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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CYTOKININS

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SUMMARY

Details are given of reversed-phase, normal-phase, and adsorption high-performance liquid chromatographic systems suitable for the separation of a wide range of naturally occurring cytokinins related to zeatin. Particular attention has been given to the development of systems to separate cytokinins which are difficult to separate by more conventional means.

INTRODUCTION

High-performance liquid chromatography (HPLC) is being used increasingly for the isolation and estimation of plant growth substances. Abscisic acid[1] and 3-indolylacetic acid[2] have been detected and measured in plant extracts by this method. A recent publication[3] has reported the use of both analytical and preparative HPLC to achieve excellent separations of a wide range of gibberellins both as the free acids and as benzyl esters.

There have been several reports on the use of HPLC for the isolation and identification of cytokinins from plant material[4–6]. However, both the compounds separated and the methods used have been somewhat limited. In general, separations have been developed using only the limited range of cytokinins readily available from commercial sources and have been exclusively confined to reversed-phase HPLC systems. In addition the identifications of cytokinins based on their elution volumes from HPLC have not generally been confirmed by more rigorous means.

As part of our research into the biosynthesis and metabolism of cytokinins it became necessary to extend the available HPLC techniques to separate efficiently a wide range of naturally occurring cytokinins related to zeatin, and their metabolites. We deemed it of particular importance to develop HPLC techniques to separate compounds exhibiting close structural similarities, *e.g.*, dihydrozeatin, the *cis* and *trans* isomers of zeatin and their corresponding ribosides and glucosides. These are compounds which are particularly difficult to separate by more conventional means.

Most of the published work on HPLC of cytokinins has involved the use of ODS (C_{18}) reversed-phase systems. This is presumably because of the ease of operation of these systems under gradient-elution conditions and because reversed-phase

systems would be expected to have the greatest utility for the analyses of these relatively polar compounds. Published work[7] describing the separations of cytokinins on pellicular polyamide and various ion-exchange materials will not be discussed here as the efficiency and resolution achieved in these systems are too low to be considered in the context of present day HPLC.

The excellent resolution of certain cytokinins on thin-layer chromatography (TLC) suggests that with the choice of suitable solvent systems adsorption HPLC should be an equally powerful tool for cytokinin analysis. Possibly because of the difficulty of finding suitable solvent systems for the adsorption HPLC of polar compounds and the relative ease with which solvent systems can be chosen for reversed-phase HPLC this area has received little attention. In addition, when attempting to purify compounds in trace quantities from natural sources it is frequently advantageous to use techniques based on as widely different mechanisms of separation as possible. Thus we have explored the use of both adsorption and normal-phase HPLC as complementary techniques to reversed-phase HPLC for the separation of cytokinins.

This paper reports on the optimisation of reversed-phase HPLC for the separation of a wide range of cytokinins and also briefly discusses suitable solvent systems and stationary phases for adsorption and normal-phase HPLC of these compounds.

MATERIALS AND METHODS

Chemicals

All chromatographic solvents used were of reagent grade and were distilled in an all-glass apparatus before use.

Triethylammonium bicarbonate (TEAB) was prepared by saturating a 2.5 *M* solution of triethylamine with carbon dioxide.

All the cytokinins used in this study were synthesized by one of us (R.H.). Product identity was established by ultraviolet, mass spectrometric and nuclear magnetic resonance analyses.

Columns and packing materials

Columns (150 × 4.5 mm I.D.) of Hypersil, Hypersil ODS and Hypersil SAS (Shandon Southern, Runcorn, Great Britain) were prepared by slurry packing at 6000 p.s.i. using a pneumatic amplifier pump (Jones Chromatography, Llanbradach, Great Britain). Methanol was used as the slurry medium for the silica and isopropanol for the reversed-phase materials. Columns of Spherisorb, 5 μm (Phase Separations, Queensferry, Great Britain) were similarly prepared. Prepacked columns of Partisil 10 and Partisil 10 PAC (250 × 4.5 mm I.D.) were purchased from Whatman (Maidstone, Great Britain).

Chromatographic equipment

Chromatography under isocratic conditions was carried out using an Altex 110 pump (Altex Scientific, Berkeley, Calif., U.S.A.) and a Cecil CE 212 variable wavelength monitor (Cecil Instruments, Cambridge, Great Britain) operating at 265 nm and fitted with a 8-μl flow-through cell. Gradient-elution chromatography was carried out on a Pye LC3X system (Pye Unicam, Cambridge, Great Britain).

Sample introduction on both chromatographs was via an Altex 905-42 syringe loading sample injector. All injections were of 30 μl and were made into a 100-μl loop.

