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Note

Reversed-phase high-performance liquid chromatography of plant hormones: some useful differences in stationary phase selectivity

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High-performance liquid chromatography (HPLC) is widely used for the isolation, purification and estimation of growth substances in plant extracts. Most studies have employed reversed-phase (RP) systems, and particularly those with a C_8 or C_{18} bonded stationary phase[1–8]. The usefulness of preparative RP-HPLC in these analyses is enhanced greatly by the availability of systems showing differences in resolution and relative retention times of both impurities and compounds of interest. Excellent purification of cytokinins, for example, can be achieved both by the manipulation of solvent composition and pH, and by sequential analysis in which differences in stationary phases are exploited[3,4,8]. Very subtle differences in stationary phase, *e.g.* ODS (C_{18}), have been used to effect considerable changes in resolution and selectivity of cytokinins[4]. We report here further examples of differences in stationary phase selectivity which have proven particularly useful in our studies on phytohormones in extracts of green plant tissues.

EXPERIMENTAL

Chemicals

HPLC-grade methanol was used throughout. Triethylammonium bicarbonate (TEAB) was prepared by saturating a 2.5 *M* solution of triethylamine with carbon dioxide. Indole-3-acetic acid (IAA), zeatin (Z) and zeatin riboside (ZR) were purchased from Calbiochem (U.S.A.), and abscisic acid (ABA) from Sigma (U.S.A.). Lupinic acid (LA), zeatin-7-glucoside (Z7G) and zeatin-9-glucoside (Z9G) were provided by Dr. D. S. Letham (A.N.U., Canberra, Australia), and zeatin-O-glucoside (ZOG) by Dr. R. Horgan (U.C.W., Aberystwyth, U.K.).

Chromatographic equipment

Prepacked columns (125 × 4.6 mm I.D.) of 5-μm Spherisorb ODS (Phase Separations) and 5-μm Hypersil ODS (Shandon Southern) were used in conjunction

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with the Laboratory Data Control HPLC system described previously[6]. A prepacked 10-μM μBondapak phenyl column (300 × 3.9 mm) and a 10-μM C_8 radial compression column (100 × 8 mm), both from Waters Assoc., were used with the Waters HPLC system described by Summons *et al.*[8], at the Australian National University, Canberra. The solvent systems used were binary mixtures of either 0.1 *M* acetic acid in methanol, or water–TEAB buffer (pH 7.0) with pure methanol (HPLC-grade).

RESULTS AND DISCUSSION

ODS reversed-phase HPLC

A comparison was made between the separation of IAA, ABA and three common cytokinins (Z, ZR and ZOG) on two similar ODS columns (Figs. 1 and 2). Using the acidic solvent system and Hypersil ODS, the acidic hormones IAA and ABA eluted last (Fig. 1A). With Sperhisorb ODS, however, and an almost identical gradient, IAA eluted first and ABA second from last, before Z (Fig. 1B). At pH 7.0, IAA and ABA eluted first, on both types of column, and were separated widely from the cytokinins (Fig. 2). Three different cytokinin elution sequences were achieved;

