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SEPARATION OF CYTOKININS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the separation and isolation of the naturally-occurring cytokinins from plant extracts. The method also separates any geometric isomers that occur.

The procedure involves tissue extraction with ethanol and the partitioning of the cytokinins into 1-butanol at pH 9.0.

Preliminary separation of the cytokinins into well defined groups is achieved by chromatography on a Sephadex LH-20 column. Final resolution of the individual cytokinins and their isomers is achieved by high-performance liquid chromatography on a column packed with LiChrosorb RP-8, 10 μm mean particle size.

INTRODUCTION

The separation, isolation and identification of the cytokinins in plant extracts has been the subject of much research[1–6]. This has resulted in the need to develop rapid and efficient methods not only to separate and to isolate the cytokinins from the interfering co-extractives but to do so reproducibly and quantitatively.

Several analytical procedures have been developed for specific cytokinins or groups of cytokinins with gas chromatography (GC)[6–9] and liquid chromatography (LC)[4,6,10–14] being employed. The major advantage in the use of LC over GC is that the cytokinins can be chromatographed without recourse to derivatization.

Of the procedures involving LC, a number have employed the reversed-phase mode of separation[6,10] using packing materials in the particle-size range 37–75 μm. More recently the use of high-performance liquid chromatography (HPLC), with microparticulate materials (5 and 10 μm particle size), has been reported[4,13,14]. Varying degrees of separation with a wide range of analysis times for a variety of cytokinins have been obtained, the highest degree of resolution and shortest analysis times being achieved with the microparticulate reversed-phase materials[13,14].

In only two of the papers cited above has the possible separation of geometric isomers been considered[4,10].

The object of the present study was to develop a rapid, efficient and reliable LC

method for the separation of the individual cytokinins and their isomers, for use in studies on cytokinin biosynthesis and metabolism.

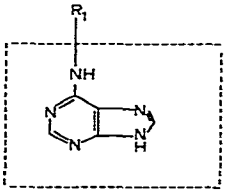
EXPERIMENTAL

Chemicals

HPLC grade methanol (Rathburn Chemicals, Walkerburn, Great Britain) was employed in the reversed-phase HPLC analysis. All other solvents were distilled unless stated otherwise.

Details of the compounds employed in this study are given in Tables I and II and these were used as received from the supplier. Standard solutions for HPLC were made up in methanol.

TABLE I
STRUCTURE OF CYTOKININ BASES



No.	*Compound*	R_1	*Supplier*
I	Adenine	H	Sigma (St. Louis, Mo., U.S.A.)
II	*trans*-Zeatin	CH_2–CH =C(CH$_3$)–CH_2OH	Sigma
III	*cis*-Zeatin	CH_2–CH =C(CH_2OH)–CH_3	Calbiochem (Los Angeles, Calif., U.S.A.)
IV	Dihydrozeatin	CH_2–CH_2–CH(CH_2OH)–CH_3	Gift
V	N^6-(Δ^2-Isopentenyl) adenine	CH_2–CH =C–$(CH_3)_2$	Sigma

Packing materials

Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was employed in the clean-up of plant extracts and LiChrosorb RP-8 (BDH, Poole, Great Britain), 10 μm mean particle size, was used for the analytical column.

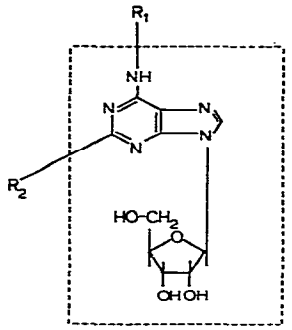
Column preparation

Clean-up column. The clean-up column, 0.45 m × 20 mm I.D., was slurry packed under gravity using a slurry concentration of *ca.* 25% (w/v) in ethanol.

Analytical column. The HPLC column, constructed from 0.20 m × 4.5 mm I.D. Apollo LC stainless-steel tubing (Magnus Scientific), was packed in the laboratory using a carbon tetrachloride slurry technique[15–17] at a packing pressure of 31.0 MPa (4500 p.s.i.g.) and employing a slurry concentration of *ca.* 10% (w/v).

