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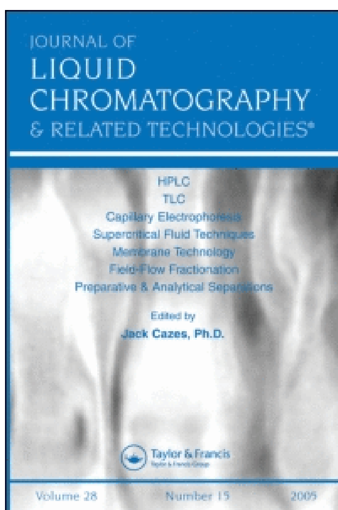

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Determination of Cytokinins by Ion Suppression-Reverse Phase High Performance Liquid Chromatography

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DETERMINATION OF CYTOKININS BY ION SUPPRESSION-REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the separation of cytokinins has been developed by the use of ion suppression-reverse phase high performance liquid chromatography (IS-RP HPLC) with high efficiency, specificity and low sensitivity. Four reverse phase chromatographic columns were compared for their ability to separate and quantify six cytokinins with respect to signal response, resolution (R_s), capacity factor (k') and efficiency, N (number of theoretical plates). The optimal aqueous and organic phase components for the resolution of these cytokinins were determined with respect to pH, ionic strength and the proportion of the organic solvents, methanol and acetonitrile. Heptane sulfonic acid was used as an organic modifier. The optimal HPLC operating conditions were applied to separate and quantify the cytokinins present in a culture supernatant of Azotobacter chroococcum.

INTRODUCTION

Cytokinins, one of the five major plant hormones, are defined as N^6-substituted amino purines and occur as a free base, ribonucleoside, ribonucleotide and glucosylated forms. The isolation and quantification of nanogram quantities of these compounds found in biological samples

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has rendered analysis difficult. Because of the limited separation of cytokinins from interfering substances, an accepted criterion for the detection of naturally occurring cytokinins is that of biological activity, as measured by several bioassays. A majority of researchers have relied upon bioassays for quantification of these compounds; however, several disadvantages exist including: limitations in sensitivity and reliability, inability to distinguish cytokinin species, and observed responses can be the net result of both inhibitory and promotive substances. Conventional analytical methods for cytokinins including solvent extraction and partitioning, ion exchange, column chromatography, TLC, GC and GC-MS have been reviewed extensively by Horgan (1) and will not be discussed here.

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High performance liquid chromatography (HPLC) offers many advantages for the separation and quantification of plant growth regulators (PGRs) including: speed of analysis, high sensitivity, efficiency, minimal sample preparation and wide scope of applications (2). HPLC of cytokinins was first introduced by Carnes and co-workers (3) and has found wide applications in the separation of free bases and their conjugates. Most of the published work on HPLC of cytokinins has involved the use of reverse phase systems because of the ease of separation of these relatively polar compounds under gradient elution. Considerable work has been performed using the μBondapack C_{18} (4), Nucleosil (5), Hypersil (4,6,7) and the octadecyl Ultrasphere packings (8,9). However, many of these studies have not reported resolution among isomers and detection limits. This is the first study to assess the Adsorbosphere-type columns for their suitability in the separation of cytokinins. There is a need to investigate the different properties of superficially similar reverse phase materials. The minor differences

in the bonded phase used in various reverse phase columns result in varied elution times of similar cytokinins, such that one column type may be more efficient than another with respect to sample type, extraction method or corresponding eluant combination. Separation of similar species on a bonded phase column can be enhanced by the use of organic modifiers (10). The equilibrium constant of the reaction for each species is also dependent upon pH, the ionic strength and the concentration of the organic solvent in the mobile phase.

This paper reports the optimization of IS-RP HPLC for the separation of cytokinins with UV detection under gradient conditions. Four reverse phase chromatographic columns [Versapack (10 μ), Adsorbosphere C_{18} (10 μ), Adsorbosphere HS C_{18} (7 μ) and Adsorbosphere Nucleoside/Nucleotide (7 μ)] were compared for their efficiency, sensitivity, signal response and resolution capabilities with respect to pH, organic solvent, organic modifier concentrations, and ionic strength.

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MATERIALS AND METHODS

Reagents

The following reagents were used: zeatin riboside (io^6Ado), dihydrozeatin riboside (H_2-io^6Ado), _t_-zeatin (_t_-io^6Ade), _c_-zeatin (_c_-io^6Ade), isopentenyl adenosine (i^6Ado), isopentenyl adenine (i^6Ade), all obtained from Sigma Chemical Co. (St. Louis, MO); ammonium acetate (NH_4OAc) from Fisher Scientific (Pittsburgh, PA); heptane sulfonic acid sodium salt (HSA) from Eastman Kodak (Rochester, NY); HPLC-grade methanol (MeOH) and acetonitrile from Burdick and Jackson (Muskegon, MI); HPLC-grade water was prepared as described by Karlson and Frankenberger (11).

