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HIGH-PERFORMANCE STERIC EXCLUSION CHROMATOGRAPHY OF PLANT HORMONES*

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SUMMARY

Details are presented of high-performance steric exclusion chromatography of plant hormones on a μSpherogel support with a molecular exclusion limit of < 2000. The speed of analysis is high compared with traditional steric exclusion chromatography and the sample capacity is well in excess of that normally encountered in other modes of high-performance liquid chromatography. The technique is, therefore, well suited for routine use at an early stage in the purification of trace quantities of endogenous hormones in plant extracts. Because of the complex nature of extracts encountered when attempting to identify any trace component from natural sources it will usually be advantageous to incorporate steric exclusion chromatography into the purification process in order to enhance the accuracy of subsequent analysis.

INTRODUCTION

The analysis of hormones such as gibberellins (GAs), cytokinins, indole-3-acetic acid (IAA) and abscisic acid (ABA) in plant extracts poses many technical problems, primarily because the amounts present are usually in the nanogram to microgram range, while their concentrations rarely exceed 1 part in 10^6 and are often as low as 1 part in 10^{10}. In such circumstances a multistep analytical sequence is necessary in order to obtain a degree of purity in the final extract which will allow an accurate determination of hormone content by methods such as gas chromatography–mass spectrometry and/or high-performance liquid chromatography (HPLC). The exact combination of procedures used is best determined by an on the spot assessment rather than the application of standardized methods since not only hormone

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levels but also the nature and amounts of contaminant vary greatly from one tissue to another. However, when deciding what particular techniques to use it is important to bear in mind two general points. Firstly, in the initial stages of purification, the substantial extract weights that are encountered necessitate the utilization of chromatographic techniques offering a high sample capacity, and secondly, purification is most effectively achieved if the individual procedures are based on widely different separatory mechanisms[1,2].

A number of methods have been used to purify hormones in plant extracts. Most are based on liquid–solid chromatography on, for example, silica gel[3], polyvinylpyrrolidone[4] or charcoal[5]. Classical liquid–liquid chromatographic techniques have also been utilized with supports of silica gel[6], Celite[7] and Sephadex LH-20[8,9]. By contrast, gel permeation or steric exclusion chromatography (SEC) is a chromatographic method where separation occurs on the basis of molecular size rather than polarity. Because of this difference in mechanism, the addition of SEC to a multistep purification procedure will enhance the overall effectiveness of the entire purification process.

The only reports on the use of SEC to purify plant hormones are those of Reeve and Crozier[1,10] who chromatographed extracts in tetrahydrofuran (THF) on a neutral polystyrene support. Despite its value, this technique in particular, and SEC in general, has not found widespread application primarily because of the large size and instability of classical gel columns. However, with the advent of macroporous cross-linked styrene–divinylbenzene copolymer supports specifically designed for HPLC[11], the procedure has been greatly simplified. SEC is now as rapid, convenient and efficient as any other mode of HPLC.

This paper reports the chromatographic properties of free GAs, gibberellin methoxycoumaryl esters (GACEs), IAA, ABA and the cytokinins, zeatin, zeatin riboside and 2-methylthio-6-(3-methylbut-2-enylamino)-9-β-D-ribofuranosyl purine (ms^2i^6 Ado) on a high-performance SEC column, and discusses areas where the application of SEC can be profitably employed in the analysis of endogenous hormones.

MATERIALS AND METHODS

Apparatus

Solvents were delivered by a Varian Model 5010 high-performance liquid chromatograph to a 300 × 8 mm μSpherogel column with a nominal pore size of 50 Å (ref. 12) (Altex Scientific, Berkeley, CA, U.S.A.) at a flow-rate of 1 ml min^{-1}. Samples in THF were applied to the column via a Perkin-Elmer Model 7105 sample injection valve. The eluent was monitored by a Waters 440 absorbance detector, a Perkin-Elmer 650-10LC spectrophotofluorimeter or a radioactivity monitor[13].

Solvents

THF was refluxed over Cu_2Cl_2 for 30 min, distilled and stored in darkness under nitrogen at 3°C prior to use. Reagent-grade ethanol, acetic acid and ethylenediamine were glass-distilled.