TABLE II

STRUCTURE OF CYTOKININ RIBOSIDES



No.	*Compound*	R_1	R_2	*Supplier*
VI	Adenosine	H	H	Sigma
VII	*trans*-Zeatin riboside	CH_2–CH =C(CH_3)–CH_2OH	H	Sigma
VIII	*cis*-Zeatin riboside	CH_2–CH =C(CH_2OH)–CH_3	H	Present as impurity in VII
IX	Dihydrozeatin riboside	CH_2–CH_2–CH(CH_3)	H	Gift
X	N^6-(Δ^2-Isopentenyl) adenosine	CH_2–CH =C–$(CH_3)_2$	H	Sigma
XI	N^6-(Δ^2-Isopentenyl)-2-methylthio-adenosine	CH_2–CH =C–$(CH_3)_2$	SCH_3	Gift

Apparatus

The liquid chromatograph was laboratory assembled from commercially available components comprising a Haskel pneumatic amplifier LC pump, 46:1 ratio (Olin Energy Systems) and a Cecil CE 212 variable-wavelength UV monitor (Cecil Instruments) operated at 260 nm, which incorporated a slightly modified cell compartment[17].

Sample was introduced by means of a Hamilton 701N Syringe, 10-μl capacity, in combination with a septum injector (HETP LC components).

Extraction procedure

A 50-g sample of *Phaseolus vulgaris* leaves was homogenized in 400 ml of ethanol in a Waring blender for 1 min at maximum speed. The resultant slurry was centrifuged at 10,000 *g* for 20 min and the pellet discarded. The supernatant was spiked with 300 μg each of adenine, adenosine, N^6-(Δ^2-isopentenyl) adenine, N^6-(Δ^2-isopentenyl)adenosine, zeatin riboside and zeatin, and then lyophilized. The resultant solid material was dispersed in 20 ml of water and centrifuged for 5 min at 10,000 g .The supernatant was removed, adjusted to pH 9.0 using 0.1 *M* NaOH and extracted four times with equal volumes of water–saturated 1-butanol, reagent grade.

The butanol extracts were combined, lyophilized and the solid material extracted three times each with 2 ml of dry methanol. The methanol extracts were combined, lyophilized, the solid material dissolved in 2 ml of water–methanol (65:35) and chromatographed on a Sephadex LH-20 column[1].

Four fractions were collected and subsequently analyzed by HPLC.

HPLC procedure

Choice of packing material. Previous work[13,14] employing reversed-phase microparticulate packings had shown promise and hence this mode of separation was chosen for the present study. Experience in this laboratory with a range of bonded-phase packings led to the choice of LiChrosorb RP-8 as the chromatographic medium.

Choice of mobile phase. The mobile phase used in the first instance was water–methanol and the composition was adjusted to give optimum separation and retention times. The pH of the solvent was approximately 5. Two alternative approaches were investigated to try and further improve the chromatographic separation. The first of these involved the replacement of methanol by acetonitrile, as differences in selectivity are often produced by such a substitution. In this instance, however, there was a marked deterioration in the chromatography of the cytokinins. The other approach involved the addition of an ion-pair reagent to the initial mobile phase and since the cytokinins are of a basic nature, sodium lauryl sulphate was chosen for this purpose. A slight reduction in retention times was produced but no significant improvement in peak shape or resolution was obtained.

Solvent preparation entailed the preliminary refluxing of the methanol and then all water–methanol mixtures were further de-gassed by placing in an ultrasonic bath for 2 min prior to use.

Analytical conditions. Standard mixtures of cytokinins (see Table III) were chromatographed on the LiChrosorb RP-8 column employing a mobile phase of water–methanol (70:30) for Group A and water–methanol (40:60) for Group B.

The isolated fractions from the plant extracts were then chromatographed under the same conditions.

TABLE III

COMPOSITION OF CYTOKININ STANDARD MIXTURES

Group A	*Group B*
Adenosine	N^6-(Δ^2-Isopentenyl) adenosine
Adenine	
trans-Zeatin riboside	N^6-(Δ^2-Isopentenyl) adenine
cis-Zeatin riboside	
Dihydrozeatin riboside	N^6-(Δ^2-Isopentenyl)-2-methylthio-adenosine
trans-Zeatin	
cis-Zeatin	
Dihydrozeatin	

RESULTS AND DISCUSSION

The chromatograms obtained from the two groups of cytokinin standards, A and B, are shown in Fig. 1 and 2.