HPLC Apparatus

The instruments used included: a Beckman Model 421 HPLC Controller, two Beckman Model 110A pumps, a silica (50 μ) [Alltech Associates, Deerfield, IL] pre-saturation column (250 x 4.6 mm), Beckman Model 210 Sample Injector, 20 μL stainless steel sample loop, an Adsorbosphere HS C_{18} (5 μ) guard column (Alltech Associates), Beckman Model 165 Variable Wavelength Detector (constant λ = 275 nm; scan 200-350 nm), Kipp & Zonen BD 40 Chart Recorder and a Hewlett Packard 3390A Integrator. Detector response was recorded in terms of peak area and was linearly proportional to the amount of cytokinins injected over several orders of magnitude.

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Stationary Phases

Four reverse-phase chromatographic columns, obtained from Alltech Associates, were tested for their separation capabilities of several cytokinins.

Versapack (10 μ) (250 x 4.6 mm; batch no. 081684-6) consists of irregularly shaped silica and according to Alltech Associates has similar properties to that of Waters Associates μBondapack C_{18} column. The silica support has a surface area of 200 m^2 g^{-1}, a carbon load of 10% and a pore diameter of 80 Å. Three modes of separation may occur while using this column. The silica support functions not only by reverse phase partition but also serves as a solid adsorbent and as a molecular size separation packing.

Adsorbosphere C_{18} (10 μ) (250 x 4.6 mm; batch no. 062585-H) is a spherical silica packing with an average pore diameter of 80 Å, 200 m^2 g^{-1} surface area, a 12% carbon load and an efficiency rated at 45,000 N m^{-1}.

Adsorbosphere HS C_{18} (7 μ) (250 x 4.6 mm; batch no. 080686-K) is a spherical silica packing with an average pore diameter of 60 Å, 350 m^2 g^{-1} surface area and a carbon load of 20%.

Adsorbosphere Nucleoside/Nucleotide (7 μ) (250 x 4.6 mm; batch no. 012887-I) is a spherical silica packing, also exhibiting a high surface area of 350 m^2 g^{-1}, average pore diameter of 60 Å and a carbon load of 20%. It has been specifically adapted for the separation of nucleosides and nucleotides.

The higher carbon load and surface areas of the Adsorbosphere HS C_{18} (7 μ) and Nucleoside/Nucleotide (7 μ) columns generally enhance separation and efficiency, lengthen column life and are particularly adapted for IS-RP HPLC.

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Mobile Phase

The ionic strength of the aqueous phase was altered via the use of ammonium acetate ranging from 5 to 30 mM, in increments of 5 mM. The concentration of the organic modifier, HSA, was altered by varying the concentration from 0.5 to 2 mM, in increments of 0.5 mM. The pH of the aqueous phase was varied between 2.5 and 7.0; both extremes being limited by the stability of the bonded phase on the columns used.

The organic phases tested for their suitability in separating cytokinins included: methanol, acetonitrile, and acetonitrile:methanol [40:60, 50:50, 60:40 (v:v)]. Several isocratic, linear and nonlinear gradient profiles were created with the organic phase ranging from 0-80% within the aqueous phase over various time periods, up to 45 minutes in duration.

Bacterial Growth

The culture of *Azotobacter chroococcum* (ATCC 9043) was grown in 100 mL of medium #14 ATCC in 250 mL side-arm culture flasks at 27°C for

72 h under dark conditions. Culture supernatants (2°C) were filtered (0.22 μ), and partitioned 3x (25 mL) with ethyl acetate (pH 2.5). The ethyl acetate fractions were pooled and extracted 3x (25 mL) with water-saturated n-butanol (pH 7.0). The butanol fractions were pooled and allowed to dry under N_2 (40°C) to prevent oxidation of the cytokinins. Samples were reconstituted in 5 mL of 1.0 M K_2HPO_4 (pH 7.0), filtered (0.22 μ) and analyzed by HPLC using the developed methodology for cytokinin separation with the Adsorbosphere Nucleoside/Nucleotide (7 μ) column, UV spectrometry and the radish cotyledon bioassay (12).

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RESULTS AND DISCUSSION

Stationary and Mobile Phases

The mobile phase consisted of binary mixtures of methanol (P', polarity index = 5.1) or acetonitrile (P' = 5.8) and ammonium acetate buffer, or a tertiary mixture of acetonitrile, methanol and the aqueous buffer. While the solvent polarity indices are similar, acetonitrile is somewhat more polar, hence being slightly more effective in eluting the polar cytokinins. For most of the stationary phases, the use of acetonitrile/NH_4OAc buffer caused the resolution of *cis*- and *trans*-zeatin to decrease. The mobile phase, methanol/NH_4OAc tended to broaden the peaks of the separated cytokinins. The tertiary mixture of acetonitrile:methanol [60:40 (v:v)]/NH_4OAc buffer was the most effective in providing symmetrical peaks for both the Adsorbosphere HS C_{18} (7 μ) and the Adsorbosphere Nucleoside/Nucleotide (7 μ) columns. While a gradual increase in the percentage of the organic solvent over time within the buffer increased the retention volume of the solutes, an increase in

efficiency and resolution were achieved in the case of all the columns tested. Figure 1 depicts optimal separation of the sample cytokinins for the four reverse phase chromatographic columns. Clearly, the Adsorbosphere Nucleoside/Nucleotide (7 μ) column provides symmetrical peaks of all six cytokinins with optimal resolution.

