Journal of Chromatography, 247 (1982) 176–179
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,109

Note

Fused-silica capillary gas chromatography of permethylated cytokinins with flame-ionization and nitrogen–phosphorus detection

ALLAN E. STAFFORD* and JOSEPH CORSE

Plant Physiology and Chemistry Research Unit, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710 (U.S.A.)

(Received June 15th, 1982)

The detection of cytokinins in plants and microorganisms has been extensively studied, initially with biological assays[1] and packed column gas chromatography of trimethylsilyl (TMS) derivatives[2]. Both methods require large sample sizes because of the low concentration of cytokinins present in biological systems, limited sensitivity and extensive purification steps. Biological assays are time consuming, lack specificity and require the absence of inhibitors for that assay. Packed column gas chromatography of cytokinins suffers from low resolution particularly at nanogram levels due to active sites which can also cause loss of compound.

In the past five years important developments in derivatization procedures[3,4], capillary column technology[5] and selective gas chromatographic detectors[6,7] have been reported. Permethylated derivatives of cytokinins are chemically stable and have exhibited many advantages over TMS derivatives at low nanogram levels[8,9] and are better suited for nitrogen–phosphorus detectors (NPDs)[10]. Fused-silica capillary columns, because of their inertness and high resolution, are ideally suited for the analysis of permethylated cytokinins. These compounds with their high nitrogen composition also lend themselves to the increased sensitivity and selectivity of the NPD.

We applied these new developments in combination to a wide range of cytokinins and report the resulting improvements in low level cytokinin detection.

EXPERIMENTAL

Reference compounds

All cytokinins used in this study were synthesized at this laboratory by reported procedures[11].

Reagents

Potassium *tert.*-butoxide (95–99%) (Alfa Products), was stored over P_2O_5. This compound was taken from below the surface, which was found to contain mainly KOH from hydrolysis. Methyl iodide (J. T. Baker, reagent grade) was freshly distilled as needed. Other reagents used were: dimethyl sulfoxide (Pierce, silylation grade), chloroform, methanol and ethyl acetate (Burdick and Jackson Labs., HPLC grade).

The methylsulfinyl carbanion solution was prepared by heating DMSO (6 ml) and potassium *tert.*-butoxide (180 mg) at 40°C for 1 h under a nitrogen atmosphere. The solution was centrifuged and stored at −30°C.

Derivatization

The cytokinin mixture (2 μg/component) was dried overnight in a reaction vial over P_2O_5 and KOH under vacuum. The methylsulfinyl carbanion solution (50 μl) was added and mixed. Methyl iodide (10 μl) was added, mixed and the mixture was heated for 30 min at 40°C. The reaction vial was removed from the heat and water (50 μl) and chloroform (100 μl) were added and mixed. The chloroform layer was removed and taken to dryness. Samples were diluted with ethyl acetate to 3.3 ng/μl for nitrogen–phosphorus detection and 80 ng/μl for flame-ionization detection.

Gas chromatography

A Hewlett-Packard 5840 equipped with a flame-ionization detector (FID) and an NPD and a Model 18835B capillary injector system was used. The fused-silica capillary column (12 m × 0.2 mm I.D.) was coated with methyl silicone fluid (Hewlett-Packard). Injector and detector temperatures were 220 and 300°C, respectively. The column was programmed from 180 to 265°C at 6°C/min with helium as carrier and make-up gas. A column flow-rate of 0.6 ml/min with a split ratio of 25:1 was used. Hydrogen and air flow-rates for the FID were 30 and 240 ml/min, respectively, with 3 and 50 ml/min, respectively, for the NPD. The NPD was operated with sufficient voltage to give a 90 mm offset and collector height was set at 0.13 cm above the jet to maximize nitrogen containing compound sensitivity. Injection volumes of 0.2 μl and 0.5 μl were used for the NPD and the FID, respectively.

