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ELECTRON-CAPTURE CAPILLARY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY OF TRIFLUOROACETYLATED CYTOKININS

MARIANNE LUDEWIG and KARL DÖRFFLING
Institut für Allgemeine Botanik und Botanischer Garten der Universität, D-2000 Hamburg 13 (G.F.R.)
and
WILFRIED A. KÖNIG*
Institut für Organische Chemie und Biochemie der Universität, D-2000 Hamburg 13 (G.F.R.)
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SUMMARY

Trifluoroacetylated derivatives of cytokinins, including N^6-(Δ^2-isopentenyl)-adenine, N^6-(Δ^2-isopentenyl)adenosine, N^6-fufuryladenine (kinetin), N^6-benzyl-adenine, *trans*-zeatin, dihydrozeatin and *trans*-zeatin riboside, were chromatographed on fused silica capillary columns and investigated by electron impact mass spectrometry. By using the "on-column" injection technique and electron capture detection, quantities as low as 1 pg of cytokinins can be detected.

INTRODUCTION

Cytokinins are potent cell division factors occurring in plant tissues and microorganisms in submicrogram quantities. Owing to their closely related structures (Fig. 1) and low concentrations, their isolation, identification and quantification raises several problems.

Various chromatographic procedures have been applied in recent years for the detection of cytokinins, including high-performance liquid chromatography (HPLC)[1–5] and gas chromatography (GC)[6–11], in some cases in combination with mass spectrometry[4–6,9–11]. In spite of this extensive work sufficient separation as well as sensitive detection of cytokinins has been achieved only rarely. The limit of detection by UV-monitoring of HPLC separations is in the range of 10 ng for pure zeatin. In GC, trimethylsilyl (TMS) derivatives, packed columns and flame ionization detection are usually employed, yielding a detection limit in the nanogram range. Even when using selected ion monitoring in mass spectrometry, 1 ng of a cytokinin was the lowest detectable amount[10]. Improved GC separations were achieved by use of glass capillary columns[12,13].

In an attempt to improve further the separation and identification procedure for natural and synthetic cytokinins, we combined the newly developed on-column injection method[14,15] with highly inert fused silica capillary columns and electron capture detection of trifluoroacetyl (TFA) derivatives of cytokinins.

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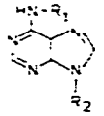


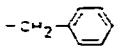
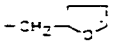
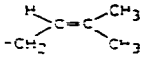
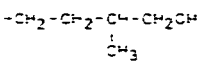
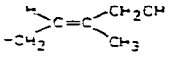
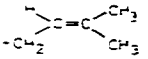
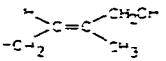
R_1	R_2	Name
Synthetic cytokinins		
	H	Benzyladenine
	H	Kinetin
Natural cytokinins		
	H	N^6-(Δ^2-Isopentenyl) adenine
	H	Dihydrozeatin
	H	trans-Zeatin
	Ribose	N^6-(Δ^2-Isopentenyl) adenosine
	Ribose	Zeatin riboside

Fig. 1. Structures of synthetic and natural cytokinins.

EXPERIMENTAL

Formation of derivatives

Amounts of 10–500 ng of pure cytokinins (Sigma, St. Louis, MO, U.S.A.) dissolved in methanol or ethanol were transferred into 1-ml vials (Microproduct L 125, Wheaton) and dried with nitrogen. After adding 200 μl dichloromethane and 50 μl trifluoroacetic anhydride, the tightly closed vials were heated for 35 min at 95°C. The excess of reagent was then removed with a gentle stream of nitrogen and the residue was dissolved in 500 μl of cyclohexane. The solution (0.1–1.0 μl) was injected into the gas chromatograph.

Gas chromatography

For GC separations, fused silica capillaries (25 m × 0.33 mm I.D.) coated with CpSil 5 (Chrompack, Berlin, G.F.R.), Carlo Erba Model 4160 and Packard 429 gas chromatographs, both equipped with an on-column injection system, and a ^{63}Ni-electron capture detector were used. The injector temperature was kept at 50–60°C; the column temperature was 180°C and was increased to 260°C at a rate of 3°C/min.

The detector temperature was 310°C. Hydrogen was used as carrier gas and nitrogen was used for the detector at a flow-rate of 20–30 ml/min.

Gas chromatography–mass spectrometry (GC–MS)

For GC–MS a Varian MAT 311 double-focusing mass spectrometer, equipped with a Carlo Erba Model 2101 gas chromatograph and Pt/Ir-interface (open coupling), was used. Electron impact (70 eV) mass spectra of TFA derivatives were recorded with a Varian MAT SS 500 data system.

