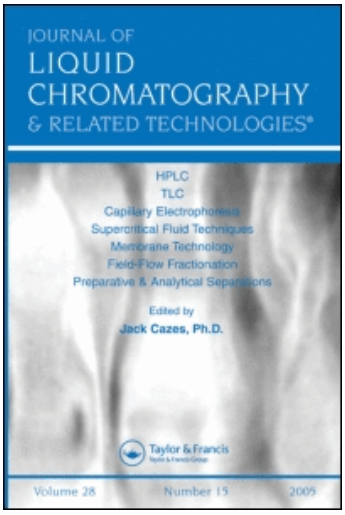

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Ernstsen, Arild and Jensen, Einar(1985) 'High Performance Liquid Chroma Tography of Cytokinins', Journal of Liquid Chromatography & Related Technologies, 8: 2, 369 — 379
To link to this Article: DOI: 10.1080/01483918508067084
URL: http://dx.doi.org/10.1080/01483918508067084

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(2), 369 - 379 (1985)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF CYTOKININS

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ABSTRACT

Details are presented of normal- and reversed-phase high performance liquid chromatographic systems suitable for the purification and the separation of a range of cytokinins. In the normal-phase mode an aminopropylsilyl column with volatile organic solvents as mobile phases was used while reversed-phase chromatography was carried out with a base deactivated octadecylsilyl column.

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 0148-3919/85/0802-0369$3.50/0

INTRODUCTION

In recent years, high performance liquid chromatography (HPLC) has come into use for the isolation and quantification of plant growth substances. Abscisic acid (1) and 3-indole acetic acid (2,3) have been isolated and measured by this method. HPLC has also been used to separate a wide range of gibberellins both as free acids (4) and as their methoxy-coumaryl esters (5).

In some of the early attempts to separate cytokinins by HPLC, ion-exchange columns were used (6), but the resins did not provide good resolution of the reference compounds, and serious difficulties were encountered when analysing endogenous cytokinins in plant extracts. Although octadecylsilyl (ODS) reversed-phase HPLC systems have been used for the separation of cytokinins (7,8) it is our experience that broad peaks and severe tailing are frequently observed. This is probably due to the binding of weakly basic cytokinins to free silanol groups on the surface of the silica support. This problem can be avoided either by deactivating the surface of the support or by adding a component to the mobile phase that competes with the solute for the binding sites.

By adding triethylammonium bicarbonate to the mobile phase, cytokinins related to zeatin were successfully separated with good peak symmetry on ODS-Hypersil columns (9). However it should be noted that care must be exercised with this system when the solvents are degassed with helium since purging the buffer phase will remove CO_2 and cause the pH to rise (9). Also, the separations achieved during an attempt to dublicate this procedure were not satisfactory (10). It therefore became necessary to extend the available HPLC techniques to purify and separate a range of cytokinins. In order to avoid the problem of tailing peaks, a deactivated ODS column was used which provides high efficiency and good peak symmetry for adsorptive basic compounds.

When attempting to purify compounds in trace quantities from complex plant extracts, it is advantageous to use HPLC procedures based on as widely different separatory mechanisms as possible (11).

Thus, we have also explored the use of an aminopropylsilyl (APS) column using volatile organic solvents as the mobile phase.

MATERIALS AND METHODS

The liquid chromatograph consisted of a M 45 and a M 6000A pump, a U6K injector, a 660 solvent programmer, a M 480 absorbance monitor (Waters Assoc.), and a LKB 2210 recorder. The HPLC columns employed were a 5 um 250 x 4.6 mm ID Supelcosil LC-18-DB (Supelco) and a 10 um 250 x 4.6 mm ID Lichrosorb 10 NH_2 (Chrompack).