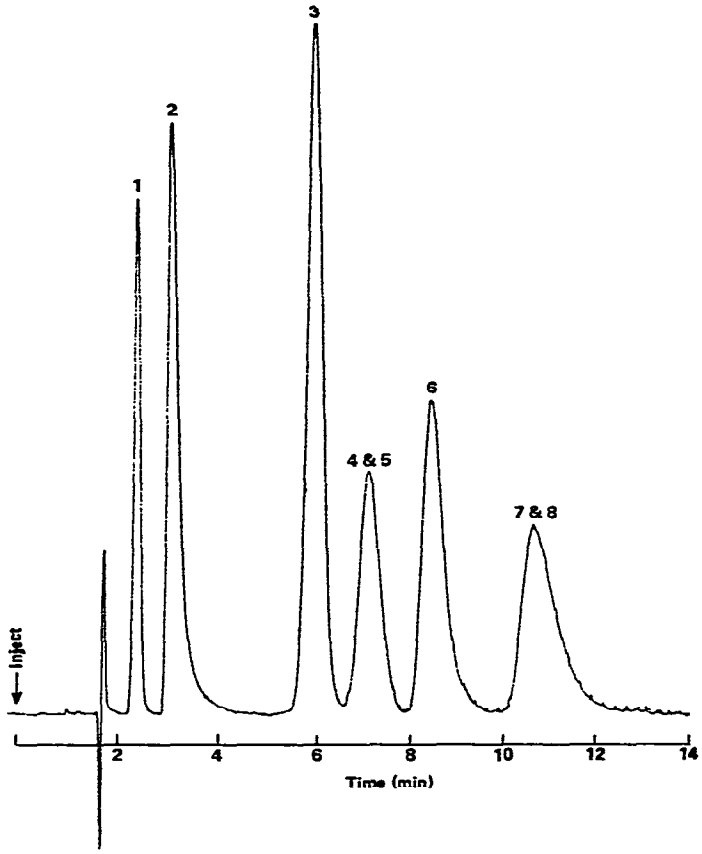


Fig. 1. Chromatogram of a synthetic mixture of cytokinin standards on a LiChrosorb RP-8 (10 μm) column. Column, 200 × 4.5 mm I.D.; Mobile phase, water–methanol (70:30); Flow-rate, 1.3 ml/min; Wavelength, 260 nm. Peaks: 1 = adenosine; 2 = adenine 3 = *trans*-zeatin riboside; 4 = *cis*-zeatin riboside 5 = dihydrozeatin riboside; 6 = *trans*-zeatin; 7 = *cis*-zeatin; 8 = dihydrozeatin.

The isomers of zeatin riboside and zeatin are well resolved (Fig. 1), however, *cis*-zeatin riboside co-elutes with dihydrozeatin riboside and similarly *cis*-zeatin co-elutes with dihydrozeatin. In both groups, A and B, the riboside elutes prior to its corresponding base. The different mobile phase systems required for the chromatography of these two groups are an indication of the differences in polarity, group B being markedly less polar than group A. Under the chromatographic conditions employed the minimum detectable quantity for the individual cytokinins ranged between 5 and 15 ng (signal-to-noise ratio 4:1).

The examination of the plant extracts required an initial clean-up, prior to HPLC analysis, in order to resolve the cytokinins present into distinct groups.

The clean-up procedure used was that described[1] for the isolation and purification of the cytokinins found in tRNA preparations and which is now used in most studies involving cytokinin fractionation and purification.