RESULTS AND DISCUSSION

Although excellent separations of TMS derivatives of cytokinins can be obtained on fused silica capillary columns[13], halogenated derivatives would be preferable for electron capture detection, in order to decrease the detection limit. Several authors attempted to use perfluoroacylated derivatives but obtained unsatisfactory results[6,16], due to the extreme sensitivity of perfluoroacyl derivatives to moisture. However, from earlier MS studies on trifluoroacetylated nucleosides, it was known that TFA derivatives can be obtained in good yields[17,18]. In fact we found that all the cytokinins could be derivatized to yield single peaks in the gas chromatogram (Fig. 2). Only the retention times of zeatin and dihydrozeatin were found to be identical and resolution was not possible under the conditions described. However, these compounds can be separated by HPLC[3,19], for example on Bondapak C_{18} columns[20]. Since intensive purification of plant extracts by HPLC is advisable prior to GC, the combination of both procedures resolves this problem without consuming additional time.

In comparing the trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl derivatives, the TFA derivatives proved to be most stable, only negligible amounts of by-products being formed. Due to the high separation efficiency of the

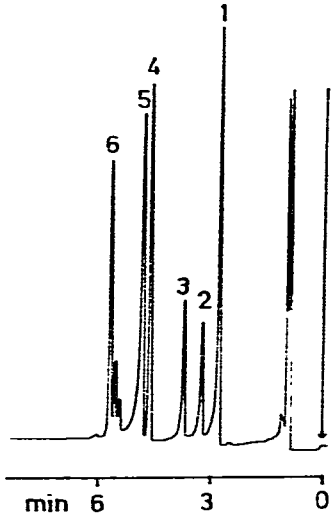


Fig. 2. Gas chromatographic separation of a standard solution of cytokinin TFA derivatives; 1 ng of each component before derivatization. Peaks: 1 = N^6-(Δ^2-isopentenyl)adenine; 2 = kinetin; 3 = zeatin; 4 = N^6-(Δ^2-isopentenyl)adenosine; 5 = N^6-benzyladenine; 6 = zeatin riboside. Electron capture detection, attenuation 512; separation conditions as in Experimental.

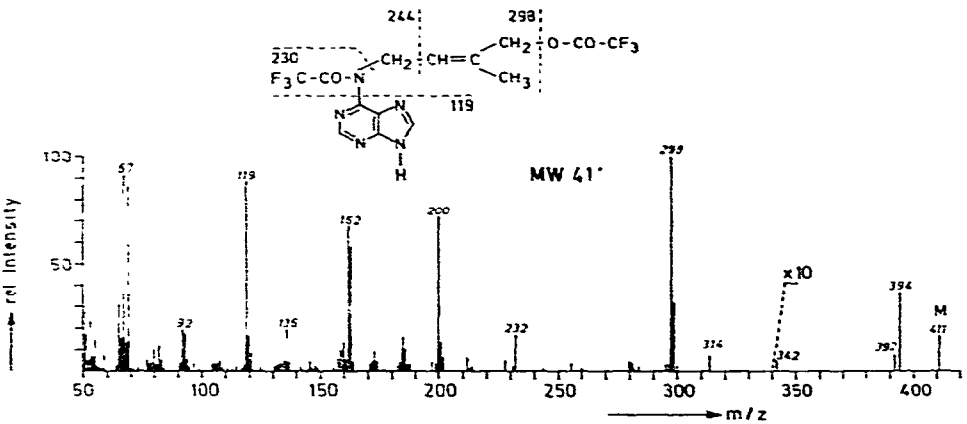


Fig. 3. 70-eV mass spectrum and fragmentation scheme of zeatin TFA derivative, GC–MS introduction.

fused silica capillary column, the excellent reproducibility of the on-column injection technique and the high sensitivity of the electron capture detector, the detection limit for all cytokinins is in the picogram range. As an example, a dose response curve for N^6-(Δ^2-isopentenyl)adenine was linear from 0.1 to 10 pg.

Although the derivatives are stable in cyclohexane solution for at least 8 h, GC analysis should be carried out shortly after derivatization.

For confirmation of the structure of the derivatives formed, mass spectra were investigated via the capillary GC inlet of the cytokinin derivatives. The mass spectra showed that the purine bases are not acylated at N-9, as indicated by the intense m/z = 119 ion of the free purine residue in the zeatin mass spectrum (Fig. 3). Intense fragment ions appear at m/z = 298 (loss of $CF_3COO\cdot$), m/z = 232 (loss of the side chain from N-6 and transfer of two hydrogen atoms to the ion), m/z = 200 (loss of CF_3COOH and CF_3CO) and m/z = 162 (loss of CF_3 from m/z = 231). In the upper mass range a molecular ion at m/z = 411 and fragments at m/z = 394 (M − OH), m/z = 392 (M − F) and m/z = 314 (M − $CF_3CO\cdot$) are observed.