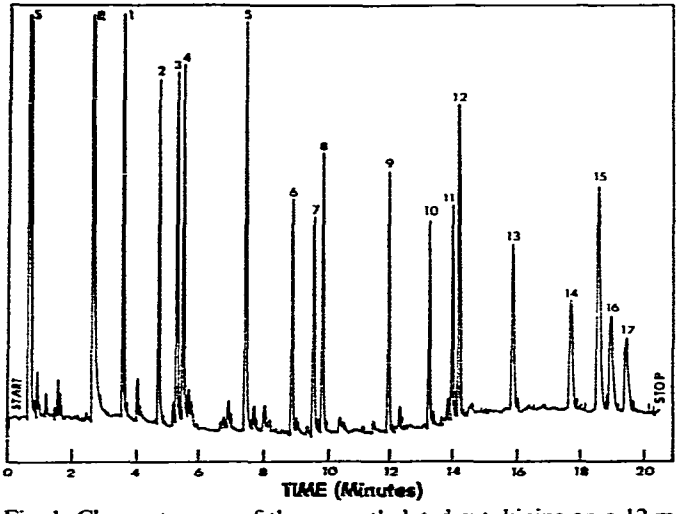

Fig. 1. Chromatogram of the permethylated cytokinins on a 12 m × 0.20 mm I.D. methyl silicone fluid fused-silica capillary column programmed from 180 to 260°C at 6°C/min. NPD, each peak contains 26 pg of the cytokinin. Peaks (for abbreviations see Table I): 1 = 2iP; 2 = DHZ; 3 = cZ; 4 = tZ; 5 = ms2iP; 6 = msDHZ; 7 = mscZ; 8 = mstZ; 9 = IPA; 10 = DHZR; 11 = cZR; 12 = tZR; 13 = msIPA; 14 = msDHZR; 15 = OHBAR; 16 = mscZR; 17 = mstZR; R = reagents; S = solvent.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 shows baseline resolution of all 17 permethylated cytokinins under the conditions described above. The performance of this column with permethylated cytokinins was superior to that reported for a 30 m SE-54 coated fused-silica capillary column which did not resolve TMS-derivatized *trans*-ribosylzeatin (tZR) from 2-methylthioisopentyladenosine (mstZR)[12]. Fig. 1 shows no significant loss of *trans*-zeatin (tZ) during permethylation and subsequent chromato-

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES, NORMALIZED RESPONSE RATIOS AND NITROGEN/CARBON RATIOS OF PERMETHYLATED CYTOKININS

Peak No	*Cytokinin*	*Relative retention*	*Normalized NPD*/FID** response ratio*	*N/C ratio* $\times 10^2$
1	Isopentenyladenine (2iP)	1.00	66	42
2	Dihydrozeatin (DHZ)	1.32	64	38
3	*cis*-Zeatin (cZ)	1.48	66	38
4	*trans*-Zeatin (tZ)	1.54	67	38
5	2-Methylthio-isopentenyladenine (ms2iP)	2.08	60	38
6	2-Methylthio-dihydrozeatin (msDHZ)	2.50	56	36
7	2-Methylthio-*cis*-zeatin (mscZ)	2.68	56	36
8	2-Methylthio-*trans*-zeatin (mstZ)	2.76	54	36
9	Isopentenyladenosine (IPA)	3.35	44	25
10	Ribosyldihydrozeatin (DHZR)	3.71	40	24
11	Ribosyl-*cis*-zeatin (cZR)	3.92	40	24
12	Ribosyl-*trans*-zeatin (tZR)	3.98	38	24
13	2-Methylthio-isopentenyladenosine (msIPA)	4.45	38	24
14	2-Methylthioribosyldihydrozeatin (msDHZR)	4.97	37	23
15	*o*-Hydroxyl-benzyladenosine (OHBAR)	5.21	34	23
16	2-Methylthioribosyl-*cis*-zeatin (mscZR)	5.33	38	23
17	2-Methylthioribosyl-*trans*-zeatin (mstZR)	5.46	34	23

* NPD response to 26.4 pg.
** FID response to 1600 pg.

graphy at low picogram levels. We have observed considerable loss of TMS-derivatized tZ at low nanogram levels on packed columns and losses have been reported on SE-54 coated fused-silica columns[12]. We also found that tailing peaks occurred during chromatography with TMS-derivatized cytokinins at picogram levels using our fused silica column and NPD.

In Table I are listed the cytokinins studied, their relative retention times, normalized NPD/FID response ratios and nitrogen/carbon (N/C) ratios. NPD response was greatest with cytokinins having the largest N/C ratios (Table I) and decreased with cytokinins containing additional sidechains, thus reducing the effective N/C ratio. These response ratios can be used as a qualitative test along with retention time in differentiating between cytokinins and other nitrogen containing compounds because the large number of nitrogen atoms (5) and the ratio of (N/C) atoms in cytokinins is unusual in small molecules.

The sensitivity and selectivity of the NPD makes it possible to reduce sample size and simplify extraction and purification procedures. This is important in the quantitative analysis of cytokinins, since such extraction and purification procedures invariably result in loss of cytokinins[13]. When used in combination with a fused silica capillary column, the NPD permitted the detection of permethylated cytokinins at low picogram levels with good resolution.

REFERENCES

1 F. Skoog and D. J. Armstrong, *Ann. Rev. Plant Physiol.*, 21 (1970) 359.
2 B. H. Most, J. C. Williams and K. J. Parker, *J. Chromatogr.*, 38 (1968) 136.
3 S. Hakomori, *J. Biochem.*, 55 (1964) 205.
4 G. C. Martin, R. Horgan and I. M. Scott, *J. Chromatogr.*, 219 (1981) 167.
5 R. D. Dandeneau and E. H. Zerenner, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 351.
6 C. A. Burgett, D. H. Smith and H. B. Bente, *J. Chromatogr.*, 134 (1977) 57.
7 S. O. Farwell, D. R. Gage and R. A. Kagel, *J. Chromatogr. Sci.*, 19 (1977) 226.
8 H. Young, *Anal. Biochem.*, 79 (1977) 226.
9 M. Claeys, E. Messens, M. van Montagu and J. Schell, in A. P. de Leenheer, R. R. Roncucci and C. van Peteghem (Editors), *Quantitative Mass Spectrometry in Life Sciences II*, Elsevier, Amsterdam, 1978, p. 409.
10 H. B. Bente, *Operating Note 5950-3536*, Hewlett-Packard, Avondale, PA, 1977.
11 J. A. Kuhnle, G. F. Fuller, J. Corse and B. E. Mackey, *Physiol. Plant.*, 41 (1977) 14.
12 T. R. Kemp and R. A. Andersen, *J. Chromatogr.*, 209 (1981) 467.
13 H. M. Dekhuijzen and E. Ch. Th. Gevers, *Physiol. Plant.*, 35 (1975) 297.