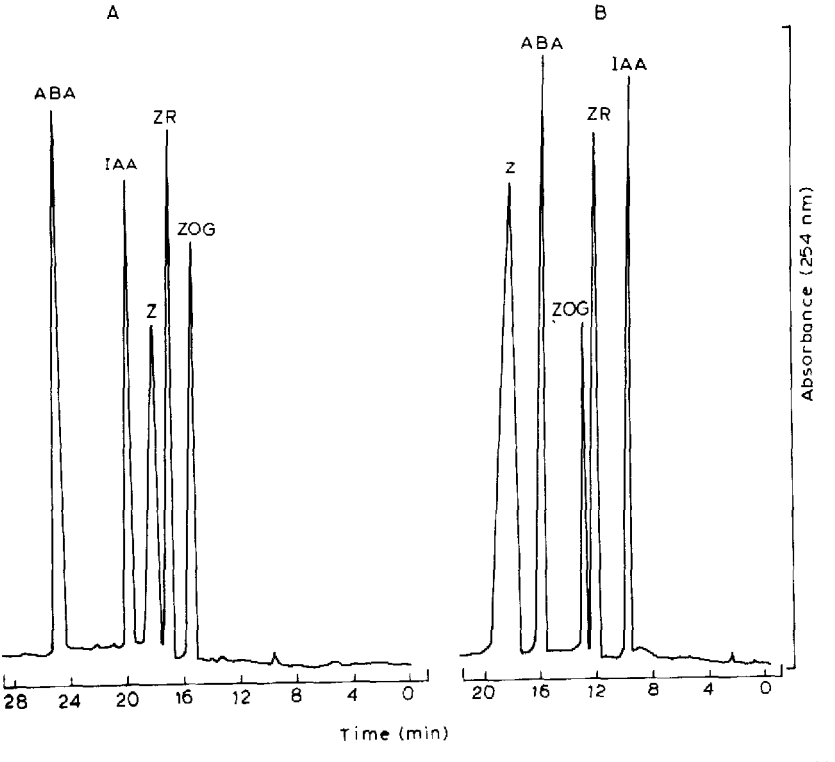

Fig. 1. Separation of IAA, ABA and three cytokinins (Z, ZR, ZOG) by RP-HPLC. (A) Column, 5-μm Hypersil ODS (125 × 4.6 mm I.D.); flow-rate, 1 ml/min; mobile phase, linear gradient, 0.1 *N* acetic acid in water to 50% 0.1 *N* acetic acid in methanol over 25 min. (B) Column, 5-μm Spherisorb ODS (125 × 4.6 mm I.D.); flow-rate, 1.5 ml/min; mobile phase, linear gradient, 0.1 *N* acetic acid in water to 50% 0.1 *N* acetic acid in methanol over 20 min. Sample injected: IAA, 5 μg; ABA, 1.5 μg; Z, ZR and ZOG, 10 μg each. For abbreviations see text.

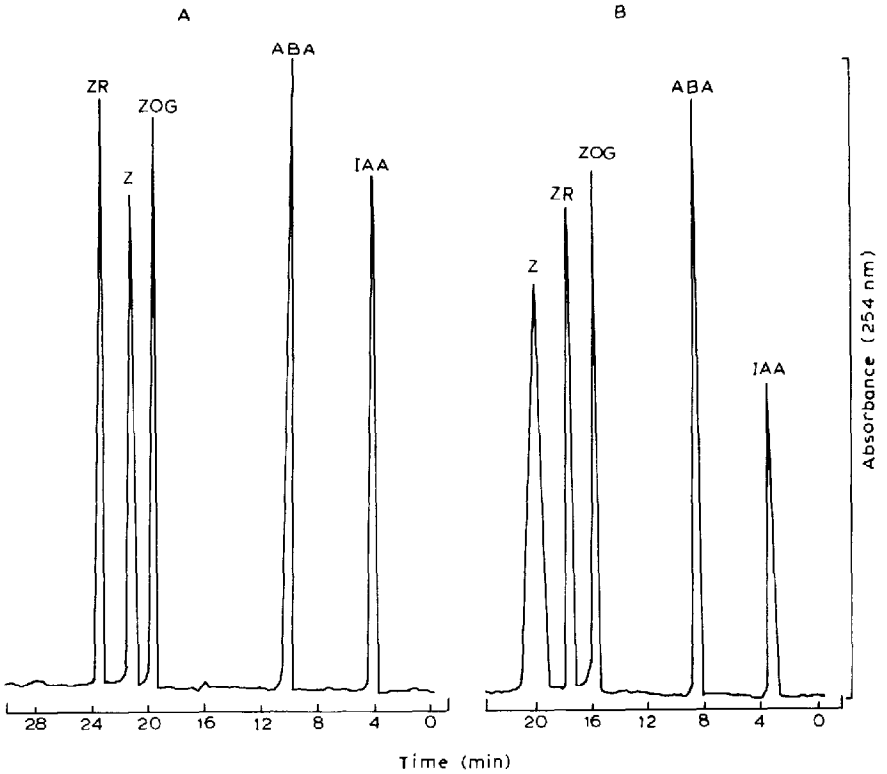

Fig. 2. As Fig. 1. (A) Column, 5-μm Hypersil ODS (125 × 4.6 mm I.D.); flow-rate, 1 ml/min; mobile phase, linear gradient, water (pH with TEAB) to 40% methanol over 30 min. (B) column, 5-μm Spherisorb ODS (125 × 4.6 mm I.D.); flow-rate, 1.5 ml/min; mobile phase, linear gradient, water (pH 7 with TEAB) to 35% methanol over 20 min. Sample injected: see Fig. 1. For abbreviations see text.

these were ZG, ZOG, Z (acidic Spherisorb ODS), ZOG, Z, ZR (neutral Hypersil ODS) and ZOG, ZR, Z (acidic Hypersil ODS or neutral Spherisorb ODS) (Figs. 1 and 2).