The elution profile of the spiked bean-leaf extract fractionated on a Sephadex LH-20 column is shown in Fig. 3. Most of the UV-absorbing material eluting prior

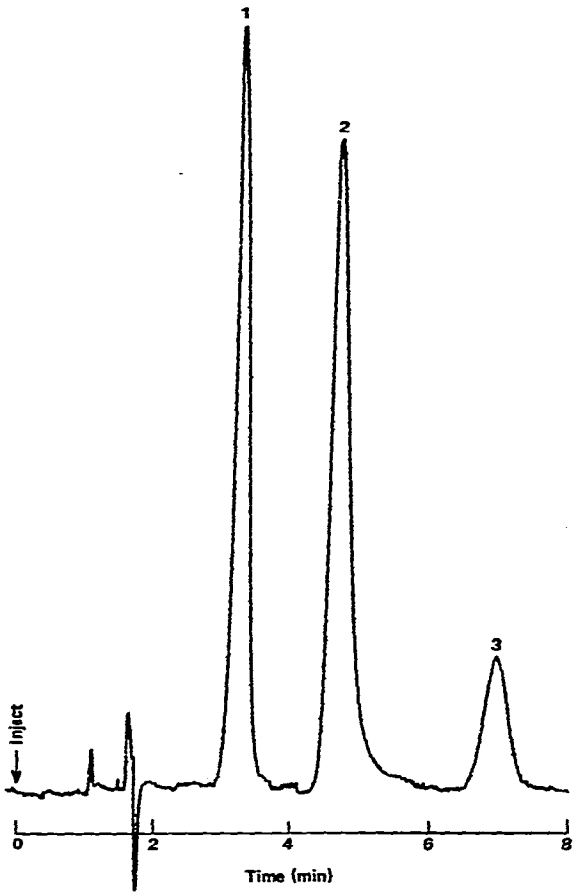


Fig. 2. Chromatogram of a synthetic mixture of cytokinin standards on a LiChrosorb RP-8 (10 μm) column. Column, 200 × 4.5 mm I.D.; Mobile phase, water–methanol (40:60); Flow-rate, 1.4 ml/min; Wavelength, 260 nm. Peaks: 1 = N^6(Δ^2-isopentenyl) adenosine; 2 = N^6(Δ^2-isopentenyl) adenine; 3 = N^6(Δ^2-isopentenyl) 2-methylthio-adenosine.

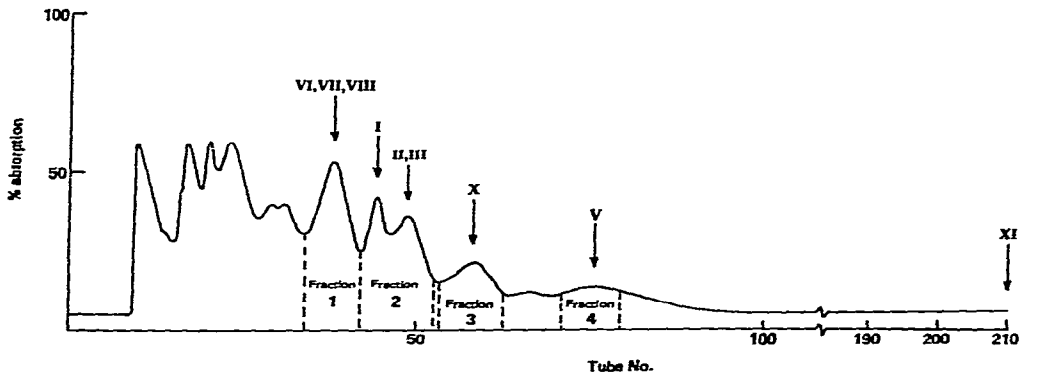


Fig. 3. Elution profile of spiked extract of *Phaseolus vulgaris* leaves on a Sephadex LH-20 column. Column, 450 × 20 mm I.D.; Mobile phase, water–methanol (65:35); Wavelength, 260 nm. Peaks: see Table I.

to the known cytokinins contains the expected cytokinin precursors and metabolites, *e.g.*, inosine, hypoxanthine, xanthine, mevalonic acid and adenosine monophosphate. The two zeatin riboside isomers co-elute with adenosine (fraction 1) and the zeatin isomers are partially separated from adenine (fraction 2). The other two cytokinins N^6-(Δ^2-isopentenyl) adenosine and N^6-(Δ^2-isopentenyl) adenine elute as discrete bands (fractions 3 and 4, respectively).