HPLC grade methanol, acetonitrile and n-hexane were supplied by Rathburn (Walkerburn, Great Britain), and ethanol was obtained from A/S Vinmonopolet (Tromsø, Norway). Adenine (A), cis- and trans-zeatin (c-Z, t-Z), kinetin (K), benzyladenine (BA), their ribosides (AR, c-ZR, t-ZR, KR, BAR) and dihydrozeatin (DHZ) (Sigma) were dissolved in dimethylformamide at a concentration of 100 ng ul^{-1}.

RESULTS AND DISCUSSION

Reversed-phase HPLC

The reversed-phase support used in this investigation, Supelcosil LC-18-DB, is specially deactivated to provide high efficiency and good peak symmetry with basic compounds. Solvent systems investigated were all binary mixtures of methanol or acetonitrile and water, and the pH was controlled by using an aqueous phase of either 20 mM ammonium acetate (pH 7.0) or 20 mM acetic acid (pH 3.5). The results are presented in Tables 1 - 4.

With methanolic solvents the free bases and their corresponding ribosides were eluted in the order A, Z, DHZ, K, BA and iP. This reflects the decreasing polarity of the 6-amino substituent of the different cyto kinins. Tables 1 and 2 also shows that each free base/riboside pair are

TABLE 1

Retention Volumes of Cytokinins. Column: Supelcosil LC-18-DB. Solvents: methanol and water, both 20 mM in ammonium acetate

Cytokinin	Retention volume (ml) as function of per cent methanol						
	60%	50%	40%	30%	20%	10%	5%
A				3.7	4.5	7.3	10.5
AR				3.9	5.8	16.2	
t-Z			5.5	9.0	25.8		
c-Z			6.3	11.1	33.7		
t-ZR			5.8	10.9			
c-ZR			6.6	13.0			
DHZ			6.2	13.0			
K		5.7	9.0	17.3			
KR		5.7	9.3	21.9			
BA	6.0	9.5	19.3				
BAR	5.6	9.2	20.8				
iP	6.7	11.6	23.7				
iPR	6.2	11.4	24.6				

TABLE 2

Retention Volumes of Cytokinins. Column: Supelcosil LC-18-DB. Solvents: methanol and water, both 20 mM in acetic acid

Cytokinin	Retention volume (ml) as function of per cent methanol							
	70%	60%	50%	40%	30%	20%	10%	5%
A				6.7	7.1	7.3	8.5	16.8
AR					5.5	6.8	12.0	28.1
t-Z			6.8	8.6	12.7	31.3		
c-Z				9.8	16.6			
t-ZR				6.5	11.8	32.9		
c-ZR				7.2	14.5			
DHZ			7.8	10.3	15.1	32.7		
K			8.3	13.8	25.5			
KR			6.1	10.7	23.1			
BA		8.1	10.3	15.3	32.2			
BAR			7.0	10.0	28.8			
iP	7.9	10.4	13.8	20.3				
iPR		6.5	7.9	11.7	26.0			

TABLE 3

Retention Volumes of Cytokinins. Column: Supelcosil LC-18-DB. Solvents: acetonitrile and water, both 20 mM in ammonium acetate

Cytokinin	Retention volume (ml) as function of per cent acetonitrile						
	30%	25%	20%	15%	10%	5%	2.5%
A						6.0	9.4
AR					4.6	11.5	29.5
t-Z				5.8	11.8		
c-Z				6.8	15.3		
t-ZR				6.9	20.2		
c-ZR				7.9	24.8		
DHZ				6.5	14.8		
K		6.0	8.9	14.1			
KR		5.8	11.2	17.5			
BA	6.6	10.1	18.7				
BAR	6.0	9.8	20.6				
iP	6.4	9.9	17.9				
iPR	5.8	9.4	19.5				

TABLE 4

Retention Volumes of Cytokinins. Column: Supelcosil LC-18-DB. Solvents: acetonitrile and water, both 20 mM in acetic acid