RESULTS

Reversed-phase HPLC

Two reversed-phase materials were used in the course of these investigations, Hypersil ODS, a 5-μm totally porous microspherical silica with a C_{18} bonded stationary phase, and Hypersil SAS, a similar material with a C_2 bonded phase.

Solvent systems investigated were all binary mixtures of methanol, ethanol or acetonitrile, and water. The effects of varying the pH and the ionic strength of the aqueous component were studied. Previously published work on reversed-phase HPLC of cytokinins has invariably involved the use of solvent systems at pH values of 2.5 to 3.5. The exact reasons for the use of these conditions are not clear. The pK_a values of most cytokinins are 4 in aqueous solution and these will be lowered somewhat in organic solvents, hence the pH of these solvent systems will be very close to the pK_a values of the cytokinins. Under these conditions maximum separation efficiencies will not be obtained. In addition the presence of a positive charge on all the compounds may tend to mask small polarity effects due to slight structural differences and lead to the inefficient separation of closely related compounds. In most cases the pH of the aqueous component of the mobile phase has been adjusted using acetic acid. It is possible that under these conditions ion-pairing effects may contribute to the separations observed. Some workers[4] have used acidic buffers as the aqueous components of their mobile phases to mask residual adsorption sites on the stationary phase. The use of acidic aqueous phases should however be avoided if possible since it has been observed in our laboratory that hydrolysis of cytokinin-O-glucosides can occur when these phases are removed by evaporation.

In view of the points made above and because the stationary phases used were virtually free of residual adsorption sites all the separations reported here were achieved at pH 7, at which pH the cytokinins are un-ionised. The use of pH values below 6 and above 8 gave inferior separations.

Since one of the objects of this study was to develop methods for the isolation of microgram quantities of cytokinins from natural sources it was considered essential to stabilise the pH of the aqueous component with a substance which could be easily and completely removed under the mildest possible conditions of temperature and pH. It was found that the addition of a few drops of 2.5 *M* TEAB to glass distilled water provided adequate stabilisation of the pH at 7. This material could be completely removed by co-evaporation with methanol at room temperature. Care must be exercised in the use of helium degassing systems with this buffer since a too vigorous purging with helium will cause the pH to rise due to the flushing out of carbon dioxide from the solvent. Provided mild and sporadic helium purging is used, the pH is stable for more than 12 h. Fig. 1 illustrates the separation of a complex mixture of zeatin-derived cytokinins on a 150 × 4.5 mm I.D. column of Hypersil ODS eluted with a gradient of increasing concentration of acetonitrile in water at pH 7 (adjusted with TEAB). The use of ethanol or methanol in place of the acetonitrile resulted in poorer separations. A similar separation was obtained for the corresponding dihydrozeatin compounds, but with each dihydrozeatin derivative

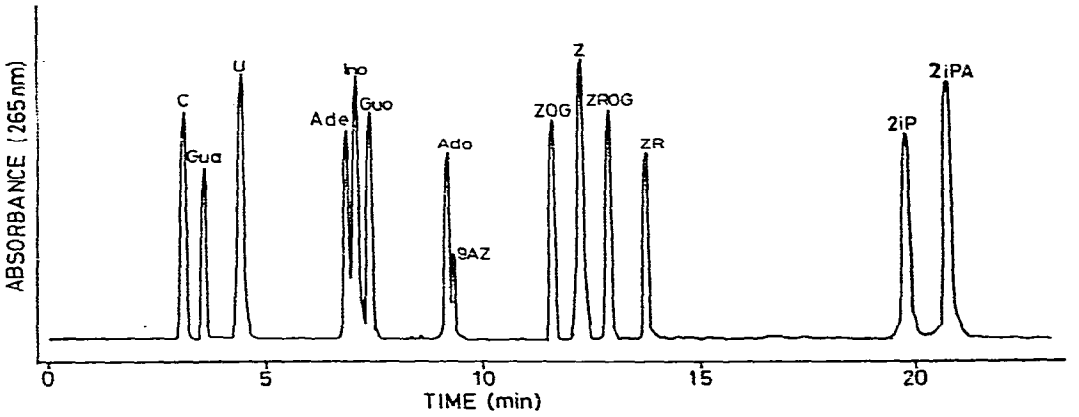


Fig. 1. Separation of a series of cytokinins related to zeatin and a number of common purines and pyrimidines by reversed-phase HPLC. Column, Hypersil ODS (150 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, 3 segment linear gradient, water (pH 7 with TEAB) to 11% acetonitrile over 7 min, 11 to 20% acetonitrile over 9 min, and 20 to 40% acetonitrile over 15 min. Abbreviations: Ade = adenine; Ado = adenosine; C = cytidine; Gua = guanine; Guo = guanosine; Ino = inosine; U = uridine; Z = zeatin; ZR = ribosyl zeatin; 9AZ = 9-alanyl zeatin; ZOG = zeatin-O-glucoside; ZROG = ribosyl zeatin-O-glucoside (all zeatin compounds are *trans* unless otherwise stated and all glucosides are the β-D-glucopyranosides); 2iP = N^6-isopentenyl adenine; 2iPA = N^6-isopentenyl adenosine.

