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IDENTIFICATION OF ABSCISIC ACID IN SHOOTS OF *PICEA ABIES* AND *PINUS SYLVESTRIS* BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

A VERSATILE METHOD FOR CLEAN-UP AND QUANTIFICATION

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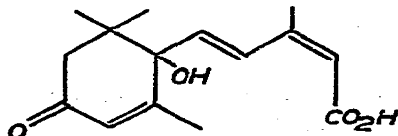
SUMMARY

cis-trans-Abscisic acid has been identified by gas chromatography–mass spectrometry in the current year's shoots of Scotch pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). Clean-up was afforded on a combined Celite–poly-N-vinylpyrrolidone–Sephadex LH-20 column. For quantitative determinations, the extracts were submitted to gas chromatography with an electron-capture detector; in this way 1-g samples of vegetative tissues of spruce and pine could be analysed for *cis-trans*-abscisic acid (as the methyl ester) with a detection limit of 1 ng/g fresh weight.

INTRODUCTION

Abscisic acid (ABA), an endogenous plant growth hormone[1], has been reported to occur in a number of species at a concentration of *ca.* 100 ng/g on a fresh-weight basis; however, only a few determinations of ABA have been made in conifers.



cis-trans Abscisic acid

Investigations of plant hormones have usually involved separate bioassays for the detection and measurement of each hormone. Such assays are of limited reliability,

both qualitatively and quantitatively, which restricts the scope of investigations that can be undertaken. Thus, chemical methods have been designed that separate the hormone from interfering compounds and quantitatively measure the amount present.

In 1969 Bonnet-Masimbert identified ABA in dormant buds of *Pinus sylvestris* by gas chromatography (GC), using the retention time on two different columns[2]. In 1972, Jenkins and Shepherd[3] identified ABA in stems of *Pinus radiata*, and Little *et al.*[4] in dormant buds of *Abies balsamea* by combined GC–mass spectrometry (MS). These reports deal only with the identification of ABA, and no quantitative method has been suggested for ABA in conifers.

In view of the low levels of growth regulators in vegetative tissues, large amounts of tissue need to be extracted, and removal of the associated impurities is both tedious and time-consuming. In 1970, Seeley and Powell demonstrated that the methyl ester of ABA has electron-capturing properties of a very high order[5]. With suitable clean-up procedures, GC with an electron-capture detector (ECD) would offer a simple technique of routine analysis for ABA in a minimal amount of vegetative tissue.

This study was undertaken in an effort to identify ABA in shoots of *Picea abies* and *Pinus sylvestris* by GC and to design an analytical method for its quantification on a routine basis.

EXPERIMENTAL

Materials

Shoots of pine and spruce were collected in Northern Sweden in September and June, respectively. The shoots were wrapped in aluminium foil, immediately frozen and stored at $-20°$.

Reagents

Methanol (redistilled), diethyl ether (p.a.), ethyl acetate (p.a.), poly-N-vinylpyrrolidone (PVP, Polyclar AT powder, GAF Corp., New York, N.Y., U.S.A.), Celite (80–120 mesh) for gas chromatography (BDH, Poole, Great Britain), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), ($\pm$)*cis-trans*-ABA (Sigma, St. Louis, Mo., U.S.A.), ($\pm$)*cis-trans*-[2-^{14}C]-ABA (New England Nuclear, Boston, Mass., U.S.A.) and scintillation liquid (Insta-Gel; Packard, Downers Grove, Ill., U.S.A.) were used.

($\pm$)*trans-trans*-ABA was prepared by illumination of a methanolic solution of *cis-trans*-ABA with UV radiation[4]. The identity of *trans-trans*-ABA was confirmed by GC–MS of its methyl ester.

Scintillation spectrometer

A Packard Tri-Carb liquid scintillation spectrometer, Model 3375, was used. All counts were corrected for background and for quenching.

Gas chromatographic system

A Pye Unicam GCV gas chromatograph equipped with an FID and an ECD was used. It was fitted with a glass column (1.8 m $\times$ 0.4 cm I.D.) packed with 1% OV-17 on Chromosorb W, AW DMCS (100–120 mesh). The operating conditions

were: injector temperature, 220°; column temperature, 200°; detector temperature, 250°; carrier gas, nitrogen at a flow-rate of 40 ml/min.

An SF-96 glass capillary column (34 m × 0.29 mm I.D.) with 122, 100 theoretical plates at $k = 5.93$ for *n*-tetradecane at 120° was used. The column was operated at 200°, with a nitrogen carrier-gas pressure of 0.6 atm resulting in average linear velocities of 35 cm/sec. The column was mounted in the gas chromatograph mentioned above with an inlet splitter (split ratio 1:20) and with nitrogen (40 ml/min) as make-up gas to the FID. A vaporizer temperature of 275° was used, and 5 μl was injected.