Cytokinin	Retention volume (ml) as function of per cent acetonitrile						
	60%	50%	40%	30%	20%	10%	7.5%
A					7.4	7.9	9.0
AR					5.2	6.5	8.3
t-Z				8.5	9.3	22.7	
c-Z				9.2	10.7	30.0	
t-ZR					7.5	18.0	
c-ZR					8.3	23.6	
DHZ			9.4	9.6	10.7	26.8	
K		7.9	8.7	11.1	17.5		
KR				5.1	9.1	50	
BA	9.3	10.8	12.6	28.5			
BAR			4.9	7.2	19.7		
iP	14.2	15.2	18.8				
iPR		5.0	5.8	8.4	19.5		

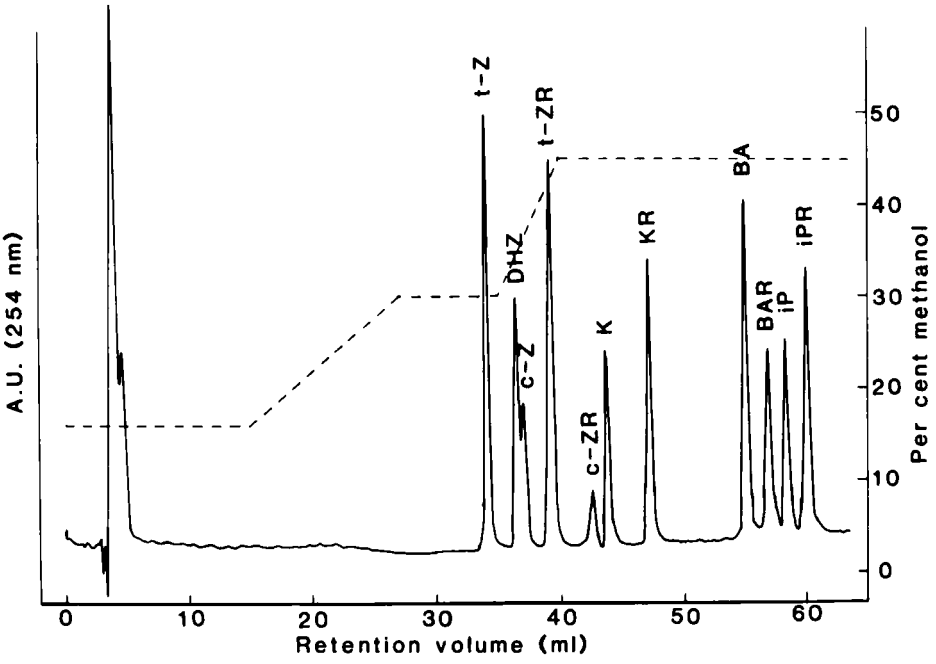

FIGURE 1

Reversed-phase Liquid Chromatography of Cytokinins.
Gradient elution with methanol and water, both 20 mM in ammonium acetate. Column: Supelcosil LC-18-DB. Gradient: 16% methanol isocratic for 15 min, 16% to 30% methanol in 12 min, 30% methanol isocratic for 8 min, 30% to 45% methanol in 5 min. Flow: 1 ml min^{-1}.

well separated from all other free base/riboside pairs. At pH 7.0 and with methanolic concentrations higher than ca. 40%, free bases eluted close to their corresponding ribosides. At lower methanolic concentrations the ribosides eluted later than their free bases. The more polar cytokinins were more rapidly separated into their free base and riboside (A/AR in ca. 6 min, iP/iPR in ca. 30 min). At pH 3.5 and with methanolic concentrations greater than ca. 30% the ribosides were eluted before and well separated from their corresponding bases. Therefore, in order to separate free base/riboside, pH 3.5 is to be preferred. For example K/KR are separated in less than 6 min at pH 3.5 and in 15 min at pH 7.0.

