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PURIFICATION OF PLANT HORMONE EXTRACTS BY GEL PERMEATION CHROMATOGRAPHY*

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Key Word Index—Gel permeation chromatography; gibberellins; indoleacetic acid; cytokinins; abscisic acid.

Abstract—Gel permeation chromatography on porous polystyrene beads has been adapted for the purification of plant extracts prior to analysis for plant hormones. The retention characteristics of gibberellins, indoleacetic acid, cytokinins and abscisic acid are presented along with chromatograms of some typical plant extracts.

INTRODUCTION

A number of methods are in use for the purification of plant extracts prior to analysis for plant hormones. Many of these techniques are based on liquid-solid chromatography and a variety of adsorptive surfaces such as silica gel [1], charcoal [2], polyvinylpyrrolidone [3] and polyamide [4] have been described. Use has also been made of classic liquid-liquid chromatographic techniques with either silica gel [5] or celite [6] as the support. A third major class of chromatographic methodology, gel permeation chromatography (GPC), differs from both partition and adsorption chromatography in that separation arises purely through molecular size differences. Thus the addition of GPC to the normal array of polarity determined separation techniques will enhance the overall effectiveness of the entire purification process, especially in the group separation of classes of compounds such as the gibberellins (GAs) where considerable variation in polarity is encountered within the group. A number of gel chromatography methods have been used for the separation and purification of plant hormones; however, very few of these could be considered to be true GPC processes.

Details of a column chromatography procedure utilising Sephadex G-10 for the purification of plant extracts prior to GA bioassay have been published [7]. Although this method should provide some separation on the basis of molecular size the purification, in fact, relies almost entirely on ion exchange-adsorption phenomena. Extensive phytochemical use has been made of Sephadex LH-20 and similar alkylated dextrans. In particular the technique of Armstrong *et al.* [8] has been of value in the separation of endogenous cytokinins, whilst MacMillan and Wels [9] have published details of a technique which gives excellent separation of GAs. However, in the former case, reverse phase partition behaviour predominates, and in the latter, straight phase partitioning is insured by the loading of the gel with an aqueous stationary phase. In general, the separation mechanism operating with this type of support usually involves either reverse phase or straight phase behaviour in addition to molecular sieving [10]. While this combination of mechanisms leads to versatility and some unique separations, it complicates the prediction of retention indices and is therefore less attractive as an initial, crude group separation procedure. In contrast, neutral polystyrene supports offer a separation that is more exclusively based on gel permeation provided a suitable solvent is used. In this regard, tetrahydrofuran is an effective solvent as it has a low viscosity, swells the polystyrene beads and readily dissolves impure plant extracts. We report below on the chromatographic behaviour of GAs, cytokinins, indoleacetic acid (IAA) and abscisic acid (ABA) on a column of porous polystyrene beads eluted with tetrahydrofuran. A number of plant extracts have also been analysed to indicate representative distributions of dry weight with respect to molecular size.

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RESULTS AND DISCUSSION

Samples were chromatographed in tetrahydrofuran on two 100 × 2.5 cm columns connected in series. The first column consisted of 50 cm of Bio-Beads SX-12 and 50 cm of SX-8, the second contained only SX-4. The gels have molecular exclusion limits of 400, 1000 and 1500 molecular weight units respectively; consequently compounds in the 0–1500 molecular weight range eluted from the column in order of decreasing molecular size.

A sample of expanded polystyrene (MW > 1500) was used to establish the void or exclusion volume (V_0) of the column while the total volume (V_T) was estimated with methanol (MW = 32). It was found that V_0 = 350 ml and V_T = 610 ml. The maximum available capacity factor (k'_{max}) is thus

$$k'_{max} = \frac{V_T - V_0}{V_0} = 0.74$$

The highest flow rate which did not cause excessive bed compression was 3.0 ml min^{-1}, and under these conditions all solutes eluted with a peak width (w) of 40 ml, giving a peak capacity (ϕ) of

$$\phi = \frac{V_T - V_0}{w} = 6.5$$

Maximum chromatographic efficiencies were achieved at V_T with values of 3600 plates and 650 effective plates being obtained for N and N_{eff} respectively using standard procedures [11]. Higher efficiencies could have been

Table 1. GPC retention characteristics of plant hormones

	Retention volume (ml)	MW
GA_3	448	346
GA_4	446	332
GA_9	492	316
GA_{13}	441	378
ABA	503	264
IAA	500	175
Zeatin	490	219
Zeatin riboside	438	351

(V_0—350 ml, V_T—607 ml, w—40 ml)

obtained by a reduction in solvent flow rate or an increase in column length, but as the technique was developed primarily as an initial, crude group separation procedure the resolution gained would not fully compensate for the increased time of analysis. On the other hand, due to the low k'_{max} value, which is typical for this type of support [12], the use of columns shorter than 200 cm would reduce the number of effective plates below the practical optimum of 500 [11]. The 200 cm column length therefore represents a compromise between resolution and the practical aspects of speed of analysis, solvent consumption and cost.

