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Note

Use of ion-pair, reversed-phase, high-performance liquid chromatography for the analysis of cytokinins

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High-performance liquid chromatography (HPLC) is a valuable tool for the separation and measurement of plant growth substances. The combination of rapid analysis, high resolution and, frequently, the elimination of a derivitization step gives HPLC a marked advantage over other methods of separation including thin-layer, low-pressure column, and gas–liquid chromatography. In some of the early attempts to separate cytokinins by HPLC, ion-exchange columns were used[1], but the resins did not provide good resolution of the reference compounds, and serious difficulties were encountered in recovering the growth regulators from crude extracts. Improved resolution and increased sample capacity have been achieved using chemically bonded, organic, stationary phases over silica supports in what is commonly referred to as the reversed-phase mode[2–5]. During separation of plant growth regulators on these columns, small amounts of acetic acid are often added to the mobile aqueous phases to improve resolution by suppressing ion formation and reducing tailing of acidic compounds[3–6]. However, the resultant positive charges on the weakly basic cytokinins may mask slight differences in polarity usually associated with small dissimilarities in structure. Under these conditions, resolution of closely related compounds such as the *cis* and *trans* isomers would be expected to decrease. In an attempt to improve the separation of cytokinins with similar polarities, Holland *et al.*[7] investigated the use of ion-pair chromatography. Shortened retention times were achieved but no improvement in peak shape or resolution resulted from the addition of sodium lauryl sulphate to a mobile phase held at pH 5. In the present work, we describe the optimization of an ion-pair system and demonstrate its potential for resolving the geometric isomers of cytokinins isolated from plant material.

MATERIALS AND METHODS

Chromatographic equipment

Gradient-elution chromatography was performed with a Spectra-Physics (Santa Clara, CA, U.S.A.) SP 8000 three-solvent system using two detectors, *viz.*, an SP 8200 dual-beam UV–visible unit and an SP 8400 variable-wavelength UV–visible unit operating at 254 and 269 nm, respectively. Samples were introduced via a nitrogen driven auto-injector port fitted with a 100-μl loop.

Prepacked columns (250 × 4.6 mm I.D. and 150 × 4.6 mm I.D.) of 5-μm

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Ultrasphere-I.P. and a semi-preparative column (250 × 10.0 mm I.D.) of 10-μm Ultrasil-ODS were purchased from Beckman (Berkeley, CA, U.S.A.). Two additional columns (250 × 4.6 mm I.D.) packed with LiChrosorb 10 RP-8 and RP-18 were obtained from Technical Marketing Associates (Mississauga, Canada).

Chemicals

All chromatographic solvents were HPLC grade (Caledon Labs., Georgetown, Canada), and all water was glass distilled and filtered through 0.2-μm MF Millipore (Bedford, MA, U.S.A.) filters.

The ion-pair reagents tested were N-1-heptanesulphonic acid, sodium salt monohydrate (Helix Assoc., Newark, DE, U.S.A.) and 1-octanesulphonic acid, sodium salt (Eastman Kodak, Rochester, NY, U.S.A.).

The cytokinin standards were purchased from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

Seeds of *Acer saccharum* Marsh. were incubated under moist conditions at 5°C for 20 days. Several 26-g samples were homogenized in 80% aqueous methanol, reduced *in vacuo* to the aqueous phase and then partitioned at pH 2.5 against ethyl acetate. After partitioning the ethyl acetate fraction against water-saturated *n*-butanol at pH 8, the butanol fraction was dried and the residue containing the cytokinins[8] was resuspended in a solution of 0.05 *M* KH_2PO_4 at pH 4 and slurried for 20 min with insoluble polyvinylpyrrolidone. The partially purified fraction was loaded onto a 3-ml C_{18} Bond Elut cartridge (Analytichem International, Lawndale, CA, U.S.A.) and washed with distilled water before eluting with methanol. The sample was then passed through a 0.2-μm filter and chromatographed on a reversed-phase semi-preparative column. Fractions with retention times corresponding to those of authentic standards were collected for further analysis by ion-pair chromatography.

