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Note

Reversed-phase high-performance liquid chromatography of several plant cell division factors (cytokinins) and their *cis* and *trans* isomers

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Several naturally occurring cell division factors (cytokinins) in higher plants, mammalian and microbial cells have been found free[1] or covalently bound as modified nucleoside constituents in tRNA[2]. These agents are derivatives of adenine or adenosine and vary among themselves according to group substitutions on the adenine moiety[3]. At present there is a need for a rapid, convenient method for the separation and analysis of mixtures of cytokinins present as trace amounts in cellular extracts and tRNA hydrolysates. It would also be desirable for the method to permit near quantitative collection and recovery of underivatized compounds for purposes such as mass spectral and bioassay studies.

Although high-performance liquid chromatography (HPLC) has been used to separate cytokinins by ion exchange[4], reverse phase techniques[5,6] or hydrogen bonding[7], there has been no HPLC method reported that permits the resolution of the geometrical isomers of the most frequently reported cytokinins, *i.e.*, zeatin and ribosylzeatin[1,3,8,9]. The purpose of this investigation was to devise a method for the separation and analysis of these prevalent cytokinin isomers along with other cytokinins that are likely to be present in a mixture.

Two reverse phase microporous columns seemed potentially useful for the development of our HPLC method, namely, the μBondapak C_{18} and Fatty Acid Analysis columns (Waters Assoc., Milford, Mass., U.S.A.)*. The former was recently used for trace enrichment and analysis of several cytokinins[5], and the latter for the separation of *cis* and *trans* isomers of fatty acids[10]. The geometrical isomers of fatty acids and isopentenyl-substituted cytokinins have common aliphatic side-chain structural differences.

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EXPERIMENTAL

High-performance liquid chromatography was performed with an ALC/GPC 204 Waters Assoc. chromatograph equipped with two 6000A pumps, a 660 solvent flow programmer, a U6K injector, a 440 UV absorbance detector adapted for 254 nm, a μBondapak C_{18} column (30 cm × 3.9 mm I.D.; 10 μm particles), and a Fatty Acid Analysis column (30 cm × 3.9 mm I.D.; 10 μm particles).

trans-Zeatin [*t*-Z or 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine], isopentenyladenine [2iP or 6-(3-methyl-2-butenylamino)purine], and isopentenyladenosine [2iPA or 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine] were obtained from Sigma (St. Louis, Mo., U.S.A.). The following compounds were supplied by Dr. J. Corse (U.S. Department of Agriculture, Berkeley, Calif., U.S.A.): *cis*-zeatin [*c*-Z or 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)purine], *cis*-ribosylzeatin [*c*-ZR or 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine], *trans*-ribosylzeatin [*t*-ZR or 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine], *cis*-2-methylthio-ribosylzeatin [*c*-msZR or 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine], *trans*-2-methylthio-ribosylzeatin [*t*-msZR or 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine], 2-methylthio-isopentenyladenosine [ms2iPA or 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine], and *o*-hydroxybenzyladenosine [*o*-OHBAR or 6-(*o*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine].

RESULTS AND DISCUSSION

Fig. 1a is a typical chromatogram showing the separation of *cis* and *trans* isomers of zeatin and ribosylzeatin and two non-polar cytokinins achieved on a Fatty Acid Analysis column using a low solvent flow-rate of 0.3 ml/min and a programmed 50-min linear gradient of 35% to 60% methanol in water. The zeatin and ribosylzeatin isomers had k' values [retention or capacity factors where k' = (peak retention volume — void volume)/void volume] that ranged from 5.8 to 7.8. The relatively non-polar cytokinins in the chromatogram represented by isopentenyladenine and isopentenyladenosine eluted much later with k' values of 11.5 and 13.0, respectively. Similar results were obtained with the μBondapak C_{18} column using the same conditions. We believe that either of these columns used with a similar slow solvent flow-rate and program conditions will be potentially useful for the analysis or semi-preparative separation of underivatized cytokinins. The relatively slow elution of separated cytokinins under the chromatographic conditions given in Fig. 1a facilitated the manual collection of cytokinin fractions. A more rapid separation of these same components on either the μBondapak C_{18} or Fatty Acid Analysis column was achieved with a solvent flow-rate of 1.0 ml/min (Fig. 1b). Although liquid[11], thin-layer[12] and gas chromatography[13] methods have been reported for the separation and analysis of zeatin and ribosylzeatin isomers, the procedures had one or more of the following disadvantages: all four compounds were not separated in one run[11–13], the chromatography required several hours[11], derivatization was required[13], and the separated components were not easily recoverable[12].