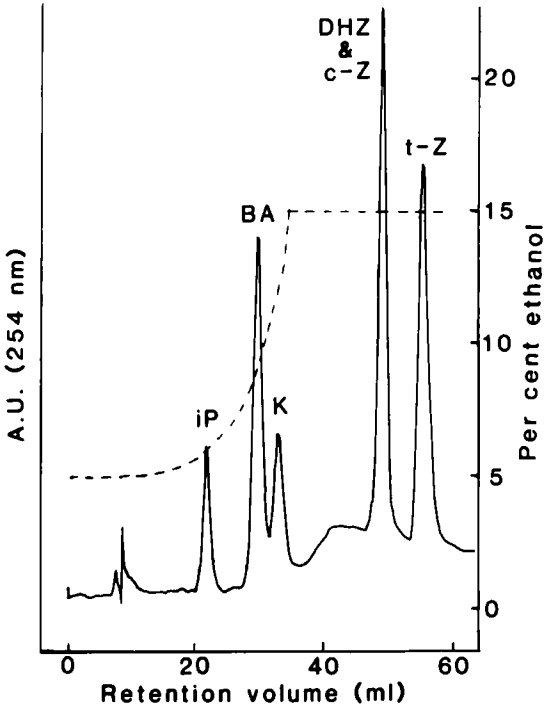

FIGURE 2

Normal-phase Liquid Chromatography of Cytokinins.
Gradient elution of free bases with ethanol (10 mM in ammonium acetate) and n-hexane. Column: Lichrosorb 10 NH_2. Concave gradient: 5% to 15% ethanol in 15 min. Flow: 2 ml min^{-1}.

The data presented in Tables 1, 2 and 3 show that the cytokinins eluted more rapidly with pH 7.0 acetonitrile based solvents than with methanolic solvents, but with a similar retention order. A different selectivity was obtained with acetonitrile based solvents at pH 3.5. At all concentrations of acetonitrile the ribosides eluted before and well separated from their corresponding free bases. Thus, when a rapid separation of riboside/free base is required, the pH 3.5 acetonitrile based solvents should be used.

TABLE 5

Retention Volumes of Cytokinins. Column: Lichrosorb 10 NH_2. Solvents: ethanol (10 mM in ammonium acetate) and n-hexane

Cytokinin	Retention volume (ml) as function of per cent ethanol							
	40%	35%	30%	25%	20%	15%	10%	5%
iP					6.4	7.6	9.8	17.2
BA					8.2	10.0	13.4	25.2
K				8.0	8.8	11.0	15.0	29.4
iPR			7.8	9.2	11.6	16.2	28.8	
DHZ		8.0	9.2	10.4	13.2	18.8		
c-Z		8.4	9.2	11.4	14.0	19.2		
BAR		8.2	9.4	11.6	14.8	21.6		
t-Z	8.0	9.2	10.8	13.2	16.6	24.0		
A	9.2	10.4	11.4	13.6	16.6	22.0		
KR	7.6	8.8	10.4	13.0	16.8	24.8		
c-ZR	8.8	10.4	13.2	17.2	25.2			
t-ZR	9.6	11.6	15.0	20.4	30.8			
AR	11.4	13.8	17.2	22.4				

TABLE 6

Retention Volumes of Cytokinins. Column: Lichrosorb 10 NH_2. Solvents: ethanol and n-hexane, both 10 mM in acetic acid

Cytokinin	Retention volume (ml) as function of per cent ethanol							
	50%	40%	30%	25%	20%	15%	10%	5%
iP					6.4	7.6	9.8	18.0
BA					8.0	10.0	13.2	26.4
K				7.6	8.6	10.8	15.0	30.0
iPR			7.6	8.8	11.2	16.0		
DHZ			8.4	10.2	13.2	18.6		
c-Z			9.0	10.8	13.8	19.4		
BAR			9.2	10.8	15.0	21.6		
t-Z		7.4	10.0	12.2	16.6	24.0		
A		8.4	10.8	12.8	16.0	21.8		
KR		7.4	10.0	12.2	16.6	24.4		
c-ZR		8.4	12.4	16.0	24.4			
t-ZR	7.0	9.4	14.4	18.8	29.8			
AR	8.4	11.2	16.8	21.0	30.8			