eluting slightly later than its zeatin counterpart. Figs. 2 and 3 illustrate the excellent resolving power of this system for mixtures of zeatin- and dihydrozeatin-derived compounds which are particularly difficult to separate by other methods. It is of

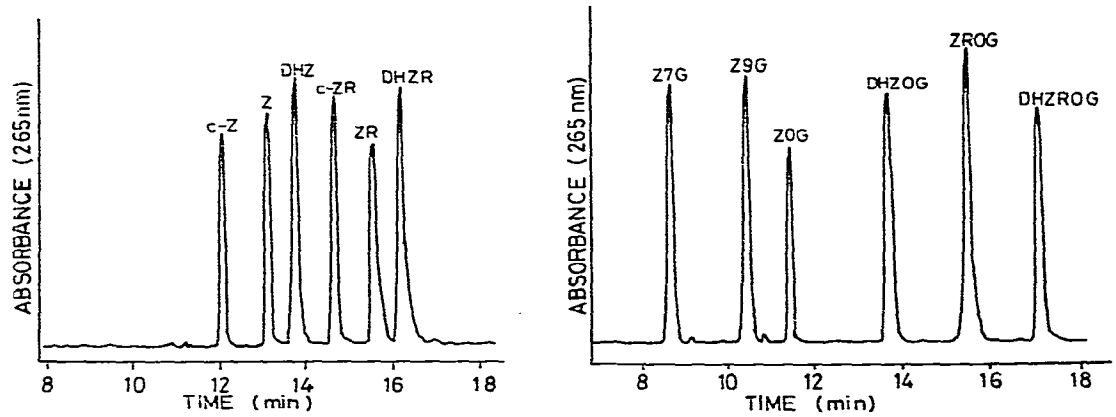


Fig. 2. Separation of a series of closely related zeatin derivatives by reversed-pahse HPLC. Separation conditions as in Fig. 1 except mobile phase, linear gradient of water (pH 7 with TEAB) to 30% acetonitrile over 30 min. Abbreviations: c-Z = *cis*-zeatin; c-ZR = ribosyl *cis*-zeatin; DHZ = dihydrozeatin; DHZR = ribosyl dihydrozeatin. Other abbreviations as in Fig. 1.

Fig. 3. Separation of a series of cytokinin glucosides by reversed-phase HPLC. Separation conditions as in Fig. 1 except mobile phase, linear gradient of 5% acetonitrile in water (pH 7 with TEAB) to 20% acetonitrile over 30 min. Abbreviations; Z9G = zeatin-9-glucoside; Z7G = zeatin-7-glucoside; DHZOG = dihydrozeatin-O-glucoside; DHZROG = ribosyl dihydrozeatin-O-glucoside. Other abbreviations as in Fig. 1.

interest to note that with the exception of 9-alanyl zeatin all the cytokinins elute later than the common purines.

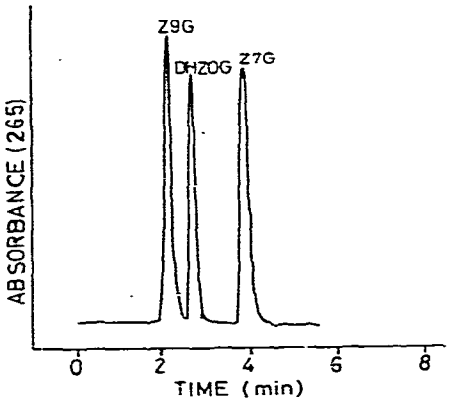
The separations achieved on Hypersil SAS were similar to those shown in Figs. 1, 2 and 3, although compounds eluted at a lower acetonitrile concentration and the resolution was somewhat inferior due to the greater amount of peak tailing exhibited by this phase. However, the magnitude of the separation between the cytokinins as a group and the common purines was greater on this material than on Hypersil ODS, and in addition 9-alanyl zeatin could be completely separated from adenosine.

