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CHROMATOGRAPHY OF 33 GIBBERELLINS ON A GRADIENT ELUTED SILICA GEL PARTITION COLUMN

R. C. DURLEY, A. CROZIER*, R. P. PHARIS and G. E. MCLAUGHLIN
Biology Department, University of Calgary, Calgary T2N IN4, Alberta, Canada

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Abstract—The elution R_f of 33 gibberellins (GAs) and abscisic acid from a gradient eluted (increasing amounts of ethyl acetate in hexane) silica gel partition column are given. Certain problems encountered with the use of silica gels for partition chromatography are discussed. An ordering of elution R_f is apparent from most of the GAs on the basis of the number and positioning of polar groups in the *ent*-gibberellane structure. Hydroxylation in rings *C* and *D* retards elution to a greater degree than hydroxylation in ring *A*. The elution of dicarboxylic GAs is not retarded to a significantly greater degree than monocarboxylic GAs, but the elution of tricarboxylic GAs is greatly retarded. Possession of an aldehydic/lactol grouping for a GA retards its elution to a greater degree than its γ-lactone counterpart.

INTRODUCTION

METHODS of separating the various gibberellins (GAs) from each other are important steps in the identification and characterization of these hormones from plant extracts. TLC is commonly used,[1] but for general analysis does not give good resolution and suffers from somewhat inefficient recovery of the GAs. Column methods such as silica gel adsorption,[2] charcoal-celite[3] and polyvinylpyrrolidone[4] chromatography give very poor resolution. Sephadex partition chromatography of the gibberellins has been recently reported,[5] but this method, whilst giving excellent separation of specific GAs normally difficult to separate by other methods, is not applicable in the general case.†

We therefore sought alternative methods to improve the separation of the GAs and found silica gel partition chromatography[6] to be an effective method. It has proven effective as well for the separation of GA-like substances in partially purified plant extracts of diverse origin,[7–10] and is a reliable, convenient and quantitative chromatographic method for

* Present address: Botany Department, University of Canterbury, Christchurch, New Zealand.

† From our own observations, this method tends to group the GAs other than those for which it was intended.

[1] (a) B. D. CAVELL, J. MACMILLAN, R. J. PRYCE and A. C. SHEPPARD, *Phytochem.* **6,** 867 (1967); (b) T. KAGAWA, T. FUKINBARA and Y. SUMIKI, *Agric. Biol. Chem.* **27,** 598 (1963).

[2] R. A. KHALIFAH, L. N. LEWIS and C. W. COGGINS, JR., *Anal. Biochem.* **12,** 113 (1965).

[3] R. C. DURLEY, J. MACMILLAN and R. J. PRYCE, *Phytochem.* **10,** 1891 (1971).

[4] J. L. GLENN, C. C. KUO, R. C. DURLEY and R. P. PHARIS, *Phytochem.* **11,** 345 (1972).

[5] (a) D. W. PITEL, L. C. VINING and G. P. ARSENAULT, *Can. J. Biochem.* **49,** 185, 1971; (b) L. C. VINING, *J. Chromatog.* **60,** 141 (1971).

[6] L. E. POWELL and K. J. TAUTVYDAS, *Nature, Lond.* **213,** 292 (1967).

[7] M. RUDDAT, R. P. PHARIS, H. AOKI and A. CROZIER, *Plant Physiol.* **43,** 2049 (1968).

[8] P. C. KOZEL and H. B. TUKEY, *Am. J. Bot.* **55,** 1184 (1968).

[9] A. CROZIER, H. AOKI and R. P. PHARIS, *J. Exptl Bot.* **20,** 786 (1969).

[10] A. CROZIER, D. H. BOWEN, J. MACMILLAN, D. M. REID and B. H. MOST, *Planta* **97,** 142 (1971).

analysis of endogenous plant GAs. For quantitative comparisons, or small scale preparative extractions, gradient elution has proven most convenient since the entire procedure can be finished within a single day, but large scale preparative extracts are best processed by stepwise elution.[6]

We have extended the original work on gradient elution by Powell and Tautvydas[6] to include 33 GAs and abscisic acid, a potent inhibitor of GA activity in plant bioassays.[11] which will be found in many plant extracts containing GA-like substances. In the process we have made some changes in the original procedures out of convenience or necessity, the details of which are given below.