Although a similar elution order was obtained with methanol and pH 7.0 acetonitrile based solvents, methanolic solvents gave a better resolution between the less polar cytokinins. The column efficiency was higher at pH 7.0 than at pH 3.5. Therefore, methanolic solvents at pH 7.0 were preferred for the analyses of the cytokinins under study (Figure 1). In Figure 1 all cytokinins, except c-Z and DHZ, are separated with baseline resolution.

Normal-phase HPLC

Extracts from plant tissues containing only trace amounts of the compound(s) of interest have to be extensively purified before quantitative analysis can be performed. In chromatographic purification steps, much time can be saved if the mobile phase is volatile organic solvents. The mobile phase used in the current study was, thus, hexane and ethanol mixtures, and pH was controlled by using solvents either 10 mM in ammonium acetate or 10 mM in acetic acid. The column was a 250 x 4.6 mm ID Lichrosorb 10 NH_2.

The results are presented in Tables 5 and 6. A similar retention order was obtained with solvents 10 mM in ammonium acetate and acetic acid, and the retention volume was only slightly influenced by pH. With the exception of iPR and BAR all the ribosides eluted as one group and later than the free bases. This is in contrast to reversed-phase HPLC where all free base/riboside pairs eluted together and well separated from all other pairs. Thus, normal-phase HPLC may provide a tool for partly separating ribosides as a group, from free bases. Also, the method is well suited to rapidly separate a riboside from its corresponding free base.

A gradient separation of the free bases is shown in Figure 2. All compounds except c-Z and DHZ were separated from each other. The ribosides were easily separated by isocratic elution. This is demonstrated in Figure 3.

The Lichrosorb 10 NH_2 column provided symmetric peaks for the various cytokinins, but the column efficiency was low. This can be improved

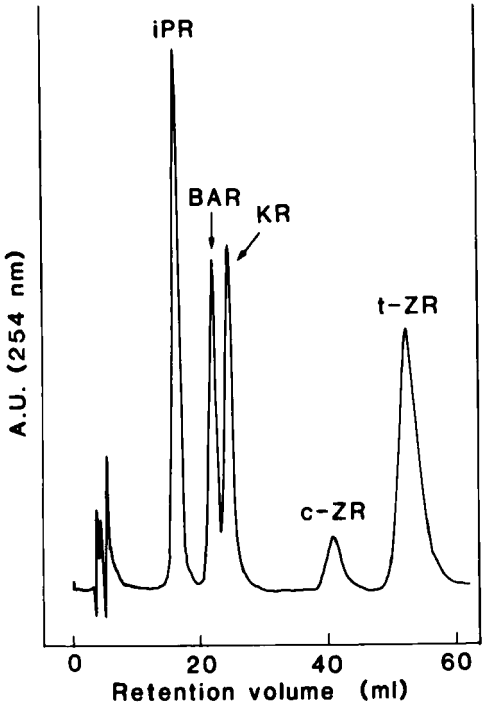

FIGURE 3

Normal-phase Liquid Chromatography of Cytokinins.
Isocratic elution of ribosides with 15% ethanol in n-hexane, both 10 mM in acetic acid. Column: Lichrosorb 10 NH_2. Flow: 2 ml min^{-1}.

by using 3u or 5u particles instead of 10u particles, but still the efficiency of such a column will be inferior to the typical reversed-phase column. Therefore, this system is not advisable to use in the final, quantitative step, but in our opinion it is a useful tool in the purification of cytokinins in plant extracts.

ACKNOWLEDGEMENT

We are grateful to Dr. Alan Crozier, University of Glasgow, for valuable comments on the manuscript.

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