Gas chromatographic–mass spectrometric system

An LKB 9000 mass spectrometer equipped with a Pye Unicam Model 84 gas chromatograph was used. The operation conditions were as indicated above, except for the carrier gas, which was helium at a flow-rate of 25 ml/min. The temperature of the connection between the gas chromatograph and the mass spectrometer was 250°. The operating conditions for the mass spectrometer were: separator temperature 250°; ion-source temperature, 270°; electron energy, 70 eV. The most abundant ion in the spectrum (*m/e* 190) was monitored for the single-ion detection.

Extraction procedure

Extracts for identification of ABA. Shoot tissue (100 g fresh weight) and [^{14}C]-ABA were homogenized (Ultra-Turrax) with cold methanol (2 × 500 ml) at −18° for 4 h. The extract was filtered and evaporated to 500 ml, then filtered through 10 g of PVP in a 5.5-cm Büchner funnel; the filtrate was concentrated to 15 ml. The extract now consisted of an aqueous solution with pH 3.3, and this was chromatographed on two Sephadex LH-20 columns in series. The first column (8 cm × 4 cm I.D.) was eluted with 0.05 *M* phosphate buffer of pH 3.0, the fractions being collected and scintillation counted. The scintillation showed the radioactivity to be eluted between 215 and 390 ml. This fraction was extracted with diethyl ether (2 × 200 ml), the extract was evaporated to dryness, and the residue was dissolved in 1 ml of methanol (part of this crude extract did not dissolve in this portion of methanol, but scintillation showed no activity in the insoluble residue). The methanol solution was applied to a second LH-20 column (60 cm × 0.9 cm I.D.) and eluted with methanol. The radioactive fraction was eluted between 35 and 39 ml. This fraction was evaporated to dryness, the residue was dissolved in 2 ml of diethyl ether plus a few drops of methanol and this solution was methylated with diazomethane in diethyl ether for 30 min. The mixture was then evaporated to dryness in a stream of nitrogen, and the residue was dissolved in 100 μl of ethyl acetate for analysis.

Smaller extracts for quantification of ABA. Shoot tissue (10 g fresh weight) and [^{14}C]ABA were homogenized with cold methanol (2 × 100 ml) at −18° for 4 h. The extract was filtered, and 10% of the solution was evaporated to 2 ml; this corresponded to material from 1 g of shoots (fresh weight). The methanol extract was chromatographed on a combined Celite–PVP–Sephadex LH-20 column with 0.05 *M* phosphate buffer of pH 3.0 as eluent. The column (28 cm × 1.1 cm I.D.) consisted of an upper layer of 1.0 cm of Celite, a middle layer of 2.5 cm of PVP and a bottom layer of 24.0 cm of Sephadex LH-20. In packing this column, the Sephadex LH-20 layer was first eluted for 30 min before the PVP and Celite were introduced. When

the fractions were collected and counted, the scintillation showed the activity to be between 50 and 70 ml. This fraction was extracted with diethyl ether (2 × 20 ml) and evaporated to small volume. After methylation as described above and evaporation to dryness, the residue was dissolved in 100 μl of ethyl acetate for analysis.

Evaporation of larger volumes of solvents was performed on an evaporator with a water-bath at 50°; smaller volumes were evaporated in a stream of nitrogen.

RESULTS AND DISCUSSION

This work was done on the current year's shoots of Scotch pine (*Pinus sylvestris*) and on elongating shoots of Norway spruce (*Picea abies*). Scintillation of added [^{14}C]-ABA was performed to monitor the ABA fraction during the clean-up procedures and to calculate the recovery. Two different clean-up procedures were developed in order to meet the requirements for ABA in the final extracts and to minimize the amount of interfering components in the final analyses.

Identification of ABA as its methyl ester was performed by GC–MS in a system equipped with an ordinary packed column. Two extracts from pine and two from spruce were used for identifications; [^{14}C]ABA was not added to these extracts so