RESULTS AND DISCUSSION

The source of silica gel used for making partition columns is an important factor, since pore size within the particles appear to be critical in the ability of the particle to accept the aqueous phase.[12] Over a period of three years we tested a number of sources among which the following were found to be most satisfactory: Mallinckrodt No. 2847 (100 mesh), Mallinckrodt SilicAR CC4 (100–200 mesh) and Woelm Silica Gel for Partition Chromatography, a product containing 20% water as stationary phase.

We originally used[7,9] Mallinckrodt SilicAR CC4 (100–200 mesh) adsorbing 0·5 M formic acid solution stationary phase on the silica prior to chromatography and employing the same gradient as that used by Powell and Tautvydas.[6] The gibberellins did not elute in groups as obtained by Powell and Tautvydas,[6] but separated in a manner (Table 1) similar to their stepwise eluted column.[6] This difference may be attributable to the fact that their column apparently utilized Mallinckrodt No. 2847 silica gel, whereas ours utilized SilicAR CC4. Although the SilicAR CC4 column was found to be very satisfactory for separating the gibberellins, Mallinckrodt's production problems forced us to discontinue its use.

TABLE 1. ELUTION R_f VALUES OF 12 GIBBERELLINS FROM A 25 FRACTION SILICAR CC4 SILICA GEL PARTITION COLUMN

Fraction No.	Gibberellin*	Fraction No.	Gibberellin*	Fraction No.	Gibberellin*
1		6	$\underline{A_5}$	12	A_{13}
2	$\underline{A_9}$	7	A_5	13	A_3 A_1
3	A_9	8	$\underline{A_6}$ A_{20}	14	A_3 A_1
		9	A_6	17	A_8
4	A_7 A_4	10		18	A_{23} $\underline{A_8}$
5	A_7 A_4	11	$\underline{A_{13}}$ A_{19}	19	A_{23}

* Underlining of a gibberellin coming in more than one fraction denotes 80% or more in that fraction. Variation in R_f of one fraction may occur.

Next we examined Mallinckrodt Silica gel No. 2847 (100 mesh), but we were plagued by sporadic 'drying out' of the column, especially with columns larger than 13 mm i.d., in fractions 2–6. Those fractions collected immediately after 'drying out' contained increased amounts of formic acid solution and spreading of the gibberellin peaks was observed.

[11] F. T. ADDICOTT and J. L. LYON, *Ann. Rev. Plant Physiol.* **20,** 139 (1969).
[12] N. S. LAMONTAGNE and D. F. JOHNSON, *J. Chromatog.* **53,** 225 (1970).

R_f of the GAs eluted from Mallinckrodt silica gel No. 2847 was similar to that reported by Powell and Tautvydas.[6]

Finally we examined Woelm 'Silica Gel for Partition Chromatography' and with this product we were able to solve the 'drying out' problem. The product contained 20% water and we were unable to satisfactorily adsorb formic acid solution on to the silica, but by eluting with solvents saturated with formic acid we found that the initial adsorption of formic acid was unnecessary. However, in order to obtain a reasonable separation of the gibberellin-like substances from plant extracts we used a solvent gradient (Fig. 1) somewhat different to that reported earlier.[6]

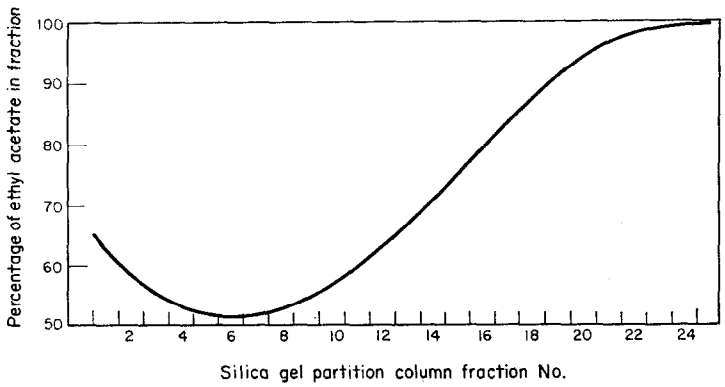

FIG. 1. PERCENTAGE OF ETHYL ACETATE IN HEXANE (BY WT) IN EACH 24 ml FRACTION LEAVING 1ST CHAMBER OF GRADIENT MIXER.