The retention volumes of a number of plant hormones are presented in Table 1. In the case of analogous compounds the expected linear relationship between elution volume and log of molecular weight is obtained. However, this is not as evident when compounds of widely differing structures are compared, presumably because of the complexity of the relationship of molecular weight to molecular size in solution. The recoveries of [^{3}H]-GA_1, [^{3}H]-GA_4, [^{3}H]-GA_8, [^{3}H]-GA_{20}, [^{14}C]-IAA, [^{14}C]-zcatin and [^{14}C]-ABA from the column were all in excess of 90%. These high recovery values, together with the fact that elution always occurred in order of descending molecular weight, indicate that selective permeation was the only separatory mechanism operating.

So as to assess the degree of purification obtained with endogenous GAs, IAA and ABA in plant extracts the acidic, ethyl acetate-soluble fraction from *Alnus* root nodules, *Phaseolus coccineus* seedlings and blackcurrant shoots were subjected to GPC. The elution points of the hormones in these extracts are identical to the values presented in Table 1. The traces illustrated in Fig. 1 were obtained by on-stream monitoring of column eluates with a differential refractometer. It was found that the response of the monitor was related to the amount of material eluting from the column. The distribution of sample weight with respect to molecular size varies greatly from species to species. The efficiency of the purification will therefore depend upon the plant material and the plant hormone under investigation. For instance, if GAs were being purified, GPC would give a 90% reduction in dry weight in the case of *Alnus* root nodules, approximately 50% with *Phaseolus* seedlings, but very little improvement would be obtained with the blackcurrant extract as the main impurities are of a similar molecular size to the GAs. It should be emphasized that these figures under-estimate the value of GPC when it is used in conjunction with other purification procedures. Certain compounds may only be separated from the hormone under investigation by GPC and although these impurities may not always contribute significantly to the total weight of the crude extracts, after purification by polyvinylpyrrolidone [3] and charcoal-celite [13] chromatography they may well represent major contaminants.

There are therefore a number of practical reasons for the inclusion of GPC as a routine purification technique in plant hormone analyses. It utilises a separatory principle quite different to that of other commonly used chromatographic systems. It is well suited for use at an early stage in the purification process because it has a large sample capacity and uses a solvent of high solubilizing power. Recovery from GPC is good and reproducibility high. Unlike other forms of chromatography all the sample is eluted from the column bed by one total volume, V_T. Consequently successive samples can be eluted without fear of overlap. Because GPC functions as a molecular sieve it has some values as a diagnostic aid in metabolism studies. When metabolites of a radioactive precursor are being purified, the presence of glucosides and other conjugates can be readily recognised because of their larger molecular size. This possible use is clearly demonstrated by the separation of zeatin and zeatin riboside (Table 1).

EXPERIMENTAL

The chromatographic column consisted of two 100 × 2.5 cm glass columns, with low dead volume end fittings, connected in series. These were packed with 50 cm of Bio-Beads SX-12 (Bio-Rad Laboratories, Richmond, California) 50 cm of SX-8 and 100 cm of SX-4 in accordance with the normal GPC practices for semi-rigid gels [9] and eluted with tetrahydrofuran.

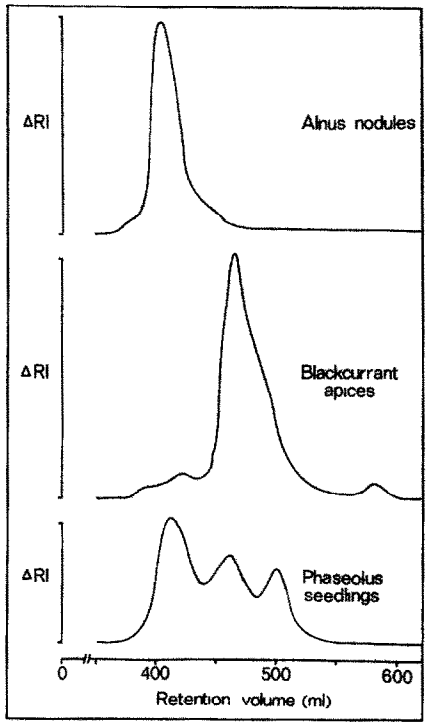

Fig. 1. GPC of the acidic, ethyl acetate soluble fraction from *Alnus* root nodules, *Phaseolus* seedlings and shoot apices of blackcurrant, as monitored with an on-stream refractometer.

The tetrahydrofuran was refluxed over Cu_2Cl_2 for 30 min and then distilled after which it was kept in darkness under N_2 prior to use. A solvent flow of 3.0 ml min^{-1} at 5 psi was maintained by means of a micro-metering pump. This was the maximum flow rate the softer SX-4 gel could tolerate without excessive compression of the bed. Samples of up to 1 g in wt and 2 ml in vol were introduced into the system through a sample injection valve. Material eluting from the column was monitored by means of a Frensel type differential refractometer linked to a flat bed recorder.

Plant extracts. The acidic, ethyl acetate-soluble fractions from *Alnus glutinosa* Gaertn. root nodules, *Phaseolus coccineus* seedlings and shoot apices of blackcurrant (*Ribes nigrum*) were obtained using standard procedures [7].

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