The effects of varying the ionic strength and pH of the aqueous phase (NH_4OAc buffer) were tested. The pK_a's of most cytokinins in aqueous solution approximate 4.0 (13). We varied the pH from 2.6 to 7.0 (the pH limitations of the columns used) and found that the signal response and peak shape were optimal at the following pH values for each respective column: 3.50, Adsorbosphere HS C_{18} (7 μ); 3.50, Adsorbosphere Nucleoside/Nucleotide (7 μ); 4.00, Adsorbosphere C_{18} (10 μ); 4.75, Versapack (10 μ). For all the columns tested, except for Versapack (10 μ), resolution improved as pH increased up to pH 4.0. At higher pH values, separated cytokinins began to coelute.

On the Adsorbosphere Nucleoside/Nucleotide (7 μ) column, the pH of the eluant affected the signal response (peak area) of ribosylated cytokinins differently than it did free base cytokinins. The peak areas of ribosylated cytokinins were greater at pH values $\leq$ 3.25, while the peak areas of non-ribosylated cytokinins were greater at pH values $\geq$ 3.25 (Fig. 2).

The acidic ammonium acetate buffer served to suppress solute ionization of the cytokinins and produced sharper zone elution profiles. Care must be taken, however, as this buffer may damage the bonded phase of the chromatographic columns at high concentrations over prolonged use. The optimal concentration of NH_4OAc for enhancing the signal response of separated cytokinins was 20 mM for the Versapack (10 μ), Adsorbosphere C_{18} (10 μ), and Adsorbosphere HS C_{18} (7 μ) columns. The

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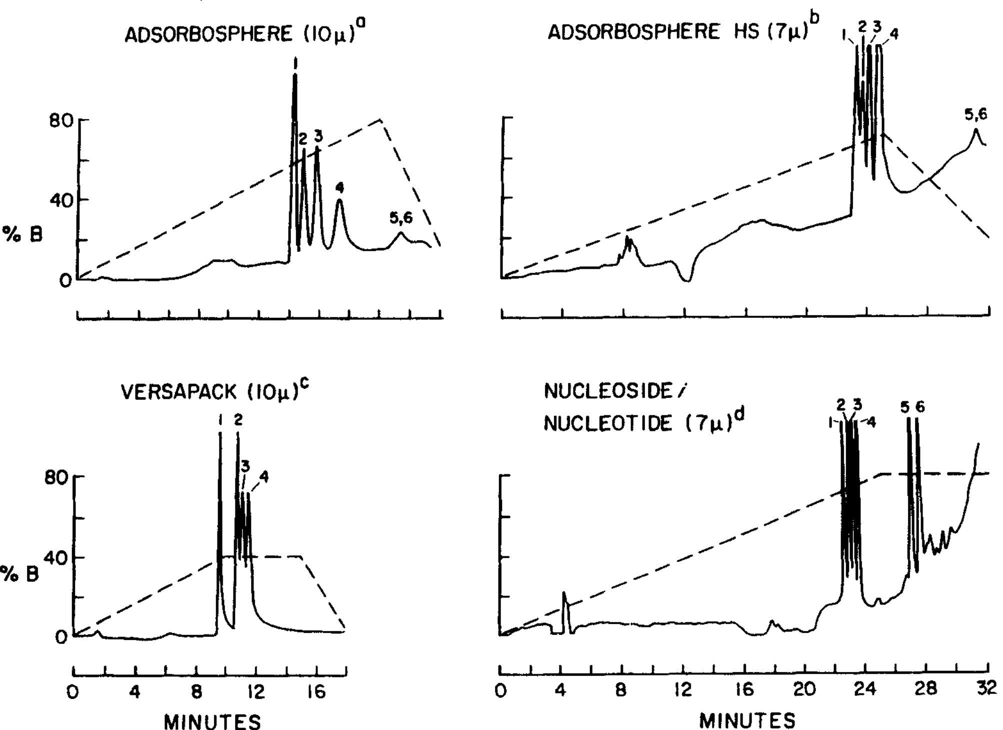

Figure 1. Typical chromatograms of cytokinins by IS-RP HPLC. Flow rate 1 mL/min; sample injected, 20 μL; detection, 275 nm. [a]Conditions: eluants, A = 0.02 M NH_4OAc + 0.001 M HSA, pH 4.00, B = MEOH; gradient: 0-80% B over 20 min; 275 nm. [b]Conditions: eluants, A = 0.02 M NH_4OAc + 0.001 M HSA, pH 3.50; B = acetonitrile:MEOH (60:40); gradient: 0-70% B over 12.5 min; 275 nm. [c]Conditions: eluants, A = 0.02 M NH_4OAc + 0.001 M HSA, pH 4.75; B = acetonitrile; gradient: 0-40% B over 10 min followed by 40% B for 5 min; 275 nm. [d]Conditions: eluants, A = 0.001 M NH_4OAc + 0.002 M HSA, pH 3.50; B = acetonitrile:MEOH (60:40); gradient: 0-80% B over 25 min followed by 80% B for 5 min; 275 nm. [1] io^{6}Ado [2] H_2-io^{6}Ado [3] t-io^{6}Ade [4] c-io^{6}Ade [5] i^{6}Ado [6] i^{6}Ade

