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# LOWER LIMITS OF MOLECULAR WEIGHTS OF COMPOUNDS EXCLUDED FROM SEPHADEX G-25 ELUTED WITH AQUEOUS ACETONE MIXTURES

## APPLICATION OF THE RESULTS TO THE SEPARATION OF THE COMPONENTS OF TANNIC ACID

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SUMMARY

Compounds of molecular weight greater than 900, 500, and 300 are excluded from Sephadex G-25 eluted with 40, 50 and 60% aqueous acetone, respectively. The lower exclusion limits were tested by showing that 40%, but not 50% or 60% aqueous acetone, separates the four phenolic components of tannic acid.

## INTRODUCTION

Our early attempts to separate the polyphenolic constituents of aqueous extracts of tree leaves on the dextran gels Sephadex G-10 and G-25, using water as eluent, were unsuccessful, some of the polyphenols being strongly adsorbed on the gels. Promising results were obtained, however, when Sephadex G-25 was used in conjunction with aqueous ethanol or aqueous acetone, each of which is known to decrease adsorptive effects[1,2]. Because with 60% aqueous alcohol Sephadex G-25 swells about half as much as with water, SOMERS[1] considered that the molecular weight of a compound completely excluded from the gel would be about 2000, assuming that a molecular weight of about 5000 is excluded with water alone. This criterion was applied to the estimation of the molecular weights of wine tannins with 50% acetone[2] as eluent, and to the polyphenols of apple peel, separated with 60% ethanol[3].

We found that the colorimetric reagent Titan Yellow (mol. wt. 695) (refs. 4 and 5) was completely excluded from Sephadex G-25 in the presence of 50% acetone, so that there seems to be no simple relationship between the extent of swelling and the molecular weight excluded. We have therefore studied the behaviour of the Sephadex G-25–aqueous acetone system.

The volumes of 50% acetone necessary to elute from the column model compounds of different types were determined. The molecular weights of compounds completely excluded were found by plotting elution volume/column bed volume ($=V_e/V_b$) against log(molecular weight). The void volume ($V_0$) of the column was

determined with Blue Dextran (mol. wt. $2 \times 10^6$). The minimum molecular weight for a compound to be excluded from the column is given by the value of log(molecular weight) at the point of intersection of the lines $V_e/V_b$ and $V_0/V_b$ (Fig. 1).

The values for minimum molecular weights excluded were much less than expected, being approximately 900, 500 and 300 for the eluents 40, 50 and 60% acetone, respectively. Confirmation of the results was sought by testing tannic acid on the column with each eluent. Tannic acid is a mixture, consisting mainly of four phenolic compounds, the relative proportions of which may vary from sample to sample: gallotannin (mol. wt. approx. 1800), trigallic acid (mol. wt. 504), *m*-digallic acid (mol. wt. 342) and gallic acid (mol. wt. 180)[6-9] (Fig. 3). As expected, 40% acetone was the only eluent that separated the components. The order in which the components were eluted was confirmed in each experiment by two-way paper chromatography.

## MATERIALS AND METHODS

### *Model compounds*

For description see key to Fig. 1. From 1 to 5 mg of the purest reagents available were dissolved in 2.0 ml of the appropriate eluent.

### *Tannic acid*

The tannic acid used was Hopkin & Williams "B.P., $C_{76}H_{52}O_{46} = 1701.2$". Because tannic acid is a mixture, neither a formula nor a molecular weight can properly be assigned to it. A large-scale separation of the components of the sample on Sephadex G-25 indicated that its composition was: gallotannin, 76.4%; trigallic acid and *m*-digallic acid, 13.8%; and gallic acid, 9.8%.

### *Acetone*

Acetone was analytical reagent grade.

### *Determination of the minimum molecular weights excluded by Sephadex G-25*

The column bed of Sephadex G-25 (Fine), volume approximately 200 ml, was made in a calibrated glass chromatographic column 35 cm × 3.25 cm I.D. The gel bed was washed to constant volume ($V_b$) with the chosen eluent and the void volume ($V_0$) determined with Blue Dextran. Effluents from the column were colllected in fractions of 2.0 ml and the elution volumes ($V_e$) at which the compounds first appeared, were measured.