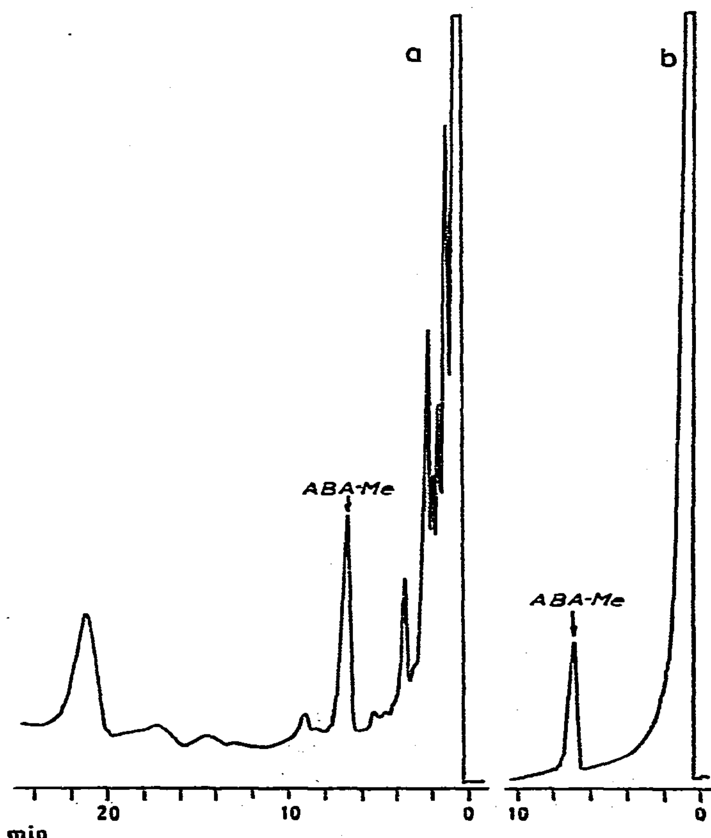


Fig. 1. Gas chromatogram (FID) of: (a) 100-g spruce extract; (b) ABA standard.

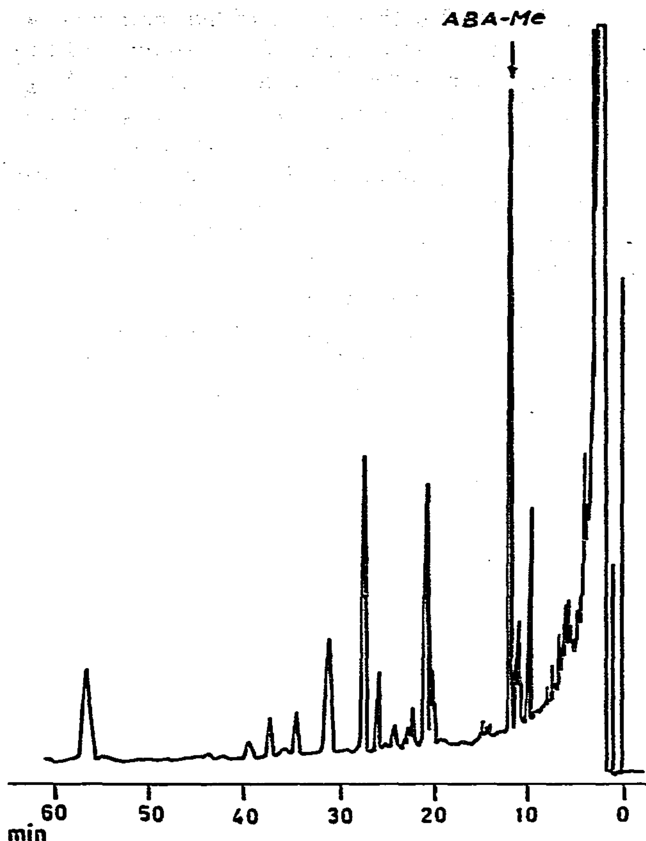


Fig. 2. The same extract as in Fig. 1 analysed on a 34-m SF-96 glass capillary column (FID).

as to avoid misleading results. Complete mass spectra were obtained and shown to be identical with those of an authentic sample and with spectra already published[3,6,7].

Under the conditions used, the isomeric esters *trans-trans*-ABA and *cis-trans*-ABA were well separated on the GC column. The retention time for *trans-trans*-ABA on a 1% OV-17 column operated at 200° was 9 min; that for *cis-trans*-ABA was 7 min. Only *cis-trans*-ABA was detected in the extracts, and, under the conditions used, no tendency on the part of *cis-trans*-ABA to isomerize into *trans-trans*-ABA was observed. Nevertheless, direct sunlight was avoided, and dim lighting and cold storage were used in the process when feasible.

The identity of *cis-trans*-ABA was further confirmed by GC on a capillary column with SE-30 as stationary phase.

For quantitative analyses of ABA in shoots, 1 g of fresh-weight tissue was used, and clean-up was performed on a combined Celite–poly-N-vinylpyrrolidone (PVP)–Sephadex LH-20 column. In this way, laborious and time-consuming handling of large volumes of solvents could be avoided. The elution volumes for the ABA fraction were shown to be relatively constant, and no [^{14}C]ABA was added to the extracts in the final determinations. By scintillation on added [^{14}C]ABA, the recovery for this clean-up procedure was found to be 85%. After methylation with diazo-

methane[8], the extracts were first subjected to GC with a conventional column and flame ionization detector (FID) in order to obtain some idea of the contents of the extract; the detection limit of ABA methyl ester under these conditions was 5 ng. The extract was then diluted at least 1000-fold and injected on to the same column attached to an ECD with a detection limit of *ca.* 5 pg of ABA methyl ester.