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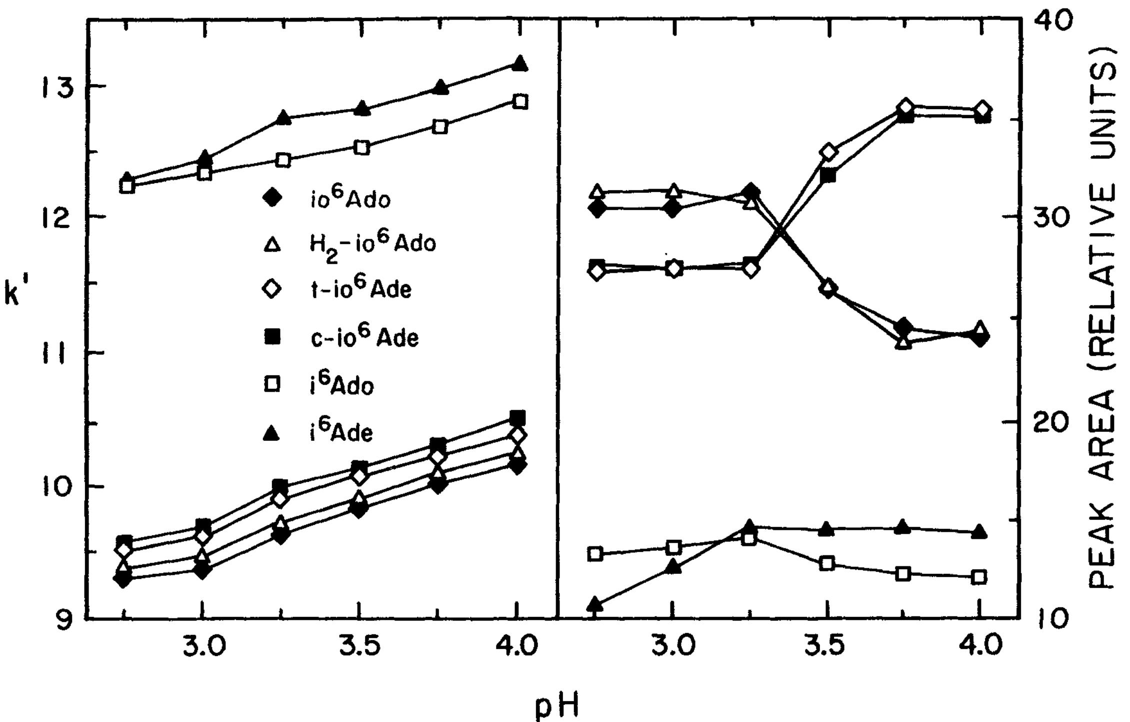

Figure 2. Influence of pH on k' and the signal response (peak area) of cytokinins detected by IS-RP HPLC. Column, Adsorbosphere Nucleoside/Nucleotide (7 μ); flow rate, 1 mL/min; Conditions: eluants, A = 0.001 M NH_4OAc + 0.002 M HSA, pH 3.50; B = acetonitrile:MEOH (60:40); gradient: 0-80% B over 25 min followed by 80% B for 5 min; sample injected, 20 μL; detection, 275 nm.

Adsorbosphere Nucleoside/Nucleotide (7 μ) column was more sensitive to the NH_4OAc concentration; 0.75 mM was optimal for enhancing the peak area of H_2-io^{6}Ado; 1 mM for i^{6}Ado, i^{6}Ade, io^{6}Ado and c-io^{6}Ade; 2 mM for t-io^{6}Ade (Fig. 3).

The organic modifier, HSA, may form a stable complex with either the cytokinins or with the bonded phase, allowing further resolution of the solutes. The concentration of HSA enhancing the signal response of most of the separated cytokinins on all the columns tested was 1 mM, except for i^{6}Ado and i^{6}Ade on the Adsorbosphere Nucleoside/Nucleotide (7 μ) column (Fig. 4). Capacity factors (k') for the separated cytokinins (i^{6}Ado, c-io^{6}Ade, t-io^{6}Ade, H_2-io^{6}Ado, and io^{6}Ado) decreased as the concentration of HSA increased. Resolution was not significantly affected over the concentration range tested, except for that between i^{6}Ado and i^{6}Ade. Without the use of HSA, coelution of several peaks occurred on all the columns tested.

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Chromatographic Parameters: [K, CV, R_s, k', N]

The distribution coefficients (K), precision of the signal response (coefficients of variation, CV) and column resolution (R_s) for each of the six solutes with the four stationary phases under their respective optimal elution conditions are shown in Table 1. Although the Adsorbosphere Nucleoside/Nucleotide (7 μ) column exhibits larger retention volumes for the cytokinins (depicted by large K values), the CV values are all $\leq$ 5.4% and resolution ranged from 1.15 to 8.48. Resolution of these compounds on the Adsorbosphere C_{18} (10 μ) column was not as effective and CV values ranged from 1.75 to 14.31. The R_s values for the Versapack (10 μ) and Adsorbosphere HS C_{18} (7 μ) are too low for reliable analytical chromatography.