Subsequent chromatography of fraction 1 on the LiChrosorb RP-8 column resolved the isomers of zeatin riboside from each other and any co-extractives (Fig. 4). Similarly, with fraction 2 the isomers are resolved and separated from the co-extractives (Fig. 5). Fractions 3 and 4, containing N^6-(Δ^2-isopentenyl) adenosine and N^6-(Δ^2-isopentenyl) adenine respectively, were satisfactorily chromatographed by increasing the methanol content of the mobile phase (Figs. 6 and 7, respectively).

The elution order obtained on the clean-up column (Fig. 3) indicates that a predominantly reversed-phase mode of separation is occurring as previously suggested by Carnes *et al.*[10]. One notable difference, as compared with the LiChrosorb RP-8 column, is the reversal of elution order for adenine and the zeatin ribosides.

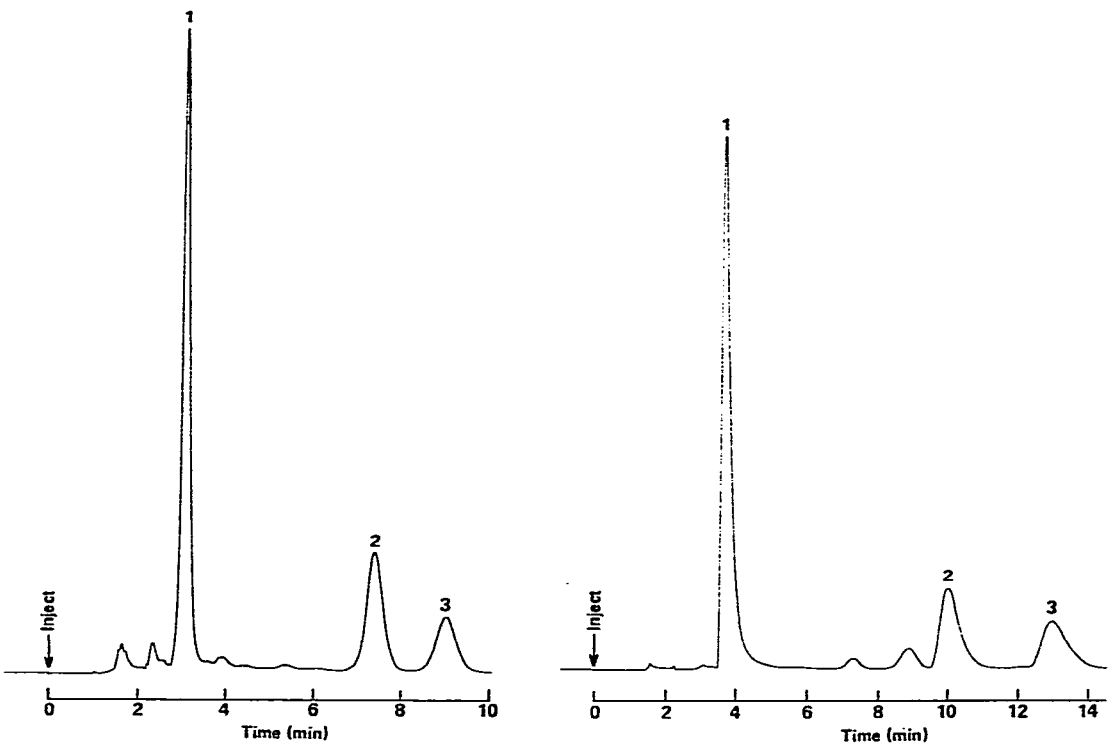


Fig. 4. Chromatogram of spiked extract of *Phaseolus vulgaris* leaves (fraction 1) on a LiChrosorb RP-8 (10 μm) column. Column, 200 × 4.5 mm I.D.; Mobile phase, water–methanol (70:30); Flow-rate, 1.1 ml/min; Wavelength, 260 nm. Peaks: 1 = adenosine; 2 = *trans*-zeatin riboside; 3 = *cis*-zeatin riboside.

Fig. 5. Chromatogram of spiked extract of *Phaseolus vulgaris* leaves (fraction 2) on a LiChrosorb RP-8 (10 μm) column. Conditions as in Fig. 4. Peaks: 1 = adenine; 2 = *trans*-zeatin; 3 = *cis*-zeatin.