RESULTS AND DISCUSSION

Several chromatographic parameters were investigated during development of the method. The data in Table I compare R_S values [resolution between two peaks, P_1 and P_2, where $R_S = 2\Delta t/(W_2 + W_1)$ and Δt = retention time of $P_2 - P_1$, W_2 = width of P_2, W_1 = width of P_1] between cytokinin pairs and demonstrate a marked improvement in resolution of a C_{18}-over a C_8-bonded stationary phase. Higher operating temperatures can also improve resolution by their effects on column efficiency, sample capacity and a decrease in k' values [capacity factor where k' = (peak retention time − void time)/void time] brought about by changes in diffusion coefficients and increased solubility of the sample components in the mobile phase[9]. Although the effects of temperature have not been considered in recent studies of methods development for cytokinins[2,3,10], the results in Fig. 1 show that a clear improvement in peak separation and a reduction in retention times was achieved by increasing column temperatures from 25 to 40°C.

Horgan and Kramers[2] concluded that resolution of cytokinins could be maximized on reversed-phase columns if the growth regulators were maintained in an unionized state at pH levels well above their pK_a values of about 4. Based on that premise, they developed an HPLC method using a triethylammonium bicarbonate

TABLE I

COMPARISON OF C_8 AND C_{18} REVERSED-PHASE HPLC COLUMNS FOR RESOLUTION (R_s VALUES, SEE TEXT) OF CYTOKININ PAIRS

Columns, Brownlee RP-18, 10 μm (250 × 4.6 mm I.D.) and Spectra-Physics RP-8, 10 μm (250 × 4.6 mm I.D.); flow-rate, 2 ml/min; temperature, 25°C. Mobile phase, a linear gradient of water (with 0.2 *M* acetic acid) to 30% acetonitrile (with 0.2 *M* acetic acid) over 30 min. Abbreviations: Z = zeatin; ZR = zeatin riboside; IPA = isopentenyl adenine; IPAR = isopentenyl adenosine.

Cytokinin pairs	*Resolution (R_s)*	
	C_8	C_{18}
trans-ZR + *trans*-Z	1.20	2.24
trans-ZR + IPA	11.23	25.36
trans-ZR + IPAR	12.06	26.25
trans-Z + IPA	11.01	24.70
trans-Z + IPAR	11.14	24.69
IPA + IPAR	0.72	0.76

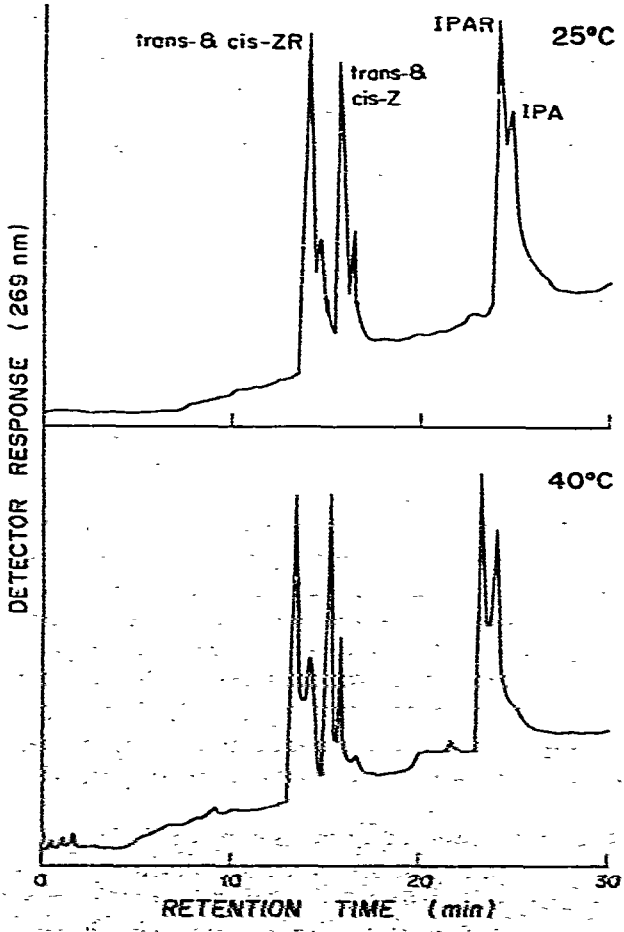

Fig. 1. Separation of cytokinin standards at ambient and elevated temperatures. Column, Brownlee RP-18, 10 μm (250 × 4.6 mm I.D.). Chromatographic conditions and abbreviations as in Table I.