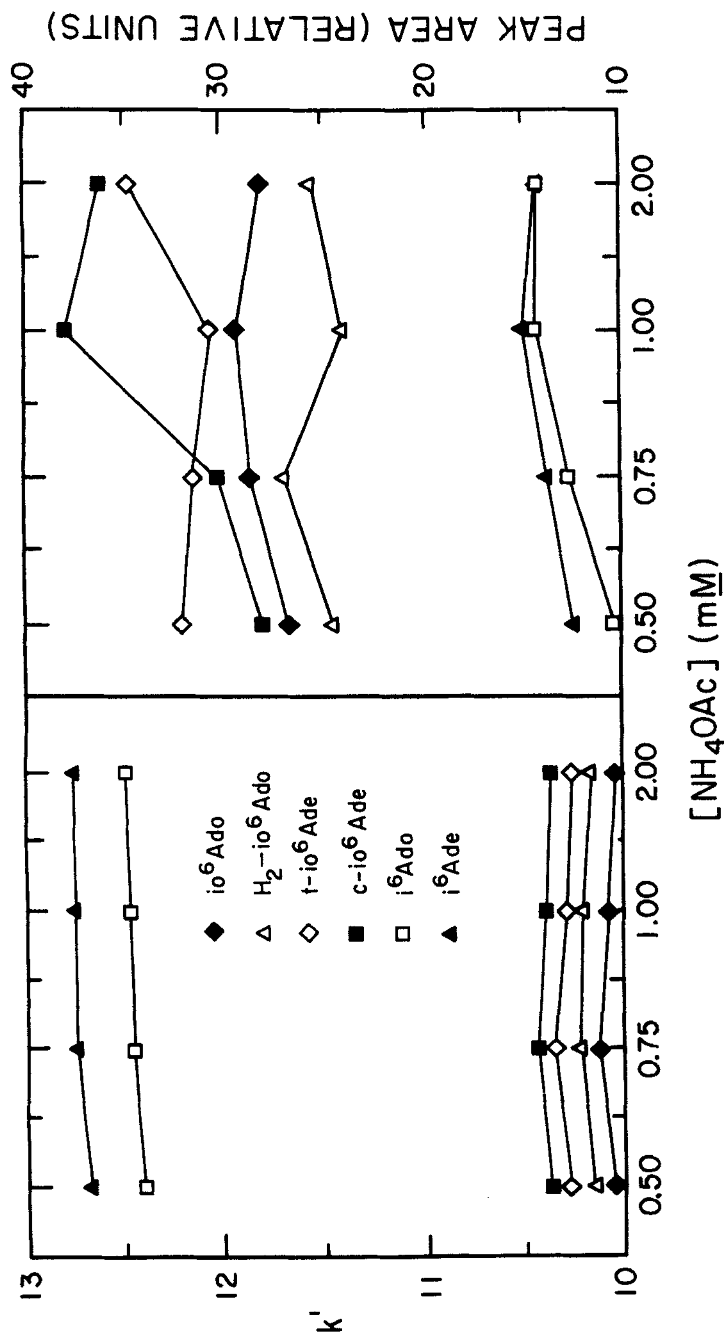

Figure 3. Influence of ammonium acetate concentration on k' and the signal response (peak area) of cytokinins detected by IS-RP HPLC. Chromatographic conditions are the same as Fig. 2.

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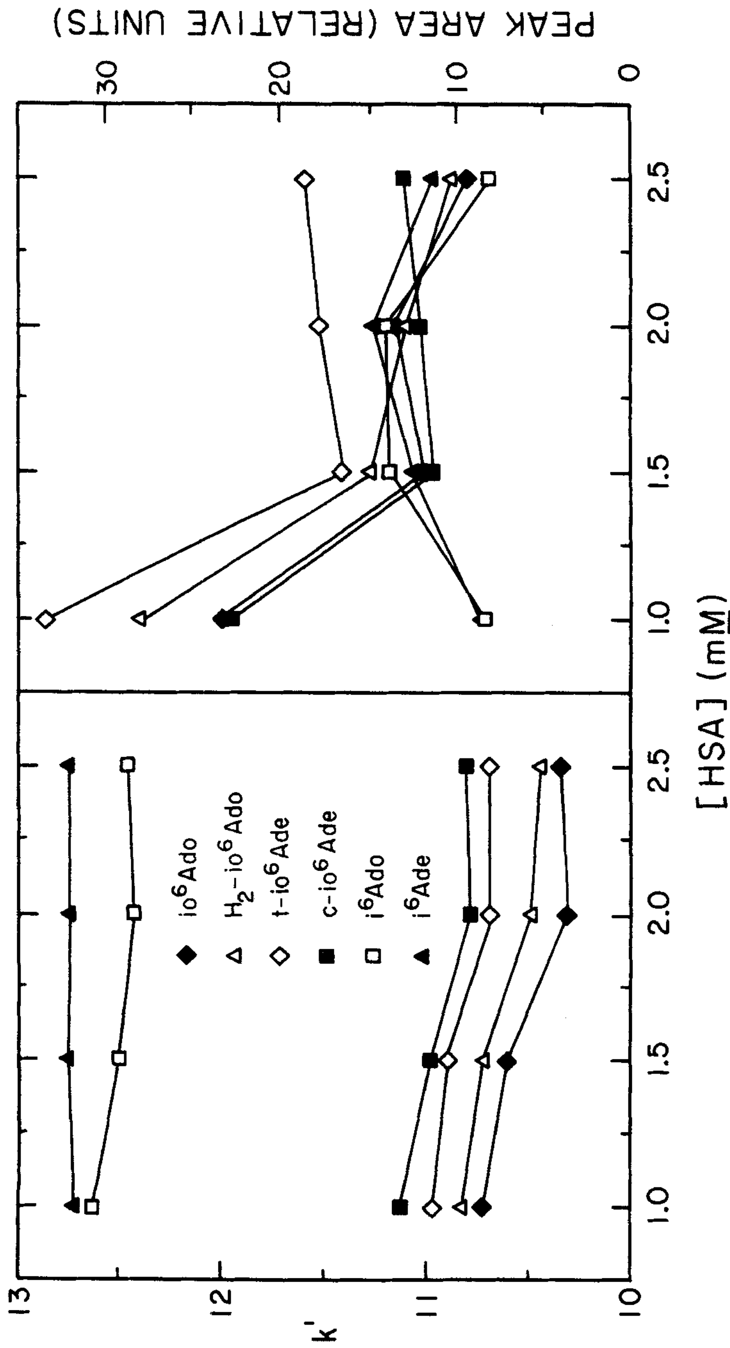

