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Note

Comparison of styrene–divinylbenzene copolymer and C_{18} silica reversed-phase packings for high-performance liquid chromatography of gibberellins

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Before high-performance liquid chromatography (HPLC) can become a routine procedure in the isolation and characterization of gibberellins, either preparatively or analytically, many alternative aspects of its use will require evaluation. Barendse *et al.*[1] made a significant contribution in their description of reversed-phase gradient chromatography for the separation of known mixtures of plant extracts. Though perhaps not directly applicable to all studies, their procedure, when combined with bioassay of collected fractions, can be used to evaluate the gibberellin content of small samples of tissues after a reasonably simple purification.

A possible alternative procedure could involve a reversed-phase column packed with styrene divinylbenzene resin (SDVB)[2,3]. This non-polar material is reputed to be more stable toward solvents and is less expensive than the commonly used C_{18} bonded microparticulate silica packings.

In this study we have compared three performance characteristics of these two reversed-phase materials as they affect gibberellin chromatography; capacity factors (k'), selectivity (α) and efficiency (N).

EXPERIMENTAL

Chromatography was carried out on a Hewlett Packard 1084 B liquid chromatograph, equipped with a variable-wavelength detector and automatic sampling system with a variable-volume injector. Mobile phase concentrations and repetitive injections were programmed.

The two reversed-phase columns used in the comparison were: (a) Hewlett-Packard RP-18 (200 × 4.6 mm I.D.), pre-packed with LiChrosorb 5 μm RP-18. This column was used in conjunction with a MPLC Guard Column (RP-18) (Brownlee Labs, Santa Clara, CA, U.S.A.). (b) Hamilton (PRP-1), styrene–divinylbenzene copolymer column 150 × 4.1 mm I.D. (Hamilton, Reno, NV, U.S.A.). Both columns had been recently purchased and had been carefully but infrequently used prior to this study.

The solvents used were: (a) HPLC grade methanol (Fisher Scientific), filtered through 0.5-μm Type FH Fluoropore PTFE filter (Millipore Corp.), and (b) acidified HPLC-grade water, prepared locally and adjusted to the required pH with 0.1 *M* H_3PO_4. The water was prepared from deionized and glass-distilled water, which was

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then pumped through a Lobar (240 × 10 mm) LiChroprep RP-8 column (E. Merck, Darmstadt, G.F.R.). Before use, the water was filtered with a 0.22-μm Type GS MF (Millipore Corp.) filter. Because of the difficulties of measuring pH of methanol–water mixtures[4], all values of stated pH represent the measured pH of the water reservoir alone and, therefore, represent an approximate value of the resultant on-column pH of the water–methanol mixture.

The gibberellins used were GA_3 (Eastman Kodak, Rochester, NY, U.S.A.), GA_7 (U.S. Biochemical Corp., Cleveland, OH, U.S.A.), and GA_9 (gift of Dr. R. P. Pharis, University of Calgary, Canada). Abscisic acid (ABA) (Calbiochem, LaJolla, CA, U.S.A.) was also used. Gibberellins and ABA were dissolved in HPLC-grade methanol at approximately $1 \cdot 10^{-3}$ *M* concentration.

TABLE I

CAPACITY FACTORS (k') FOR GIBBERELLIC ACID (GA_3) AND GIBBERELLIN A_7 (GA_7) CALCULATED FROM CHROMATOGRAPHIC RUNS ON PRP-1 AND RP-18 COLUMNS AT DIFFERENT pH VALUES AND DIFFERENT METHANOL CONCENTRATIONS

pH	*Methanol (%)*	*PRP-1*		*RP-18*	
		GA_3	GA_7	GA_3	GA_7
2.5	40	12.04	–	2.4	22.1
	50	4.97	53	1.4	7.3
	70	1.8	6.6	0.8	1.6
	90	0.6	2.0	0.6	0.8
3.0	40	11.5	–	2.4	22.2
	50	4.7	58.7	1.4	7.4
	70	1.8	6.7	0.8	1.6
	90	1.2	2.0	0.6	0.7
4.0	40	7.9	0	1.7	19.6
	50	3.1	52	0.9	5.6
	70	2.04	5.0	0.67	1.4
	90	0.75	1.0	0.25	0.5
5.0	40	1.1	15.5	0.59	8.73
	50	1.0	6.4	0.35	3.43
	70	0.8	2.0	0.27	1.85
	90	0.5	0.9	0.5	0.7

Performance parameters were calculated from isocratic chromatography with different mixtures of methanol and acidified water at different pH values. Calculations were based on mean retention time values from a minimum of three chromatograms. All sample volumes were 20 μl of the appropriate gibberellin solution. Mixtures of gibberellins (50-μl samples) were separated by gradient chromatography. In all experiments, the flow-rate was 1 ml/min, the oven temperature was 40°C, and UV absorption was measured at 210 nm. The parameters were calculated according to the following equation:

capacity factor, $k' = \frac{t_R - t_0}{t_0}$; selectivity, $\alpha = \frac{k' GA_7}{k' GA_3}$; and efficiency, N plates/m where $N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$, where t_R retention time, t_0 = retention time of an unretained compound, and $w_{1/2}$ is the peak width at half height.