Samples

[^{3}H]GA_9 (47 Ci mmol^{-1}) was prepared by selective hydrogenation of $\Delta^{2,3}$ GA_9 (ref. 14). [^{3}H]GA_{43} (*ca.* 0.5 Ci mmol^{-1}) was synthesized from DL-2[^{3}H]mevalonic acid (176 mCi mmol^{-1}) using a liquid endosperm preparation from immature seed of *Cucurbita maxima*[15]. Methoxycoumaryl esters of GA_1, GA_3, GA_4, GA_5, GA_7, GA_8, GA_9, GA_{13}, GA_{14}, GA_{16}, GA_{20}, GA_{25} and GA_{36} were prepared by incubation of each of the free acids with an equimolar amount of 4-bromomethyl-7-methoxycoumarin (BMMC), a one tenth molar equivalent of 18-Crown-6 (Aldrich, Milwaukee, WI, U.S.A.) and a crystal of K_2CO_3 in 100 μl of dry acetonitrile at 60°C for 2 h. The reaction mixture was taken to dryness, water was added and the GACEs were extracted into chloroform prior to purification by reversed-phase HPLC. Acidic, dichloromethane extracts from sap (270 ml), shoots (25 g) and callus (28 g) of Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) were obtained by standard procedures[16].

RESULTS AND DISCUSSION

The μSpherogel support has a nominal exclusion limit of < 2000 daltons and compounds under this limit elute from the column in order of decreasing size. The exclusion volume (V_0) was established by chromatography of polystyrene (average mol. wt. 3000) in THF, while acetone (mol. wt. 58) was used to determine the total volume (V_t). It was found that $V_0 = 5.5$ ml and $V_t = 9.5$ ml. The maximum available capacity factor ($k'_{max.}$) is therefore:

$$k'_{max.} = \frac{V_t - V_0}{V_t} = 0.73$$

Under optimum solvent conditions all solutes eluted with peak widths (w) of 0.4 ml, yielding a peak capacity (φ) of:

$$\varphi = \frac{V_t - V_0}{w} = 10.0$$

At V_t chromatographic efficiencies were calculated to be 9000 plates and 1600 effective plates for N and N_{eff}, respectively using standard procedures[17].

The elution characteristics of a number of GACEs, BMMC, IAA, ABA, GA_9 and GA_{43} on μSpherogel are presented in Table I. The retention volumes (V_R) of the GACEs in THF ranged from 6.3–6.8 ml. Although the technique provides only partial resolution of individual GACEs it is of value in separating these compounds from unreacted BMMC (V_R 7.7 ml) in 18-Crown reaction mixtures. When endogenous GAs are derivatized SEC will also separate the GACEs from many of the impurities in the extracts.

Substantial band broadening, non-Gaussian peak shapes and retentions in excess of V_t were evident when free acids were chromatographed in THF. The phenomenon was readily suppressed by the addition of acetic acid to the THF to a final concentration of 100 mM. When this was done ABA and IAA eluted at 6.6 and 7.1 ml, respectively. The extremes of the molecular weight range of the free GAs were

TABLE I

SEC RETENTION CHARACTERISTICS OF GACEs, BMMC, GAs, ABA AND IAA

Compound	*Mol. wt.*	*Solvent**	*Retention volume (ml)*
$GA_{13}CE$	942	1, 2	6.3
$GA_{25}CE$	926	1, 2	6.3
$GA_{36}CE$	738	1, 2	6.3
$GA_{14}CE$	724	1, 2	6.3
GA_8CE	552	1, 2	6.3
$GA_{16}CE$	536	1, 2	6.5
GA_1CE	536	1, 2	6.4
GA_3CE	534	1, 2	6.4
GA_4CE	520	1, 2	6.5
$GA_{20}CE$	520	1, 2	6.5
GA_5CE	518	1, 2	6.5
GA_7CE	518	1, 2	6.5
GA_9CE	504	1, 2	6.8
BMMC	270	1, 2	7.7
GA_{43}	394	2	7.0
GA_9	316	2	7.6
ABA	264	2	6.6
IAA	175	2	7.1

* Solvent 1 = THF; solvent 2 = 100 m*M* acetic acid in THF.

represented by [^{3}H]GA_9 and [^{3}H]GA_{43}. The V_R of GA_{43} was 7.0 ml and that of GA_9, 7.6 ml. In both instances *w* was 0.4 ml. Thus, collection of the 6.8–7.8 ml zone provides a very effective and simple method of separating endogenous GAs as a group from the complex of substances typically present in plant extracts. SEC is well equipped to handle the high sample weights that are routinely encountered with such extracts since this particular column-packing combination has a sample capacity of > 100 mg (ref. 12) and THF has a high solublizing power. Recoveries were always $> 90\%$.

In the case of GACEs, the expected relationship held between the elution volume and the log of the molecular weight. However, the relationship did not hold for compounds of diverse structure. For instance, GA_{43} eluted much later than ABA although it has a higher molecular weight than ABA. It is a matter of conjecture whether this is consequence of the complexity of the relationship between molecular weight and molecular size or the involement of separatory mechanisms other than selective permeation.