Figure 4. Influence of 1-heptane sulfonic acid concentration on k' and the signal response (peak area) of cytokinins detected by IS-RP HPLC. Chromatographic conditions are the same as Fig. 2.

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TABLE 1

Distribution (K), Precision (CV) and Resolution (R_s) of Some Cytokinins in Relation to Various Stationary Phases

	Stationary phase											
	Versapack (10 μ)[a]			Adsorbosphere C_{18} (10 μ)[b]			Adsorbosphere HS C_{18} (7 μ)[c]			Adsorbosphere Nucleoside/ Nucleotide (7 μ)[d]		
Cytokinin	K (min)[e]	CV[f]	R_s[g]	K	CV	R_s	K	CV	R_s	K	CV	R_s
io^6Ado	9.16	4.12		13.08	7.87		12.56	5.98		20.20	4.33	
			0.896			0.854			0.256			1.15
H_2-io^6Ado	10.52	8.44		13.04	8.39		12.77	4.63		20.43	5.36	
			0.688			1.02			0.313			1.50
t-io^6Ade	10.92	5.98		13.76	14.31		13.05	12.92		20.73	4.05	
			0.786			1.34			0.490			1.25
c-io^6Ade	11.61	16.11		14.84	1.75		13.29	4.57		20.98	4.34	
			--			3.22			4.22			8.48
i^6Ado	N.D.[h]	N.D.		20.13	9.65		15.51	8.56		24.94	4.81	
			--			N.A.[i]			N.A.			1.22
i^6Ade	N.D.	N.D.		20.13	9.65		15.51	8.56		25.43	4.95	

[a]conditions: eluants, A = 0.02 M NH_4OAc + 0.001 M HSA, pH 4.75; B = acetonitrile; gradient: 0-40% B over 10 min followed by 40% B for 5 min; 275 nm

[b]conditions: eluants, A = 0.02 M NH_4OAc + 0.001 M HSA, pH 4.00; B = MEOH; gradient: 0-80% B over 20 min; 275 nm

[c]conditions: eluants, A = 0.02 M NH_4OAc + 0.001 M HSA, pH 3.50; B = acetonitrile:MEOH (60:40); gradient: 0-70% B over 12.5 min; 275 nm

[d]conditions: eluants, A = 0.001 M NH_4OAc + 0.002 M HSA, pH 3.50; B = acetonitrile:MEOH (60:40); gradient: 0-80% B over 25 min followed by 80% B for 5 min; 275 nm

[e]$K = t_R - t_m$, where t_R is the retention time of the solute and t_m is the retention time of the solvent front

[f]$CV = (\sigma/\mu)$, where σ is the standard deviation and μ is the arithmetic mean

[g]$R_s = [2(\{t_R\}_y - \{t_m\}_x)]/[W_x + W_y]$, where x and y denote two solutes and W is the width of the peak at baseline expressed in units of time

[h]N.D., not determined

[i]N.A., not applicable due to coelution of i^6Ade with i^6Ado

The k' values of the cytokinins on the four stationary phases ranged from 5.09 to 12.78 (Table 2). Column efficiency was calculated in terms of theoretical plates (N) for each of the cytokinins on the four chromatographic columns. The Adsorbosphere Nucleoside/Nucleotide (7 μ) column was the most efficient in terms of N, by at least one order of magnitude, in comparison to the other chromatographic columns. For this column, the number of theoretical plates ranged from 71,856 to 216,039. The better columns in terms of efficiency for the separation of cytokinins when considering column length are ranked as follows: Adsorbosphere Nucleoside/Nucleotide (7 μ) >> Adsorbosphere HS C_{18} (7 μ) > Adsorbosphere C_{18} (10 μ) > Versapack (10 μ).

