Journal of Chromatography, 325 (1985) 425–429
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

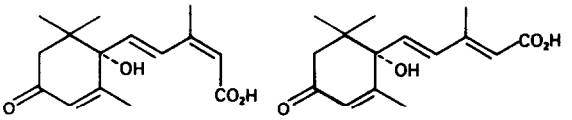
CHROM. 17 718

Note

Less hazardous derivatization procedure for gas chromatography of plant hormone abscisic acid

DIETMAR TIETZ*,*
Tropical Research and Education Center, University of Florida, Homestead, FL 33031 (U.S.A.)
and
ARNO TIETZ
Institut für Allgemeine Botanik und Botanischer Garten der Universität, Ohnhorststrasse 18, D-2000 Hamburg 52 (F.R.G.)
(Received March 12th, 1985)

Abscisic acid (ABA, see Fig. 1) is involved in many plant growth and developmental processes and seems to play a vital role during stress conditions[1–3]. Of many procedures for quantitative hormone analysis[4] gas chromatography (GC) is one of the most important. The trimethylsilyl derivative[5] or the *p*-nitrobenzylester[6] of ABA can be formed and a few nanogram are detectable. A small-scale methylation with etheral diazomethane[7] in combination with electron-capture detection allows more sensitive and selective determinations sometimes even in the lower picogram range. The reaction is quantitative, by-products occur only in a rather small amount and number. However, diazomethane is a very carcinogenic and unstable compound and is not commercially available. Spontaneous explosion during carefully performed synthesis is reported[8].



a b

Fig. 1. Structures of abscisic acid: (a) *cis–trans* isomer, the predominant compound in plants; (b) *trans* isomer, normally found in plants only in small amounts. It may be formed also during the purification procedure.

In this study an attempt is made to replace diazomethane by Methyl-8 (Pierce). Its effective ingredient is dimethylformamide dimethylacetal, a substance recommended earlier by Carns and Christiansen[9] for rapid and effective ABA derivatization. Since no experimental data are given in this short abstract and no further details are

* Present address: Section on Macromolecular Analysis, Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205, U.S.A.

0021-9673/85/$03.30

available[4], the optimum conditions for this reaction are determined and its effectiveness compared with diazomethane.

EXPERIMENTAL

Synthetic racemic ABA (Sigma) and Methyl-8 (2 mequiv. ml^{-1} in pyridine, Pierce, Rockford, IL, U.S.A.), either undiluted or 1:10 diluted with dried pyridine, were heated in small Reacti-vials (Pierce) closed by a septum (Tuf-Bond, PTFE/silicone disks obtained by Pierce). Temperature and reaction time were varied and the amount of methylated ABA formed was monitored by use of a Varian Model 3700 gas chromatograph equipped with a pulsed electron-capture detector. The conditions were: 2 m × 1/4 in. glass column packed with 3% OV-17 on Gas Chrom Q, 80–100 mesh; carrier gas flow, 30 ml nitrogen per min; injector temperature, 250°C; detector temperature, 300°C; oven temperature program: 160°C constant for 3 min, then increase by 4°C min^{-1}, final temperature 210°C; injection volume, 1 μl of sample (Methyl-8 reaction mixture) containing 0.1 to 10 ng ABA per μl. All reactions were performed in the absence of moisture.

In addition to synthetic ABA, plant extracts were also examined. A 20-g amount of leaves of *Argyroderma fissum* (Haw.) L. Bol. (Mesembryanthemaceae) and 14 g of water-stressed *Pisum sativum* L. sprouts (2 h stress, 19% loss of weight) were frozen by liquid nitrogen, separately homogenized by mortar and pistil and extracted with 96% ethanol. After dilution with water the ethanol was removed by distillation *in vacuo*. The aqueous residue was frozen, then thawed. This causes precipitation of most of the chlorophyll which can be removed by filtration. Afterwards the extract was adjusted to pH 9 and two times partitioned against ethyl acetate, re-adjusted to pH 3, one time partitioned against hexane and three times against ethyl ether. The acidic etheral phase contains ABA. It was reduced in volume and subjected to thin-layer chromatography using pre-coated 20 × 20 cm silica gel 60 F_{254} plates (Merck) and solvent system toluene–ethyl acetate–glacial acetic acid (50:30:4). The ABA-containing silica gel identified by marker ABA (blue spots under UV light) was scraped off and eluted with ethyl acetate. These eluates were devided in two equal parts respectively. One half or, for comparison, abscisic acid standard solutions (ABA purchased from Serva) were transferred to Reacti-vials. After removal of the solvent the open vials were dried in a desiccator for two days (or one day in case of standards) and were then closed with screw cap and septum. A 100-μl volume of 1:10 diluted Methyl-8 was injected through the septum into each vial which was then heated in an aluminium block at 100°C for 30 min. Plant extracts changed colour, however, it seems to have no effect on the derivatization procedure.

