar ABA levels. Also, differences in ABA concentrations in the four treatment groups are obvious.

Plant	*Treatment*	*Sample*	*ABA* (*ng g^{-1} D.W.*)	$\bar{X}$ (*ng g^{-1} D.W.*)	*S.D.* (*ng g^{-1} D.W.*)	*R.S.D.* (*%*)
A	Control	A-1	256.39	267.35	12.52	4.68
		A-2	264.67			
		A-3	280.99			
B	2 days stress	B-1	929.33	922.10	22.02	2.39
		B-2	939.60			
		B-3	897.37			
C	3 days stress	C-1	1759.90	1780.54	137.36	7.71
		C-2	1927.05			
		C-3	1654.67			
D	4 days stress	D-1	3031.54	3291.95	240.48	7.31
		D-2	3338.70			
		D-3	3505.63			

The precision is good because several injections of the same sample into the HPLC system gave the same result, as did analyses of different samples that had been subjected to the same water stress conditions (Table I). Further the reproducibility is very good. The relative standard deviation (R.S.D.)[10] was 6.22% with a range from 5.26% to 7.18% (n = 21) over nine days.

Mass spectrometry was used for peak confirmation. After separation of ABA from other plant substances and collection of this fraction, mass spectrometry gave satisfactory confirmation of the peak substance (Figs. 3 and 4).

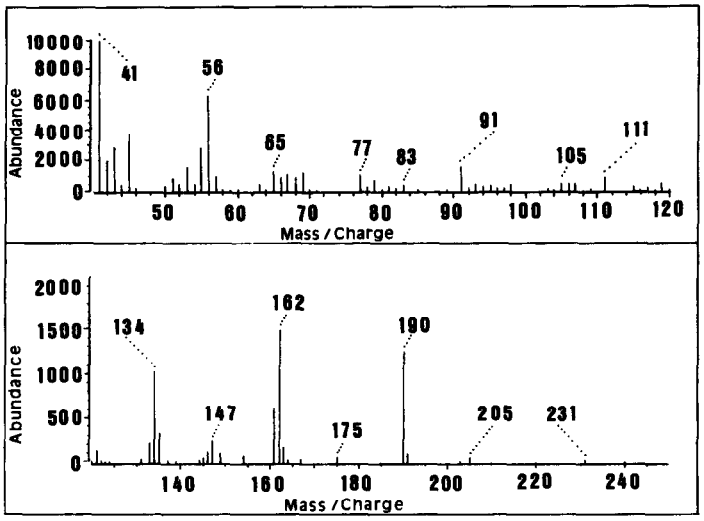

Fig. 3. Electron impact mass fragmentation patterns of ABA standard.

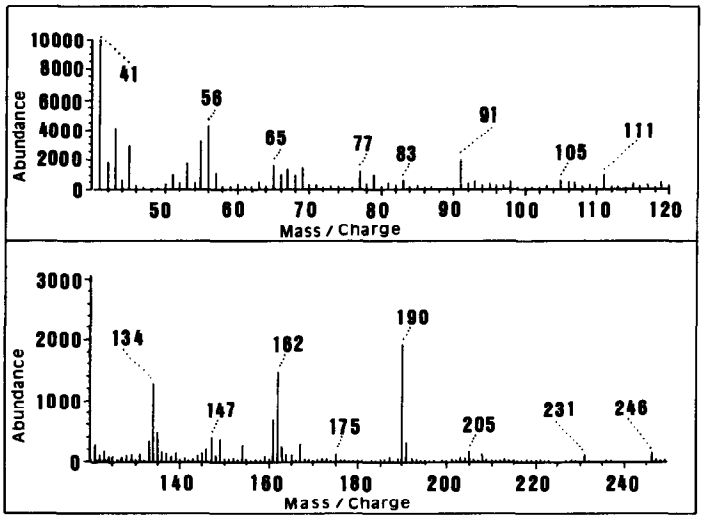

Fig. 4. Electron impact mass fragmentation of ten peaks collected from the HPLC column after ten injections of *Fatsia japonica* leaf extracts.

Recovery and limit of quantitation

We determined the recovery by adding a solution of ABA standard to leaf extracts. The recovery was 62-69 ± 16.89% (standard error).

The limits of quantification for ABA standard and for endogenous ABA were different. For ABA standard the limit was 159 pg μl^{-1}, whereas for endogenous ABA the limit was *ca.* 53 ng g^{-1} dry weight, calculated for a peak signal-to-noise ratio of 2. This is because baseline resolution is not achieved completely in the region of the chromatogram where ABA elutes.