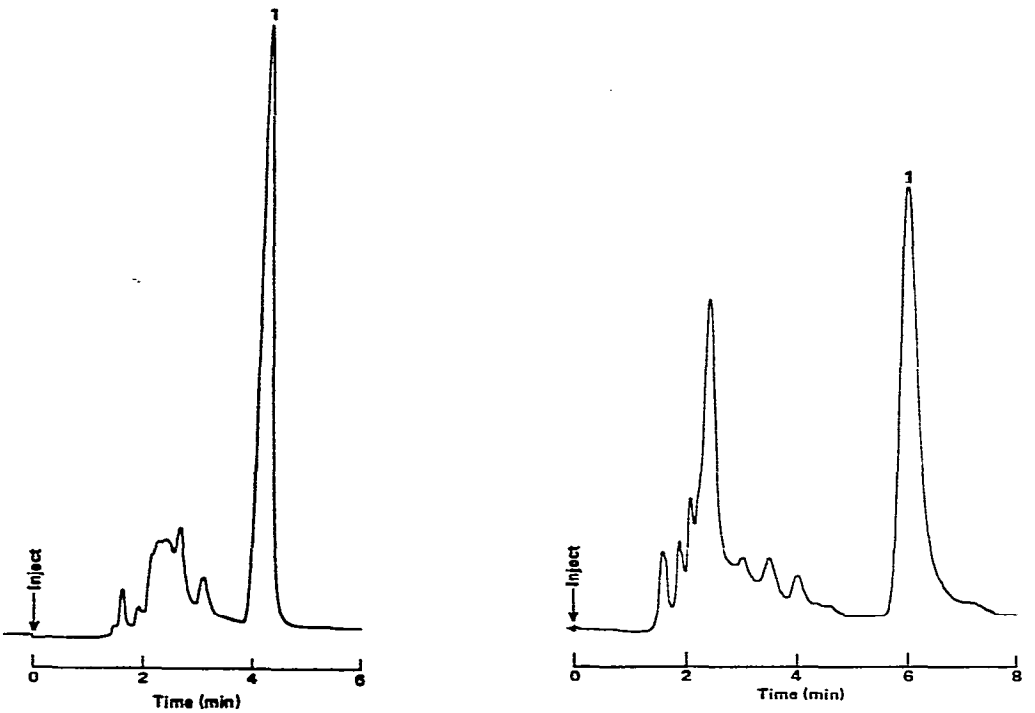


Fig. 6. Chromatogram of spiked extract of *Phaseolus vulgaris* leaves (fraction 3) on a LiChrosorb RP-8 (10 μm) column. Column, 200 × 4.5 mm I.D.; Mobile phase, water–methanol (40:60); Flow-rate, 1.1 ml/min; Wavelength, 260 nm. Peak: 1 = N^6-(Δ^2-isopentenyl) adenosine.

Fig. 7. Chromatogram of spiked extract of *Phaseolus vulgaris* leaves (fraction 4) on a LiChrosorb RP-8 (10 μm) column. Conditions as in Fig. 6. Peak: 1 = N^6-(Δ^2-isopentenyl) adenine.

If dihydrozeatin riboside and dihydrozeatin had been present in the plant extracts the Sephadex LH-20 clean-up column would not have separated them from *cis*-zeatin riboside and *cis*-zeatin respectively. However, by employing a Sephadex G-10 column for the clean-up[18] these co-eluting cytokinins would be resolved.

In the above studies the leaf extract was spiked with a relatively large amount of the naturally occurring cytokinins. More recent studies on cytokinin biosynthesis using cytokinin-autonomous tobacco callus[19] have demonstrated that 10 μg of each of the cytokinins, when added to 200 g of callus tissue, is more than adequate for the UV detection of the cytokinins eluting from the HPLC column.

CONCLUSIONS

The present study demonstrates the ability of chemically bonded, reversed-phase microparticulate material to separate a wide range of cytokinins and in particular to resolve their geometric isomers.

The advantages of the short retention times and low elution volumes of the HPLC system make it a valuable technique for the separation and isolation of unknown components for subsequent identification by spectroscopic techniques.

In combination with a prior Sephadex clean-up the HPLC procedure can readily be used both qualitatively and quantitatively in both biosynthetic and metabolic studies.

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