RESULTS AND DISCUSSION

Comparison of the k' values of GA_3 and GA_7 chromatography with different methanol concentrations at different pH values on the two columns is presented in Table I. For comparable conditions at low pH and low methanol concentrations, the PRP-I column showed significantly higher k' values than the RP-18 column. The ratios of k' values for PRP-1 to k' values for RP-18 were between 5.0 and 1.0 for GA_3 and between 9.28 and 1.08 for GA_7.

Moreover, for GA_7 and GA_3, the PRP-1 column gave higher selectivity factors (α) under comparable conditions (Fig. 1A and B). Except for solvent combinations of high methanol values at high pH (≥ 5), where only small differences in α were observed, the PRP-1 column gave α values 2 and 3 times higher than the RP-18 column.

Though greater k' and α values were achieved for the PRP-1 column under the conditions used, the peaks were broader and therefore the efficiencies were lower. This result is predictable from Majors' work[2]. Sample values for GA_7 at pH 3 and 70% methanol were 12,000 plates/m on RP-18, compared to 4000 plates/m for the PRP-1 column. Similarly, with another plant growth regulator, ABA, which possesses similar chromatographic characteristics but a superior UV absorption spectrum, up to 16,000 plates/m were observed with the RP-18 column, compared to 5000

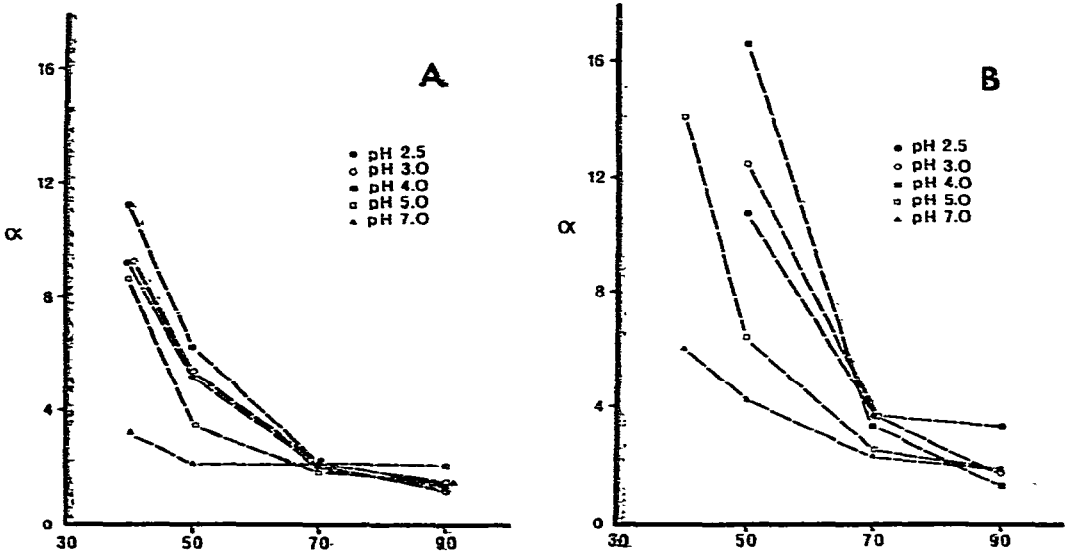

Fig. 1. Selectivity values (α) calculated for the RP-18 column (A) and for the PRP-1 column (B) at different solvent concentrations (%methanol) and at different pH values.

plates/m for the PRP-1 column. Whether these values apply only to the columns tested or whether they could be altered under other chromatographic conditions, is not known.

The significance of these observations for the chromatography of mixtures of gibberellins from extracts is that for gradient elution patterns comparable to those developed on the RP-18 column, higher initial methanol concentrations and steeper gradients are required for the PRP-1 column. Thus, to produce adequate separations of the free-acid gibberellins we use a 45–60% methanol gradient over 25 mins. with the RP-18. Comparable separations on the PRP-1 were obtained with a 50–90% methanol gradient over 30 min.

For each set of experiments, considerable testing of alternative protocols will be necessary. For example, the use of formic acid[5] in place of H_3PO_4 (ref. 1) in ion suppression should prove beneficial. The present study indicates that the SDVB material in the PRP-1 column could prove useful in some applications, especially in view of the lower initial cost, potentially higher flow-rates, greater retention and higher selectivity. Moreover, equilibration time is up to 3 times shorter for the PRP-1 column than for the RP-18 column. For more precise analysis the RP-18 column which shows higher efficiencies may be required.

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