DISCUSSION

This method has good precision, good recovery and high reproducibility. It should be noted that the important criterion of a method is the extent of variation that occurs from sample to sample; the recovery do not reflect the quality of the method[10,11]. This is an important consideration when it is necessary to measure ABA levels during daily stress cycles and subsequent recovery. When one of these processes is to be studied, a large amount of sample is collected for further ABA analyses. It is then necessary to have a simple method in order to carry out large numbers of analyses and avoid handling of samples.

The total recovery of ABA with the present method was 62.69%, which is similar to those reported by other investigators[8,12–14].

The R.S.D. for the entire system was 6.22%, which is similar to the value reported by Bousquet *et al.*[7] and comparable to the valve of 10% reported by Kling *et al.*[10] for indoleacetic acid (IAA). We cannot compare the reproducibility of our method with that of other techniques because many workers did not report the R.S.D.

Previously it was necessary to work with a large amount of leaves per sample

(1–20 g dry weight), and the extraction process was tedious and the purification step very difficult. Only small amounts of leaf material per sample are needed in the present method and we can also use the same leaf for other biochemical and ecophysiological analyses. Further, small amounts of plant material lead to less extensive chromatographic interferences.

In contrast to Kling *et al.*[10], in our method an SAX column provides a good means for quantification of ABA. There is no variability of retention time, but the life of the column is shorter than that of other types of column. With a SAX column we could determine ABA in 190 leaf extracts and 82 standards before renewal of the packing material became necessary.

We consider the method described here to be selective for ABA because the putative ABA peak is symmetrical without shoulders, the detector was set for the wavelength of maximum absorption of ABA and the identity of the presumed ABA peak from leaf extracts of *Fatsia japonica* recorder by the detector was confirmed by mass spectrometry. Comparison of the mass spectra of the ABA from the samples with an ABA standard showed them to be identical.

This method, in its present form, is valid only for the determination of ABA; no other plant hormones such as IAA can be determined in this way because, taking into consideration results in the literature and our experience, the plant material that we use is lyophilized and not fresh, when the aqueous phase after extraction is dried *in vacuo* IAA can be lost by sublimation and the measurement of UV absorbance is much less specific than fluorescence for IAA.

We believe that this method can be used in a wide range of experiments which require the quantification of ABA. Routine analyses of ABA may be performed in the near future using this method.

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REFERENCES

1 S. J. Neil and R. Horgan, in L. Rivier and A. Crozier (Editors), *The Principles and Practice of Plant Hormone Analysis*, Vol. I, Academic Press, London, 1987, Ch. 3, p. 111.
2 J. A. D. Zeevaart and R. A. Creelman, *Annu. Rev. Plant Physiol. Mol. Biol., 39 (1988) 439.*
3 *R. C. Durley, T. Kannangara and G. M. Simpson, Can. J. Bot.*, 56 (1978) 157.
4 S. M. Norman, V. P. Maie and L. C. Echols, *J. Liq. Chromatogr.*, 5 (1982) 81.
5 T. Kannangara, G. M. Simpson, K. Rajkumar and B. D. Murphy, *J. Chromatogr.*, 283 (1984) 425.
6 G. T. Vaughan and B. V. Milborrow, *J. Chromatogr.*, 336 (1984) 221.
7 E. Bousquet, N. A. Santagati and G. Romeo, *J. Chromatogr.*, 354 (1986) 503.
8 G. Guinn, D. L. Brummett and R. C. Beier, *Plant Physiol.*, 81 (1986) 997.
9 E. Vermeer, E. Knegt and J. Bruinsma, *J. Chromatogr.*, 404 (1987) 346.
10 G. J. Kling, L. M. Perkins, P. E. Cappiello and B. A. Eisenberg, *J. Chromatogr.*, 407 (1987) 377.
11 A. Majcherczyk, L. Rakoczy and A. Hüttermann, *J. Chromatogr.*, 357 (1986) 399.
12 M. Hein, M. L. Brenner and W. A. Brun, *Plant Physiol.*, 76 (1984) 951.
13 J. A. D. Zeevaart, *Plant Physiol.*, 71 (1983) 477.
14 K. C. Hall, S. J. Chapman and D. G. Christian, *J. Sci. Food Agric.*, 37 (1986) 219.