The other half of both plant extracts was methylated with diazomethane[7] and then dissolved in 100 μl isooctane.

GC conditions: Varian Model 1400 gas chromatograph equipped with an electron-capture detector; 2 m × 1/4 in. glass column, 3% OV-17 on Chromosorb W HP, 80–100 mesh, injection volume, 1 μl sample; carrier gas (nitrogen) flow-rate, 40 ml min^{-1}; injector temperature, 230°C; oven temperature, isotherm at 190°C; detector temperature, 235°C.

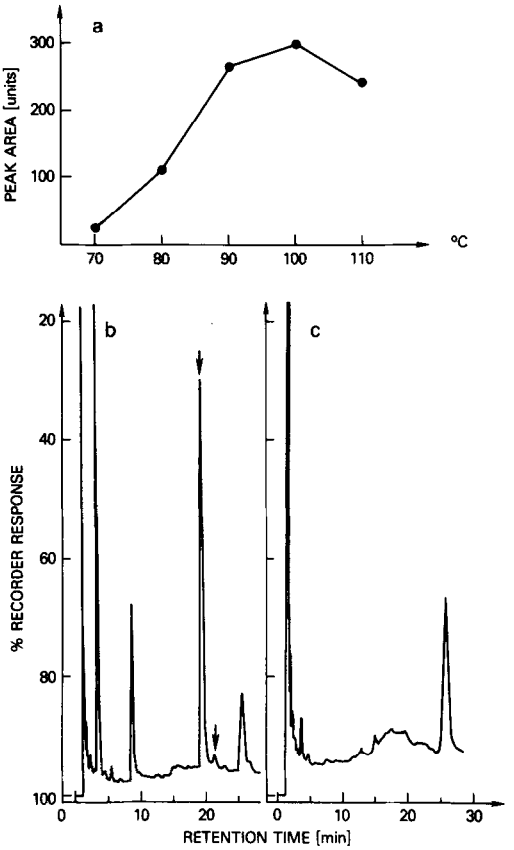


Fig. 2. Derivatization of synthetic ABA with 1:10 diluted Methyl-8. (a) Data of a representative experiment where samples (concentration 1 ng μl^{-1}) were treated at different temperatures for 30 min each. The ABA peak area is plotted *vs.* the reaction temperature. (b, c) Representative gas chromatograms of ABA (100°C treatment, attenuator 128 range 9) and a control sample (heated Methyl-8, attenuator 64 range 9). *cis*- and *trans*-ABA are indicated by arrows.

RESULTS AND DISCUSSION

Following the manufactorer's suggestion for fatty acids dried ABA samples were heated with Methyl-8 at 60°C for 15 min, but this led to unsatisfasctory sensitivity and ABA peak shape. Therefore different derivatization temperatures and times were investigated. Treatment with undiluted Methyl-8 at 90°C for 30 min produced the largest peaks (results not shown). The sensitivity could be further improved by 1:10 dilution of Methyl-8 with pyridine which lowered the background level of the electron-capture detector. Maximum peak area of samples containing 1 ng ABA per μl was then obtained at 100°C as shown in Fig. 2a. Typical gas chromatograms of ABA and a control sample (heated Methyl-8) are presented in Fig. 2b and c. As little as 100 pg of abscisic acid were easily detectable without special attention to optimization.