The MS fragmentation was confirmed by exact mass measurements at high resolution. The mass spectrum of trifluoroacetylated zeatin riboside (Fig. 4) is very similar to the fragmentation of adenosine and adenosine derivatives[17,18]. Major fragments arise from cleavage of the base moiety B (m/z = 410) by loss of 97 mass units (B + H − $CF_3CO\cdot$; m/z = 314) and loss of 113 mass units (B + H − $CF_3COO\cdot$; m/z = 298). Further loss of a trifluoroacetic acid molecule from m/z = 314 gives rise to m/z = 200. The sugar residue (m/z = 421) easily loses two molecules of trifluoroacetic acid to yield m/z = 193. The molecular ion at m/z = 831 splits off a fluorine radical (m/z = 812) and $CF_3COO\cdot$ (m/z = 718). From the molecular ion, a $CF_3CO\cdot$ radical (m/z = 734) and an additional CF_3COOH (m/z = 620) are also lost. The ions at m/z = 232 and m/z = 162 originate from a fragmentation similar to that for the zeatin derivative.

Promising results were obtained when this new GC procedure was applied in conjunction with electron capture detection to the identification and quantification of cytokinins from plant extracts. Fig. 5 shows a gas chromatogram from 14 ml of sunflower exudate obtained by decapitating young seedlings and collecting the liquid from the cut surface of the hypocotyl stump. The exudate was prepurified by

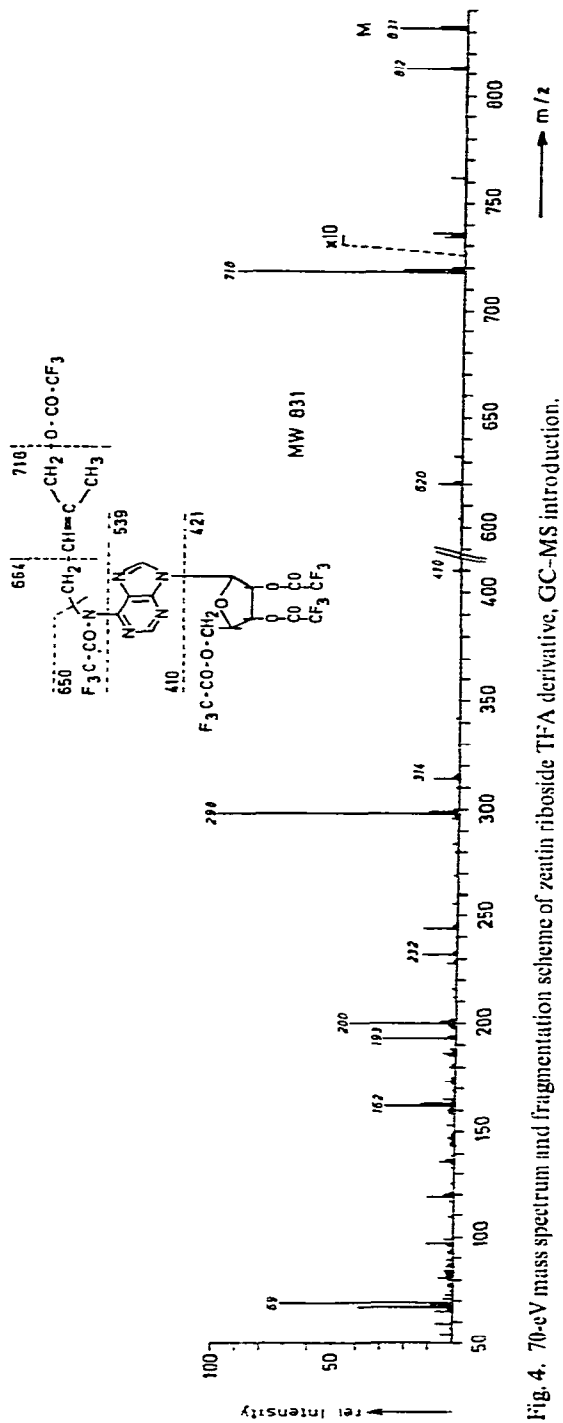


Fig. 4. 70-eV mass spectrum and fragmentation scheme of zeatin riboside TFA derivative, GC–MS introduction.

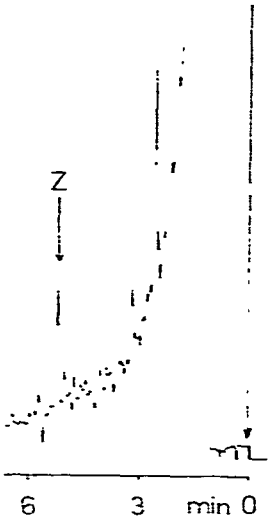


Fig. 5. Detection of zeatin (Z) from 14 ml of sunflower exudate, TFA derivative; signal corresponds to 15 pg zeatin. Electron capture detector, attenuation 16; separation conditions as in Experimental. Column temperature: 180 C.

HPLC[20]. Besides zeatin, N^6-(Δ^2-isopentenyl)adenine and the ribosides of the two cytokinin bases were identified.

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