The elution R_fs of 33 gibberellins are given in Table 2. It can be seen that this gradient resulted in the grouping of a large number of GAs in fractions 2–5. Separation of these GAs by silica gel partition chromatography will therefore require a revised gradient.[13] Incidentally, none of the gradient-eluted silica gel columns we examined gave gibberellin elution patterns similar to that reported by Khalifah *et al.*[2] Providing the column height remains the same, the column diameter can be increased to accommodate extracts of high dry wt (see Experimental) with no significant variation in GA R_fs. $GA_{1,3,4,5,7,9,\&13}$ were examined quantitatively by GLC, and recovery was 90+% for $GA_{4,5,7\&9}$ and 80+% for $GA_{1,3\ \&\ 13}$.

An ordering of elution R_f is apparent for most of the GAs on the basis of the number of polar groups and positioning of the group in the *ent*-gibberellane skeleton. Hydroxylation in rings *C* and *D* retard the GA to a greater extent than hydroxylation in ring *A* (for example compare GA_1 and GA_2 to GA_{16} and A_{34}; and GA_{13} to GA_{17}). The elution of the dicarboxylic GAs (e.g. $GA_{12,14,18}$) is not retarded to a significantly greater degree than the elution of similar monocarboxylic GAs, whereas the elution of tricarboxylic GAs (e.g. $GA_{13,28}$) is greatly retarded, more so (GA_{13}) on the Woelm than on the Mallinckrodt silica gel. The possession of an aldehydic/lactol grouping ($GA_{19,23,24}$) also retards the elution of GA over its γ-lactone counterpart ($GA_{20,1,9}$).

[13] E. A. PETERSON and H. A. SOBER, *Analyt. Chem.* **31**, 857 (1959).
[14] R. C. DURLEY and R. P. PHARIS, *Phytochem.* **11**, 317 (1972).

This partition column provides a useful and convenient means for the partial analysis of GAs in plant extracts. Although this method does not give a complete separation of all the GAs, its resolving powers, particularly for the more 'polar' GAs, are superior to those of TLC systems[1] presently in extensive use. Furthermore, we find a greater recovery of the GAs from the partition column than we are able to achieve from TLC.

TABLE 2. ELUTION R_f VALUES OF 33 GIBBERELLINS FROM A 25 FRACTION WOELM SILICA GEL PARTITION COLUMN

Fraction No.	Gibberellin*	Fraction No.	Gibberellin*
		13	$\underline{A_1}\ \underline{A_3}\ A_{30}$
2	$\underline{A_9}\ A_{12}$	14	$A_1\ A_3\ A_{19}$
3	$A_9\ A_{11}$† $A_{14}\ A_{24}\ A_{14}\ A_{31}$	15	$A_2\ A_{13}\ A_{19}$
4	$A_4\ A_5\ \underline{A_6}\ A_7\ A_{14}\ \underline{A_{15}}$	16	$A_2\ \underline{A_{13}}\ A_{22}$
	$A_{20}\ A_{25}\ A_{31}$ ABA	17	$A_{18}\ A_{22}\ A_{26}\ A_{29}$
5	$A_6\ \underline{A_{10}}\ A_{15}$	18	$A_{18}\ A_{26}\ A_{29}$
6	A_{10}	19	$\underline{A_{17}}$
8	$A_{27}\ A_{34}$	20	$A_{17}\ A_{23}$
9	$A_{16}\ A_{27}\ A_{34}$	21	$\underline{A_{21}}\ A_{23}$
10	$\underline{A_{16}}\ A_{27}\ A_{34}\ A_{33}$	22	A_{21}
11	A_{33}	23	$\underline{A_8}\ A_{28}$
12	$A_1\ A_3\ A_{30}$	24	$A_8\ A_{28}$

* Underlining of a gibberellin coming in more than one fraction denotes 80% or more in that fraction. Variation in R_f of one fraction may occur.