Using the most effective derivatization conditions plant extracts were exam-

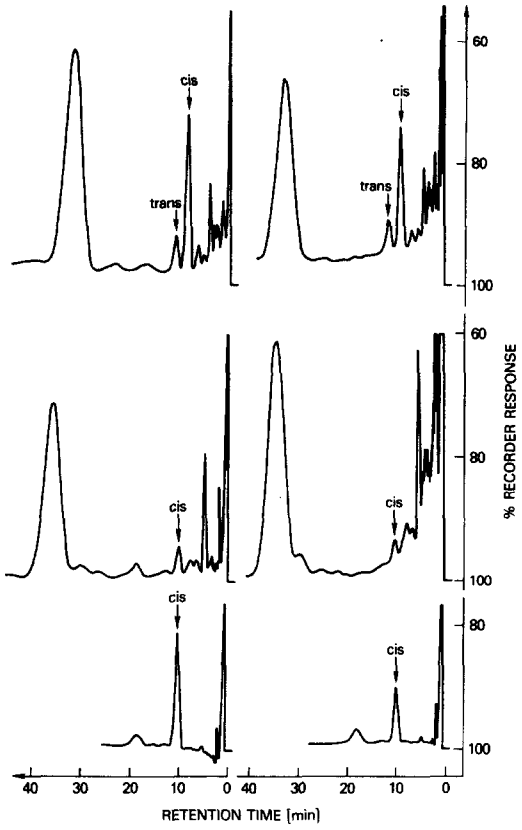


Fig. 3. Gas chromatograms of extracts from *Pisum* (upper panel) and *Argyroderma* (middle panel) derivatized with Methyl-8 (on the left) or diazomethane (on the right). Lower panel: synthetic ABA (Serva) treated with Methyl-8, concentration 2 ng μl^{-1} (left) or 1 ng μl^{-1} (right). All curves are obtained at attenuator 4 range 9, ABA peaks are indicated by arrows.

ined. In Fig. 3 results of Methyl-8 treatment are compared to those obtained with diazomethane. Pea plants, the ABA content of which was increased by water stress, have a higher ABA level than the material of the succulent plant *Argyroderma*. Comparing extracts with equal ABA amounts both reagents produce hormone peaks in the same size range. This indicates that Methyl-8 unaffected by plant extract ingredients converts ABA to its methyl ester. Moreover, the advantage of Methyl-8 is a reduced amount of visible impurities with lower retention times in the chromatogram.

CONCLUSION

Methyl-8 for abscisic acid analysis in *Pisum* and *Argyroderma* plants gives at least as good results as diazomethane. On the one hand, the Methyl-8 derivatization needs more care for elimination of water. On the other hand, Methyl-8-treated extracts are ready for injection, whereas the etheral diazomethane solution needs to be evaporated. Methyl-8 in contrast to diazomethane is commercially available and less dangerous to handle. No explosion-proof fume hood is necessary.

Quarrie[10] described a method of ABA determination by use of ethylated ABA as an internal standard. It remains to be tested whether Ethyl-8 (dimethylformamide diethylacetal, Pierce) could be a good substitute for diazoethane, a less stable compound than diazomethane.

Rivier *et al.*[11] introduced hexadeuterated ABA as an internal standard in combined GC–mass spectrometry, a very powerfull method for ABA analysis. It might be possible to introduce trideuterated methyl abscisate produced by use of Tri-Deuter-8 (Pierce) for internal standardization.

ACKNOWLEDGEMENTS

This research was supported in part by TREC-Homestead and a travel fellowship of the Deutsche Forschungsgemeinschaft to D.T.

Plant material of *Argyroderma* was kindly supplied by Dr. Heidrun Hartmann.

REFERENCES

1 T. A. Mansfield, A. R. Wellburn and T. J. S. Moreira, *Philos. Trans. R. Soc. London, Ser. B*, 284 (1978) 471–482.
2 K. Dörffling, *Das Hormonsystem der Pflanzen*, Thieme Verlag, Stuttgart, 1982.
3 D. Tietz and A. Tietz, *Biol. Unserer Zeit*, 12 (1982) 113–119.
4 K. Dörffling and D. Tietz, in F. T. Addicott (Editor), *Abscisic Acid*, Praeger Special Studies, New York, 1982, pp. 23–77.
5 L. A. Davis, D. E. Heinz and F. T. Addicott, *Plant Physiol.*, 43 (1968) 1389–1394.
6 J. Velasco, G. Ram Chandra and N. Mandava, *J. Agr. Food Chem.*, 26 (1978) 1061–1064.
7 M. Schlenk and J. L. Gellermann, *Anal. Chem.*, 32 (1960) 1412–1414.
8 Anonymous, *Sich. Chemiearbeit*, 34, No. 1 (1982) 4.
9 H. R. Carns and M. H. Christiansen, *Plant Physiol. (Suppl.)*, 51 (1973) 47.
10 S. A. Quarrie, *Anal. Biochem.*, 87 (1978) 148–156.
11 L. Rivier, H. Milon and P.-E. Pilet, *Planta*, 134 (1977) 23–27.