The results obtained for the separation of a mixture of 1.5 μg of each of several relatively non-polar cytokinins on a μBondapak C_{18} column with a flow-rate

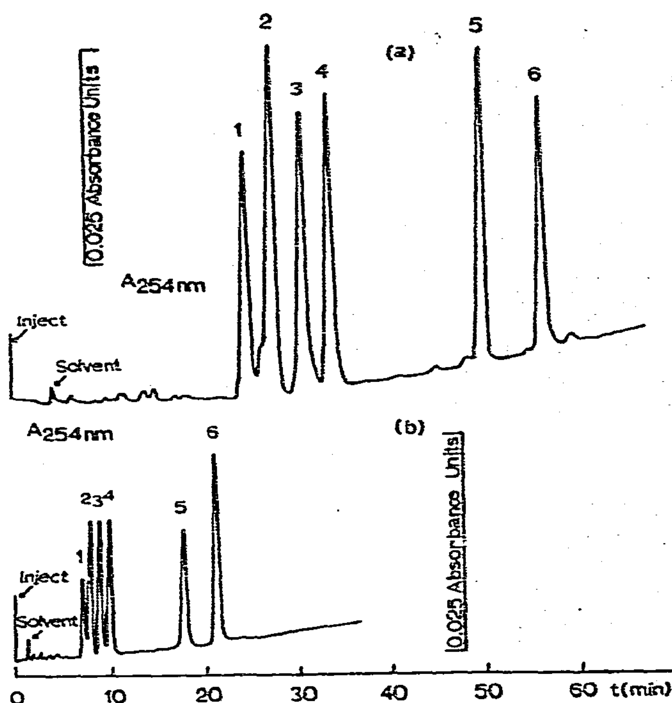

Fig. 1. Chromatograms for reversed-phase HPLC separation of cytokinins on a Fatty Acid Analysis column. Linear gradient 35:65 to 60:40 methanol–water from 0 to 50 min. (a) Flow-rate 0.3 ml/min. Injection of mixture in 4 μl methanol. Peaks: 1 = *t*-ZR (0.5 μg); 2 = *c*-ZR (0.3 μg); 3 = *t*-Z (0.25 μg); 4 = *c*-Z (0.25 μg); 5 = 2iPA (0.25 μg); 6 = 2iP (0.25 μg). (b) Flow-rate 1.0 ml/min. Injection of mixture in 3 μl methanol. Peaks: 1 = *t*-ZR (0.3 μg); 2 = *c*-ZR (0.2 μg); 3 = *t*-Z (0.2 μg); 4 = *c*-Z (0.2 μg); 5 = 2iPA (0.2 μg); 6 = 2iP (0.2 μg).

of 1.0 ml/min under programmed conditions are given in Table I. These same chromatographic conditions failed to resolve a mixture of *c*-msZR, *t*-msZR and 2iPA on either the μBondapak C_{18} or Fatty Acid Analysis columns. Subsequently, the isomers of methylthio-ribosylzeatin separated from 2iPA when the solvent flow-rate was reduced to 0.3 ml/min, but *c*-msZR and *t*-msZR were not resolved by any of the conditions or columns used.

TABLE I

HPLC SEPARATION OF NON-POLAR CYTOKININS ON A μBONDAPAK C_{18} COLUMN

A linear gradient of 10:90 to 60:40 methanol–water from 0 to 40 min was used with a 1.0 ml/min flow-rate.

Compound	*k' (retention)*
Acetophenone (internal standard)	17.5
t-Z	18.5
o-OHBAR	23.2
t-msZR	25.2
2iP	26.9
ms2iPA	32.8

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