We have used Hypersil SAS extensively in our laboratory for studies into the biosynthesis of cytokinins. Using a 150 × 4.5 mm I.D. column of this material and eluting isocratically with 5% acetonitrile in water (pH 7 with TEAB) it is possible to completely remove the common adenine metabolites, which usually constitute 99% of the labelled compounds extracted from tissue fed with radioactively-labelled compounds extracted from tissue fed with radioactively-labelled adenine, from the very minute quantities of cytokinins produced. In practice the column is eluted with the above solvent until the eluate reaches background radioactivity and the cytokinins are then eluted with pure methanol. We have found that a column of this size can efficiently effect this sort of group separation with sample sizes of up to 100 mg.

Adsorption HPLC

No detailed studies of cytokinins on adsorption HPLC have been published. The excellent resolution of quite complex mixtures of cytokinins by TLC indicates considerable potential for this technique although the solvent systems used are in most cases inappropriate for HPLC. However, as stated in the Introduction, when attempting to isolate small quantities of natural products in the pure state there is considerable advantage in the sequential utilisation of several separation techniques operating by as widely different mechanisms as possible. We have therefore conducted a preliminary investigation into the potential of adsorption HPLC for cytokinin analysis with a view to using it as a complementary technique to reversed-phase HPLC for the isolation and identification of cytokinins from plant sources.

The HPLC adsorbants investigated were Partisil 10, Spherisorb 5 μm and Hypersil. The utility of these materials was related to their specific surface areas. The most active of these, Partisil 10, with specific surface area of 400 m^2/g, was found to be useful only with solvent systems having a high proportion of a polar component (water, ammonium hydroxide or acetic acid). It was found to be particularly useful for the separation of closely related cytokinin glucosides using tetrahydrofuran–water mixtures, an example of which is shown in Fig. 4. Spherisorb 5 μm and Hypersil with specific surface areas of 300 and 200 m^2/g, respectively, exhibited similar properties but the lower activity Hypersil was found to be the most satisfactory material for the widest range of compounds. We attempted to develop a solvent system for HPLC which showed similar properties to the *n*-butanol–ammonia (sp.gr. 0.880)–water (6:1:2, upper phase) system which is useful in the TLC analysis of cytokinins. This work led to solvent systems based either on chloroform or acetonitrile with polar components of methanol and/or water and containing ammonium hydroxide. With solvent systems of this type all the compounds used in this investigation could be chromatographed satisfactorily. For example a mixture of zeatin-7- and zeatin-9-



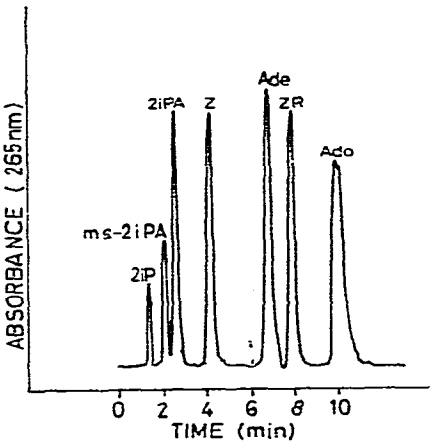


Fig. 4. Separation of three cytokinin glucosides by adsorption HPLC. Column, Partisil 10 (250 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, tetrahydrofuran–water (9:1). Abbreviations as in Figs. 1 and 2.

Fig. 5. Separation of adenine, adenosine and a series of cytokinins by adsorption HPLC. Column, Hypersil (150 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, ammoniacal chloroform (1 l of chloroform was satuarted by shaking with 250 ml of ammonia (sp.gr. 0.880) and allowed to stand overnight)–methanol–water (100:5:0.3). The column was eluted with 100 ml of mobile phase before starting the analysis. Abbreviations: ms-2iPA = 2-methylthio-isopentenyl adenosine. Other abbreviations as in Fig. 1.

glucosides could be well separated on a 150 × 4.5 mm I.D. column of Spherisorb 5 μm using a solvent system of acetonitrile–methanol–ammonium hydroxide (sp.gr. 0.880) (100:10:1). Fig. 5 illustrates an example of a cytokinin separation on Hypersil with a chloroform-based solvent system. This particular adsorbent/solvent combination has been used very successfully in our laboratory for the final purification of samples of zeatin and zeatin riboside after initial purification by reversed-phase HPLC. It should be pointed out however that when using solvent systems of this type that are effectively 100% water saturated, at least 20 column volumes should be allowed to pass through the column to ensure that stable capacity factors (k') are obtained.

The results presented above illustrate just a few of the solvent systems which we have found to be useful for the isolation and purification of cytokinins from natural sources. They do demonstrate however the potential of adsorption HPLC for cytokinin analysis and we hope will offer guidelines to other workers to develop systems to help solve their particular purification problems.