buffer at pH 7 as the mobile phase. The separations achieved during our attempts to duplicate their procedure were not satisfactory due, apparently, to adsorption of cytokinins to the support matrix as evidenced by a drop in peak areas during repeated injections. Good separations were obtained by using ion-pair chromatography with a highly acidic solvent system at a pH of 2.65 and heptanesulphonate as the counter-ion. At higher pH levels, resolution was reduced and the peaks merged (Fig. 2). The use of buffer salts was avoided and the requisite pH maintained by making the solvent system 0.2 *M* with respect to acetic acid. Concentrations of 3–6 m*M* heptanesulphonate provided optimal separation of peaks while a 10 m*M* concentration prolonged analysis time without additional benefits. A larger counter-ion, *i.e.*, a longer alkyl chain, also increased retention time and k' values (see also ref. 11), but separation of the cytokinins was nevertheless reduced (Table II). These observations may explain the report by Holland *et al.*[7] that a C_{12} counter-ion dissolved in a mobile phase at pH 5 failed to provide any significant improvement in resolution or peak shape.

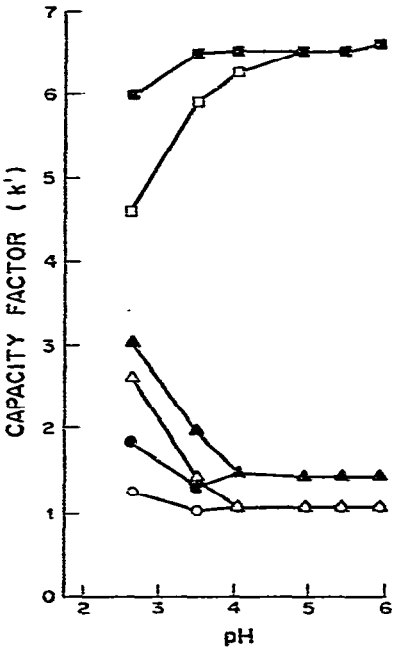

Fig. 2. Effect of pH on the capacity factor. Column, Ultrasphere-I.P., 5 μm (150 × 4.6 mm I.D.); flow-rate, 1 ml/min; temperature, 40°C; pH adjusted with 0.2 *M* sodium acetate buffer. A three-solvent mobile phase with a linear gradient: (A) 75% water with 0.2 *M* acetic acid, pH 2.65, and 6 m*M* heptanesulfonic acid; (B) 6% acetonitrile with 0.2 *M* acetic acid; (C) 19% methanol with 0.2 *M* acetic acid, held for 2 min, then programmed to 55% A, 6% B, 39% C over 6 min and held for 10 min. Symbols: ⊙ = *trans*-zeatin riboside; ● = *cis*-zeatin riboside; △ = *trans*-zeatin; ▲ = *cis*-zeatin; □ = isopentenyl adenosine; ■ = isopentenyl adenine.

Acetonitrile was chosen as the major organic modifier since it proved superior to both methanol and tetrahydrofuran for separation of UV-absorbing contaminants from cytokinin peaks. A microprocessor controlled ternary-gradient system gave

TABLE II

EFFECTS OF TWO COUNTER-IONS WITH DIFFERENT CHAIN LENGTHS ON THE CAPACITY FACTOR (k', SEE TEXT)

Column. Ultrasphere-I.P., 5 μm (250 × 4.6 mm I.D.); flow-rate, 0.8 ml/min; temperature, 40°C. A three-solvent mobile phase with a two segment linear gradient: (A) 93% water with 0.2 *M* acetic acid, pH 2.65, and 6 m*M* heptanesulphonic acid; (B) 5% acetonitrile with 0.2 *M* acetic acid; (C) 2% methanol with 0.2 *M* acetic acid programmed to 70% A, 25% B, 5% C over 30 min, to 50% A, 45% B, 5% C over 8 min and held for 7 min. Abbreviations as in Table I.

Cytokinin	*Capacity factor (k')*	
	Heptanesulphonic	*Octanesulphonic*
trans-ZR	1.26	3.17
cis-ZR	1.85	3.58
trans-Z	2.65	4.12
cis-Z	3.09	4.39
IPAR	4.60	5.12
IPA	6.01	7.39

excellent flexibility in programming the composition of the mobile phase and also minimized solvent waste during methods development.