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Sensitivity

Detection limits varied with each cytokinin on the four stationary phases tested from 1.0 μ*M* for the Versapack (10 μ) to 0.1 μ*M* for the Adsorbosphere Nucleoside/Nucleotide (7 μ) column. Figure 5 depicts a sensitivity diagram for the Adsorbosphere Nucleoside/Nucleotide (7 μ) column and the elution sequence of the six cytokinins: io^6Ado > H_2-io^6Ado > *t*-io^6Ade > *c*-io^6Ade > i^6Ado > i^6Ade. The detection limit of the Adsorbosphere Nucleoside/Nucleotide (7 μ) column, calculated by a three-fold signal-to-noise ratio (S/N = 3) at the baseline, ranged from 44 to 93 ng ml^{-1}.

Detection of Cytokinins by HPLC in an Azotobacter Culture

Cytokinin-like activity was found in the culture supernatant of *Azotobacter chroococcum* using the radish cotyledon bioassay. A logarithmic standard curve (R^2 = 0.98) predicted approximately 0.02 μg of zeatin equivalent activity per mL of liquid culture, corresponding to 91.2 n*M* of zeatin-like compounds.

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TABLE 2

Column Capacity Factor (k') and Efficiencies (N) of Four Stationary Phases in Detection of Cytokinins

Cytokinin	Stationary phase							
	Versapack (10 μ)[a]		Adsorbosphere C_{18} (10 μ)[b]		Adsorbosphere HS C_{18} (7 μ)[c]		Adsorbosphere Nucleoside/Nucleotide (7 μ)[d]	
	k'[e]	N[f] (plates)	k'	N	k'	N	k'	N
io^6Ado	5.36	18694	7.02	7207	5.09	5972	10.36	90279
H_2-io^6Ado	5.56	8007	7.34	7798	5.18	3461	10.52	210497
t-io^6Ade	5.79	5951	7.82	4274	5.30	6389	10.68	216039
c-io^6Ade	12.32	1686	8.63	3017	5.47	4938	10.82	112189
i^6Ado	N.D.[g]	--	10.81	5319	7.12	5969	12.54	71856
i^6Ade	N.D.	--	C.E.[h]	C.E.	C.E.	C.E.	12.78	74494
Average		8584		5523		5345		129225

[a]conditions: eluants, A = 0.02 *M* NH_4OAc + 0.001 *M* HSA, pH 4.75; B = acetonitrile; gradient: 0-40% B over 10 min followed by 40% B for 5 min; 275 nm

[b]conditions: eluants, A = 0.02 *M* NH_4OAc + 0.001 *M* HSA, pH 4.00; B = MEOH; gradient: 0-80% B over 20 min; 275 nm

[c]conditions: eluants, A = 0.02 *M* NH_4OAc + 0.001 *M* HSA, pH 3.50; B = acetonitrile:MEOH (60:40); gradient: 0-70% B over 12.5 min; 275 nm

[d]conditions: eluants, A = 0.001 *M* NH_4OAc + 0.002 *M* HSA, pH 3.50; B = acetonitrile:MEOH (60:40); gradient: 0-80% B over 25 min followed by 80% B for 5 min; 275 nm

[e]$k' = (t_R - t_m)/t_m$, where t_R is the retention time of the solute and t_m is the retention time of the solvent front

[f]N (number of theoretical plates) = $16\,(t_R/W)^2$, where W represents peak width expressed in units of time

[g]N.D., not determined

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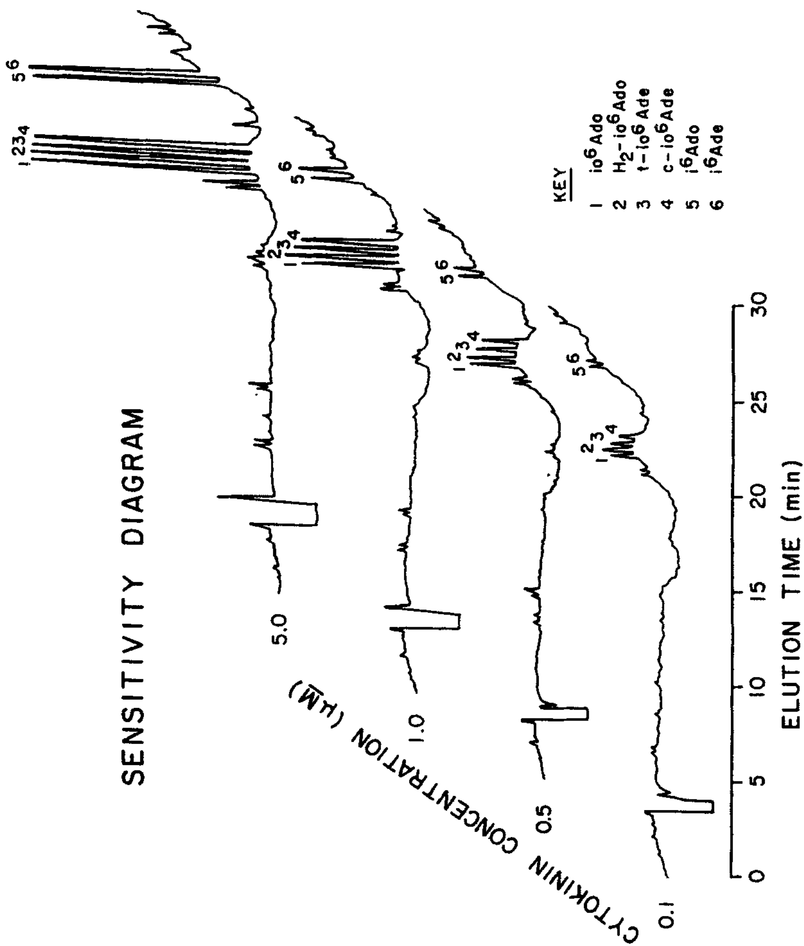