Normal-phase partition HPLC

No details of normal-phase partition HPLC of cytokinins have been published. We have briefly investigated the use of Partisil 10 PAC, a polar phase material consisting of cyanopropyl groups bonded to a microparticulate silica, for this purpose. This material appears to have good potential for the separation of the polar cytokinins which are more difficult to chromatograph on adsorption systems. Thus it could be used to complement reversed-phase chromatography for these compounds. Fig. 6 illustrates the separation of zeatin-7- and zeatin-9-glucosides on this material using a solvent system of acetonitrile–water (9:1). It should be noted that the separation of these compounds in this system is better than that achieved in any of the other HPLC systems studied in this investigation.

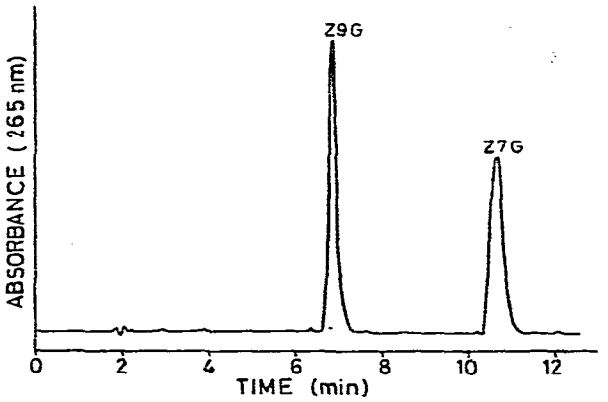


Fig. 6. Separation of isomeric zeatin N-glucosides by normal-phase partition HPLC. Column, Partisil 10 PAC (250 × 4.5 mm I.D.); flow-rate, 2 ml/min; mobile phase, acetonitrile–water (9:1). Abbreviations as in Fig. 1.

DISCUSSION

The results of this investigation show that reversed-phase HPLC is probably the most powerful single chromatographic technique for the purification of cytokinins from plant material. The suitability of reversed phases for operation under gradient-elution conditions with simple solvent systems, their high sample capacities under these conditions and their ability to separate a very wide range of compounds makes reversed-phase HPLC the obvious first step method for cytokinin purification. Reversed-phase HPLC has been viewed as a substitute for the more time consuming reversed-phase chromatography on Sephadex LH-20. In our view there are many situations where the two techniques should be considered complementary. With care, LH-20 columns can be constructed on almost any scale and can provide an initial purification step when large sample sizes make immediate preparative HPLC impracticable.

It should be borne in mind that although the results presented here demonstrate the excellent resolving power of Hypersil ODS for complex mixtures of naturally occurring cytokinins they do not necessarily indicate the usefulness of this material for the purification of cytokinins in plant extracts.

Preliminary work in our laboratory does however indicate that the reversed-phase system described in this paper performs well for the isolation of cytokinins from plant material. Using a 150 × 10 mm I.D. column of Hypersil ODS and the solvent system used in Fig. 1 it has been possible to isolate a pure sample of zeatin riboside from the ethyl acetate soluble, basic material obtained from 260 g fresh weight of *Vinca rosea* crown gall tissue. This was the only chromatographic step used in the procedure.

The elution order of the cytokinins on Hypersil ODS suggests that this material is not operating exclusively by a reversed-phase mechanism. In spite of the fact that the number of residual adsorption sites is minimal, evidenced by a k' value for nitrobenzene in heptane of <0.2 (ref. 8), the more polar ribosides elute later than the corresponding bases. This sort of anomaly has been noted for other reversed-phase materials[9].

As a result of our preliminary investigations into the adsorption and normal-phase HPLC of cytokinins, we believe that these techniques are of considerable value as second-stage methods to reversed-phase HPLC. They have received little attention to date, probably due to the difficulty of finding suitable solvent systems and the belief, mistaken we believe, that all the problems of cytokinin purification will be solved by the use of reversed-phase techniques. Obviously workers in this field will have to develop solvent systems suitable for their particular applications. However, we believe that the adoption of these techniques in combination with reversed-phase HPLC will eventually lead to a more efficient utilisation of HPLC as the major tool for the isolation of cytokinins from plant material.

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NOTE ADDED IN PROOF

Two recent publications by Andersen and Kemp[10] and Holland *et al.*[11] have described reversed-phase chromatography of cytokinins on reversed-phase materials other than those described here. The very different separations reported in these papers highlight the different properties of superficially similar reversed-phase materials. These are possibly due to minor differences in the extent of bonded phase coverage and the exact nature of the support material.

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