Bond Elut tubes provided a fast and effective means of removing pigments and UV-absorbing contaminants in the butanol fraction of the seed extracts prior to semi-preparative HPLC. For some applications, sample clean-up with these reversed-phase, disposable cartridges is sufficiently effective to proceed directly to an analytical column. In the case of ion-pair chromatography, however, semi-preparative, reversed-phase HPLC was useful for removing compounds that could interfere with or act as counterions. Fractions from the semi-preparative column that had retention times corresponding to authentic standards (Fig. 3) were bioassayed with the oat-leaf senescence test[12] to confirm cytokinin activity, and the unused portions were then pooled and loaded onto the 5-μm ion-pair column. The estimates of cytokinin content in the seeds indicated concentrations of 78 and 17 ng of *trans*- and *cis*-zeatin and 70 and 24 ng of *trans*- and *cis*-zeatin riboside per gram fresh weight of tissue (Fig. 4). Peaks that co-chromatographed with authentic isopentenyl adenine or isopentenyl adenosine were not observed.

The UV-absorbing contaminants evident in Fig. 4 decrease the reliability of the previous measurements, and more stringent purification procedures for use prior to analytical HPLC are currently under development. Nevertheless, the cytokinin fractions are well-separated and clean enough for further confirmation of both identity and amounts by gas chromatography–flame-ionization detection[13], bioassay or gas chromatography–mass spectrometry. The potential of the ion-pair method is clearly demonstrated by resolution of authentic geometric isomers of cytokinin bases and their ribosyl derivatives at 254 and 269 nm (Fig. 5). The absorbance ratios obtained by monitoring cytokinin levels at these two wavelengths supplement co-injection with authentics and improve the likelihood of correct identification during HPLC of tissue extracts.

Before proceeding to bioassays and other methods of confirmation, the counter-ions from the ion-pair reagent must be removed from the cytokinin fractions.

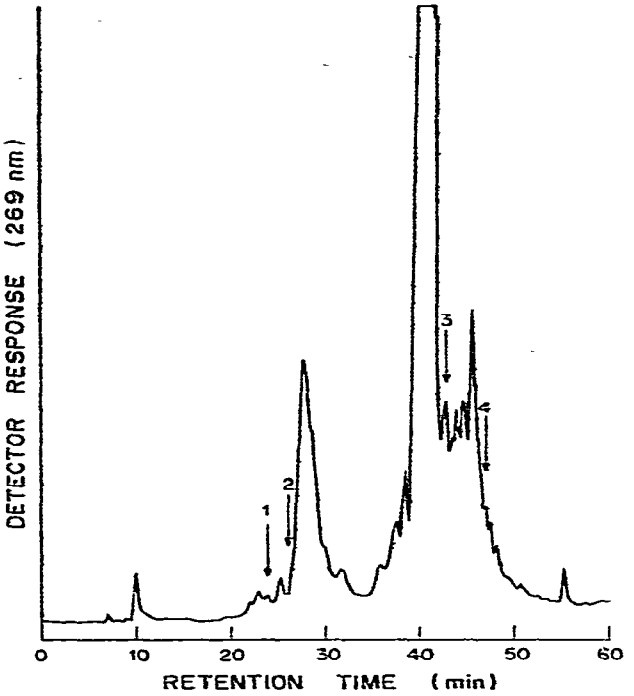

Fig. 3. Fractions collected from a partially purified extract of *Acer saccharum* seeds. Column, Ultrasil-ODS, 10 μm (250 × 10.0 mm I.D.); flow-rate 2 ml/min; temperature, 40°C. A three-solvent mobile phase with a linear gradient: (A) 85% water with 0.2 *M* formic acid: (B) 5% acetonitrile with 0.2 *M* formic acid; (C) 10% methanol with 0.2 *M* formic acid programmed to 10% A, 5% B, 85% C over 50 min. Peaks: 1 = fraction containing *trans* isomers of zeatin and zeatin riboside; 2 = fraction containing *cis* isomers of zeatin and zeatin riboside; 3 = fraction containing isopentenyl adenosine; 4 = fraction containing isopentenyl adenine.