These marked differences in selectivity in both solvent systems are difficult to understand, particularly as these two types of spherical porous silica packing materials are so closely related[9]. Similar differences were observed in an earlier study, where the separation of Z from dihydrozeatin-O-glucoside at pH 7.0 was difficult to achieve on Hypersil ODS, but relatively simple on Spherisorb ODS[4]. The properties of reversed phases based on silica depend on many parameters[10]. Effects of the eluent composition can be ruled out. Presumably, differences in the amount of unreacted and accessible silanol groups could at least partly explain these effects[11].

C_8 and μBondapak phenyl reversed-phase systems

Another interesting effect, and one which is also difficult to explain, is the profound difference in cytokinin selectivity which were shown between a C_8 radial compression column and a μBondapak phenyl column in acidic solvent. The relative retention times of the polar cytokinins LA, Z7G, Z9G and ZOG were quite different in the two systems (Fig. 3). We have found these sytems to be particularly useful in

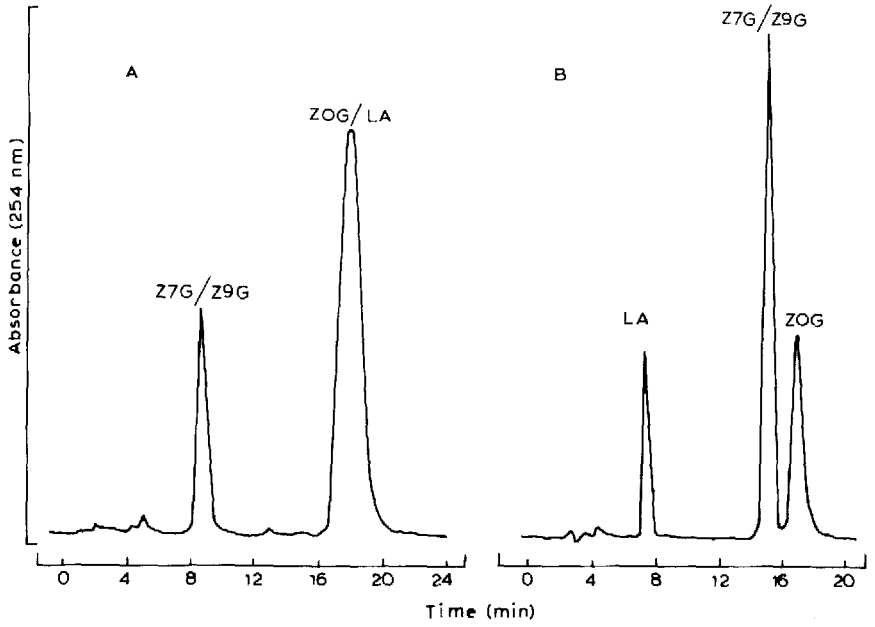

Fig. 3. Separation of Z7G/Z9G, ZOG and LA by RP-HPLC. (A) Column, 10-μm Waters 8CN 10 (C_8) (RCM, 100 × 8 mm I.D.); flow-rate, 3 ml/min; mobile phase, isocratic, 0.1 *N* acetic acid in 20% methanol. (B) Column, 10-μm Bondapak phenyl (300 × 3.9 mm I.D.); flow-rate, 2 ml/min; mobile phase, linear gradient, 0.1 *N* acetic acid in water to 10% 0.1 *N* acetic acid in methanol over 20 min. Sample injected: ZOG, 0.5 μg; Z7G/Z9G, 0.25 μg (A) or 0.5 μg (B) of each; LA, 0.5 μg (A) or 0.25 μg (B). For abbreviations see text.

the purification of LA (9-alanyl-zeatin), which seems to elute very early from most reversed-phase columns, and is often difficult to separate from non-cytokinin purines and other early-eluting contaminants[3].

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