† Detection of GA_{11} in fraction No. 3 was not conclusive, and chromatography could not be repeated due to insufficient quantities of GA_{11}. However, based on its chemical structure, elution should occur here.

EXPERIMENTAL

Chromatography. Mallinckrodt* No. 2847 silica gel or SilicAR CC4 (100–200 mesh) was prepared and eluted by the method of Powell and Tautvydas.[6] Woelm Silica Gel for Partition Chromatography† (20 g) was preslurried with a 0·5 M HCO_2H saturated solution of 10% EtOAc: 90% hexane, poured into the column and packed to a size of 20 × 1·3 cm.‡ This column size is suitable for a partially purified extract containing up to 100 mg dry wt. However, for extracts of greater dry wt columns as large as 20 × 4·1 cm have been employed successfully. Extracts were introduced by means of adsorbing them on 1–3 porous chromatographic discs§ (pre-washed extensively in EtOAc) and placing these discs carefully on top of the column. Elution solvents were saturated with 0·5 M HCO_2H solution to increase the degree of partitioning and prevent adsorption. The column was gradient-eluted with EtOAc-hexane, mixing being achieved with a Varigrad system[13] with four chambers in series, chambers 1–4 containing respectively EtOAc–hexane 65:35

* Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.

† Waters Associates Inc., 61 Fountain St., Framingham, Mass., U.S.A.

‡ Over a 2-yr period lots of 20% H_2O Woelm Silica Gel for Partition Chromatography contained approx. 20% H_2O, and 20 g of silica gel packed to approx. 20 cm in a 13-mm (i.d.) column. Recently, however, lots of this silica gel have been purchased which contain 24–26% H_2O, and 20 g of this silica gel packs to 24–26 cm. Woelm has since provided lots for testing which contain 17–20% H_2O, but 20 g of these lots still pack to 23–24 cm. R_f of eluted GAs is influenced by use of these more recent lots, a column made with 20 g and packing to 23–26 cm will elute GA_4, GA_5, GA_7 and GA_9 one to two fractions, GA_1 and GA_3 four to five fractions, GA_{13} two to three fractions and GA_8 one to two fractions later than shown in Table 2. Use of a lesser amount of silica gel in order to pack a 20-cm column lessens the degree of retardation, but does not result in elution R_f values noted for earlier lots (Table 2). No explanation has been provided by Woelm for this change in packing and elution characteristics of the silica gel. We would recommend that users of Woelm Silica Gel for Partition test each lot of silica gel with standard GAs covering a wide range polarities (i.e. GA_9, GA_5, GA_3, GA_{13}, GA_8) before use.

§ Scientific Glass Apparatus, Inc., Bloomfield, N.J., U.S.A. Catalogue No. F-2332X.

(129 ml), 20:80 (147 ml), 100:0 (114 ml), 100:0 (114 ml) respectively. This gradient (Fig. 1) was determined experimentally so as to provide the maximum spacing of bioassay peaks of GA-like substances in plant extracts. A total solvent vol. of 514 ml (including 10 ml of 10% EtOAc: 90% hexane) was collected in 20 ml fractions. Finally the column was eluted with two fractions of MeOH or four fractions of EtOAc in order to remove highly polar GA-like substances which may have adsorbed on the silica rather than being partitioned as intended.

Analysis. 10–100 μg of GA were placed on the discs in solution, and the solvent evaporated prior to placing the discs on the column. Detection and quantitative estimation was by GLC using a dual column F & M Model 402 with heated injectors and flame ionization detectors, and GLC procedures and preparation of derivatives were the same as those reported earlier.[10] 5 separate silica gel columns were run to avoid overlapping of certain of the GAs on the GLC columns during detection. Gibberellins $A_{1,3,4,5,7,8\&13}$ were used as reference standards between partition columns, although no one GA could be run on all five columns. As well, gibberellins $A_{1,3,4,5,7,9,\&13}$ were run on three replicate columns to verify the reproducibility of the system.

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