It is well known that an ECD is easily contaminated or overloaded, with consequent severe analytical problems; however, it offers a great advantage in this analysis, as ABA methyl ester has a very low detection limit. The FID was first used as a safety precaution so as to avoid contamination problems.

Despite the clean-up procedures, many components still remain in the final extract (see Figs. 1 and 2). However, as can be seen in Figs. 3 and 4, after further dilution, most of these components disappear on the ECD chromatogram, making this method well suited to trace analysis for ABA in small amounts of conifer tissue.

Fig. 3. Gas chromatogram (ECD) of spruce extract. (a) From Fig. 1, diluted 1000 times; (b) 100 pg of reference standard.

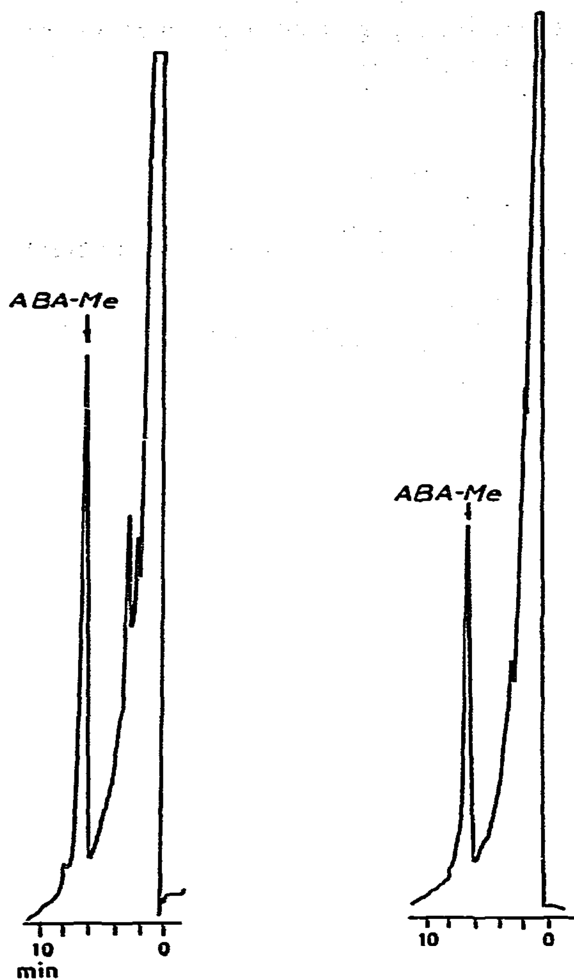


Fig. 4. Gas chromatogram (ECD) of spruce extract from: (a) 1 g of shoot tissue; (b) 50 pg of ABA standard.

By this method, the amounts of *cis-trans*-ABA in the extracts were 170 ± 10 ng/g for pine and 320 ± 10 ng/g for spruce (fresh-weight basis).

The quantification of ABA on a conventional column agreed well with that on the capillary column, and this was further demonstrated by MS with single-ion detection on the main fragment (*m/e* 190)[9,10]. However, the amount of ABA ester in the extracts as determined by this method was about 10% higher than that found in the determinations using ECD. This might be explained by contribution to the ion at *m/e* 190 from interfering natural products in the extracts with the same retention time as ABA ester on a conventional column.

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REFERENCES

1 B. V. Milborrow, *Ann. Rev. Plant Physiol.*, 25 (1974) 259.
2 M. Bonnet-Masimbert, *Ann. Sci. Forest*, 26 (1969) 511.
3 P. A. Jenkins and K. R. Shepherd, *New Phytol.*, 71 (1972) 501.
4 C. H. A. Little, G. M. Struntz, R. la France and J. M. Bonga, *Phytochemistry*, 11 (1972) 3535.
5 S. D. Seeley and L. E. Powell, *Anal. Biochem.*, 35 (1970) 530.
6 B. H. Most, P. Gaskin and J. MacMillan, *Planta*, 92 (1970) 41.
7 R. T. Gray, R. Mallaby, G. Ryback and V. P. Williams, *J. Chem. Soc. Perkin Trans. II*, (1974) 919.
8 J. de Boer and H. J. Backer, *Org. Syn., Collect. IV*, (1963) 250.
9 I. D. Railton, D. M. Reid, P. Gaskin and J. MacMillan, *Planta*, 117 (1974) 179.
10 L. Rivier, H. Milon and P.-E. Pilet, *Planta*, 134 (1977) 23.