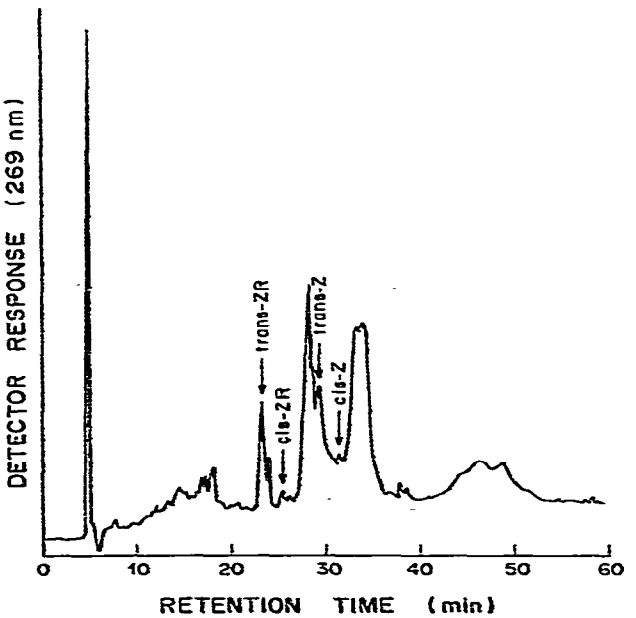

Fig. 4. Ion-pair chromatography of combined fractions 1 and 2 from Fig. 3. Chromatographic conditions as in Table II. Abbreviations as in Table I.

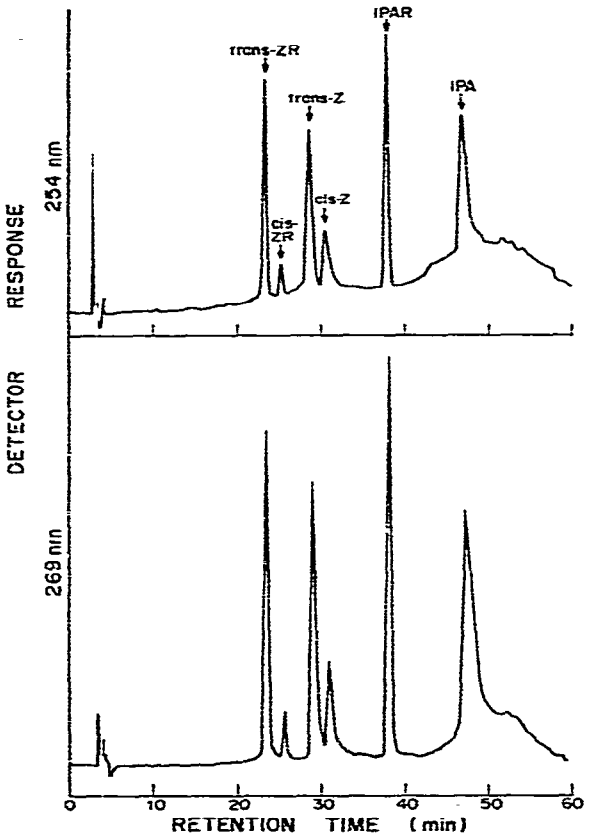

Fig. 5. Separation of cytokinin standards by ion-pair chromatography. Chromatographic conditions as in Table II. Abbreviations as in Table I. Amounts injected: *trans*-zeatin riboside = 4.6 μg, *cis*-zeatin riboside = 0.4 μg; *trans*-zeatin = 4.5 μg, *cis*-zeatin = 0.5 μg, isopentenyl adenosine = 5.0 μg, isopentenyl adenine = 5.0 μg.

This was achieved by adjusting the fractions to pH 8 with 0.1 *M* NaOH and loading onto Bond Elut cartridges. Raising the pH neutralized the charged cytokinins and allowed the counter-ions to be washed from the cartridges with distilled water. Subsequent elution with methanol gave cytokinin recoveries in the range of 90% and higher. To ensure that molecular integrity of the cytokinins was maintained during elution of the ion-pair reagent, chromatographic behaviour, UV-absorption patterns and biological activities were used to compare standards from which counter-ions were removed (treated) to standards not previously exposed to the ion-pair reagent (untreated). Thin-layer chromatography on both cellulose and silica gel plates gave identical R_F valves for treated and untreated standards and the UV absorption patterns measured from 230 to 280 nm were also the same. The oat-leaf senescence test[12] confirmed these results, and for all dilutions tested, over the range of 10^{-3} to 10^{-5} *M*, the biological activity of the cytokinins eluted from the Bond Elut columns remained unchanged.

The present work demonstrates that ion-pair chromatography is readily adaptable for the separation and measurement of closely related cytokinins and that

subsequent removal of the ion-pair reagent to facilitate more definitive identification poses no special problems.

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