Since cytokinins are sparingly soluble in THF a THF–ethanol (95:5) mobile phase was used. The data are presented in Table II. Both zeatin riboside and ms^2i^6Ado eluted before zeatin which chromatographed with a much lower efficiency. As zeatin is a purine the poor efficiency for this compound could have been due to the presence of an ionizable proton at the N-9 position. In keeping with this possibility, the chromatographic efficiency for zeatin increased fourfold when 1 m*M* ethylenediamine was added to the solvent. Although this change in mobile phase composition did not affect the retention volume of zeatin riboside, there was a small shift in the retention of zeatin while ms^2i^6Ado was much more strongly retained than had previously been the case. While such changes in column selectivity offer versatility and may allow some unique separations they do indicate that effects other than molecular sieving also play a role in the separation process.

TABLE II

SEC RETENTION CHARACTERISTICS OF ZEATIN RIBOSIDE, ZEATIN AND ms^2i^6 Ado

Compound	*Mol. wt.*	*Solvent**	*Retention volume (ml)*	*Efficiency (theoretical plates)*
Zeatin riboside	351	3, 4	6.5	4200
Zeatin	291	3	7.2	950
		4	6.8	4600
ms^2i^6 Ado	382	3	6.6	4350
		4	7.7	4800

* Solvent 3 = THF–ethanol (95:5); solvent 4 = 1.0 m*M* ethylenediamine in THF–ethanol (95:5).

In order to assess the type of results likely to be obtained with plant extracts, the acidic dichloromethane-soluble fractions from sap, shoots and callus of Douglas-fir were subjected to SEC in THF–acetic acid. The output from the column was monitored with a spectrofluorimeter. In addition, successive 0.2-ml fractions were collected, dried and weighed. The traces are illustrated in Fig. 1. Except for very-low-molecular-weight components, the fluorimeter output was related to the amount of material eluting from the column. The distribution of sample with respect to molecular size was different for each of the tissues analysed, although not as diverse as previously observed when extracts from different plant species were analysed by conventional SEC procedures[10]. Fig. 1 also shows that small amounts of material eluted after V_t (9.5 ml). This is an indication that, for at least a minor portion of the extract, retention is not determined solely on the basis of molecular size. Despite this, the speed of analysis is high as successive extracts can be analysed at intervals of *ca.* 10 min without fear of sample overlap. This may be contrasted with run-times of 320 min for classical

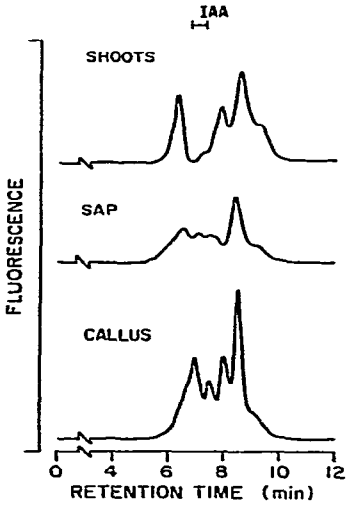

Fig. 1. High-performance SEC of acidic dichloromethane extracts from sap, shoots and callus of Douglas fir. Column: 300 × 8 mm μSpherogel 50 Å; exclusion limit: $2 \cdot 10^3$; solvent: 100 m*M* acetic acid in THF; flow-rate: 1 ml min^{-1}; detector: spectrofluorimeter, excitation 280 nm, emission 350 nm.

SEC on neutral polystyrene beads[10] and the associated increase in solvent consumption.

The effectiveness with which SEC is able to reduce gross contaminants in the fractions of interest will depend both upon the plant material and the hormone in question. The three samples illustrated in Fig. 1 contain IAA as a trace component[18]. Collection of the IAA zone (6.9–7.4 ml) resulted in a 9-, 25- and 8-fold reduction in the dry weights of the sap, shoot and callus extracts, respectively. Such degrees of purification enhance the subsequent analysis of endogenous IAA by techniques, such as reversed-phase HPLC, which have a much lower sample capacity than SEC. Even if lower purification figures are obtained, SEC can still be of practical value. Certain contaminants may only be separated from the hormone under study by SEC. Although they may not always contribute significantly to the total weight of the crude extract, they could become major contaminants if not removed by subsequent purification techniques. Provided that molecular sieving is the dominant separatory mechanism, there are also theoretical grounds for using SEC as a purification step prior to analysing trace quantities of any substance from natural sources. The use of SEC places an upper limit on molecular size and thereby restricts the number of compounds potentially present in an extract. This simplifies the analytical situation considerably and, as a consequence, the degree of uncertainty associated with the accuracy of analysis is markedly reduced. These theoretical considerations are discussed in detail elsewhere[19,20]. Practically, the use of SEC allows the application of high resolution procedures much earlier in an analytical protocol than would otherwise be possilbe.

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