Figure 5. Sensitivity diagram of cytokinin separation by IS-RP HPLC. Chromatographic conditions are the same as Fig. 2.

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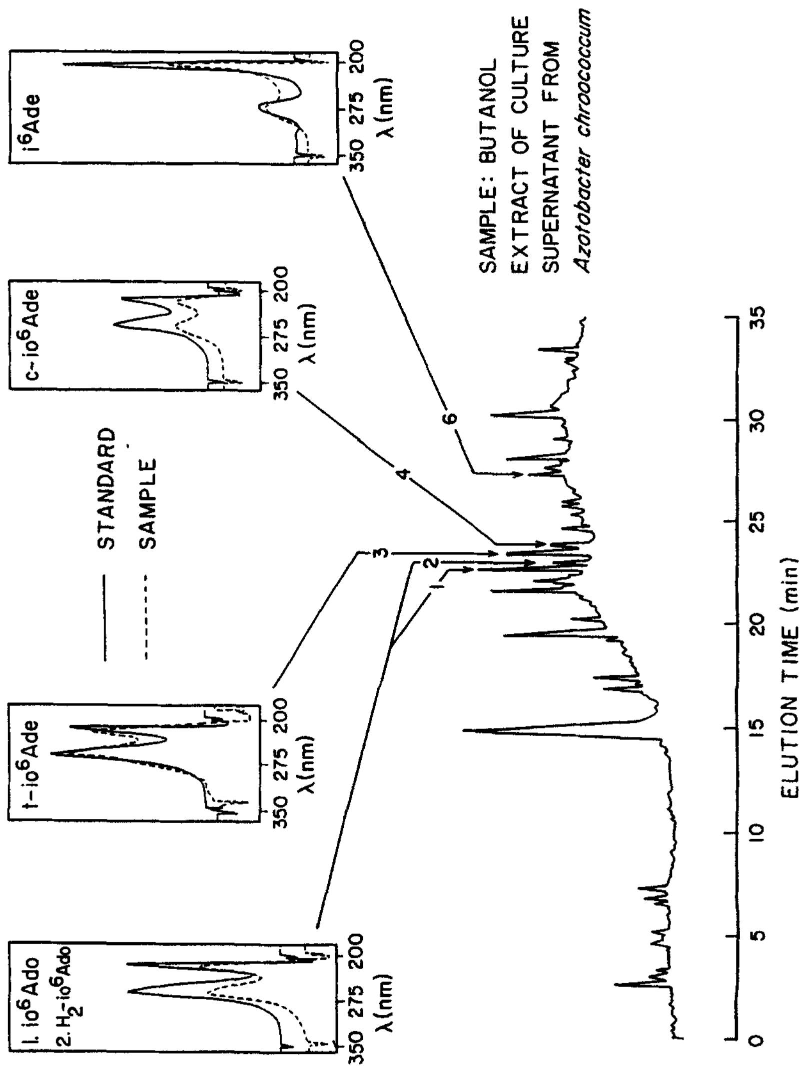

Figure 6. HPLC chromatogram of a culture supernatant of Azotobacter chroococcum. Chromatographic conditions are the same as Fig. 2.

The developed method described with the Adsorbosphere Nucleoside/ Nucleotide (7 μ) column was used to speciate and quantitate cytokinins in the bacterial supernatant (Fig. 6). The following quantities of cytokinins were detected, expressed in molarity of the culture supernatant: io^6Ado (88.5 nM), H_2-io^6Ado (9.18 nM), *t*-io^6Ade (61.8 nM), *c*-io^6Ade (0.505 nM), and i^6Ade (6.70 nM). Identification was verified by matching UV spectra of the solutes with standard references.

CONCLUSIONS

This paper highlights the chromatographic properties of four reverse phase materials in the separation of cytokinins. The methodology described for ion suppression-reverse phase high performance liquid chromatography (HPLC) using the Adsorbosphere Nucleoside/ Nucleotide (7 μ) column allows the immediate separation and quantitation of the multiple cytokinins present in biological samples. The procedure is highly sensitive, specific, precise and exhibits high efficiency. Isopentenyl adenine, *cis*-and *trans*-zeatin as well as their ribosylated and dihydro-derivatives are readily resolved from each other. The cytokinins are eluted with a linearly increasing concentration of acetonitrile:methanol (60:40) in an acidic acetate buffer, using heptane sulfonate as the organic modifier. Analysis time was approximately 35 minutes.

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