The ratios $V_0/V_b$ and $V_e/V_b$ were plotted against log(molecular weight). Fig. 1 shows graphs for the three eluents.

### *Separation of the components of tannic acid*

Tannic acid (10.0 mg in 2.0 ml of the appropriate eluent) was applied to the column and eluted with aqueous acetone. After diluting where necessary, each fraction of the eluent was treated with 1 ml Folin–Denis reagent and 2 ml saturated sodium carbonate solution, and after 45 min the optical densities of the solutions were measured in 1-cm cells at 725 nm. The optical density of each fraction, corrected for the initial dilution where necessary, was plotted against its elution volume (Fig. 2).

The order in which the components were eluted was confirmed by paper chromatography of the combined and concentrated fractions constituting a peak using Whatman No. 2 chromatography paper. The solvents applied were: first way, 6% acetic acid; second way, *sec.* butanol–acetic acid–water (14:1:5). The positions of the phenols were shown by dipping the chromatograms in a mixture of equal volumes of 0.3% ferric chloride solution and 0.3% potassium ferricyanide solution.

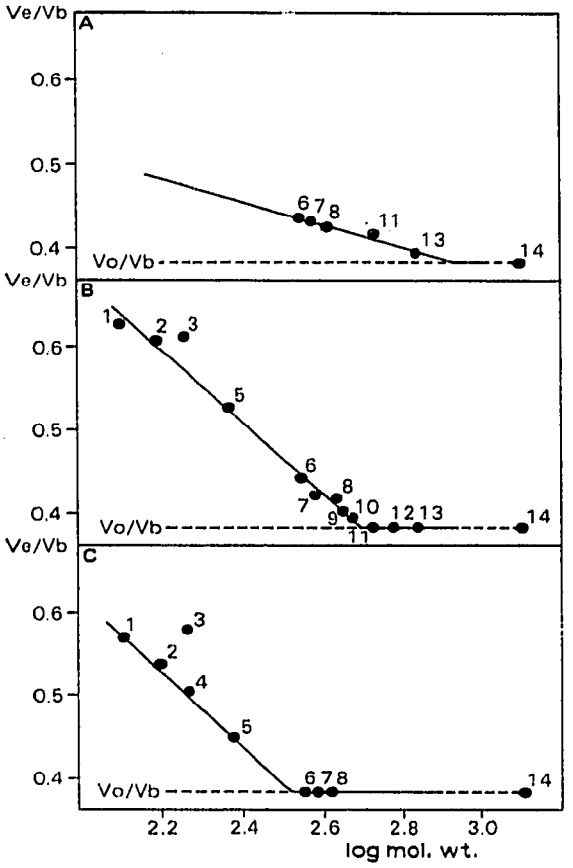


Fig. 1. Graph showing the lowest molecular weight for compounds to be excluded by Sephadex G-25 (Fine) with (A) 40%, (B) 50% and (C) 60% aqueous acetone as eluent. Model compounds: (1) pyrogallol (mol. wt. 120), (2) 2,2′-dipyridyl (mol. wt. 156), (3) gallic acid (mol. wt. 180), (4) glucose, (mol. wt. 180), (5) 4-(2-pyridylazo)resorcinol Na-salt (PAR, mol. wt. 237), (6) Phenol Red (mol. wt. 354), (7) *m*-Cresol Purple (mol. wt. 382), (8) Chlorophenol Red (mol. wt. 423), (9) Rhodamine 6G (mol. wt. 450.5), (10) Thymol Blue (mol. wt. 466), (11) Bromocresol Purple (mol. wt. 540), (12) Bromothymol Blue (mol. wt. 624), (13) Titan Yellow (mol. wt. 695), (14) Blue Dextran (mol. wt. $2 \times 10^6$). Indicators were detected by making the solutions alkaline; phenolic compounds by reacting with ferric chloride solution; 2,2′-dipyridyl was detected by adding a dilute solution of ferrous sulphate, followed by ammonia. The colorimetric reagents PAR and Titan Yellow were visible as such. Column bed volume ($V_b$), 187 ml; void volume ($V_0$), 72 ml; $V_0/V_b = 0.383$.

## RESULTS AND DISCUSSION

The minimum molecular weights of the model compounds excluded by Sephadex G-25 when eluted with 40, 50 and 60% acetone are shown by the graphs of $V_e/V_b$

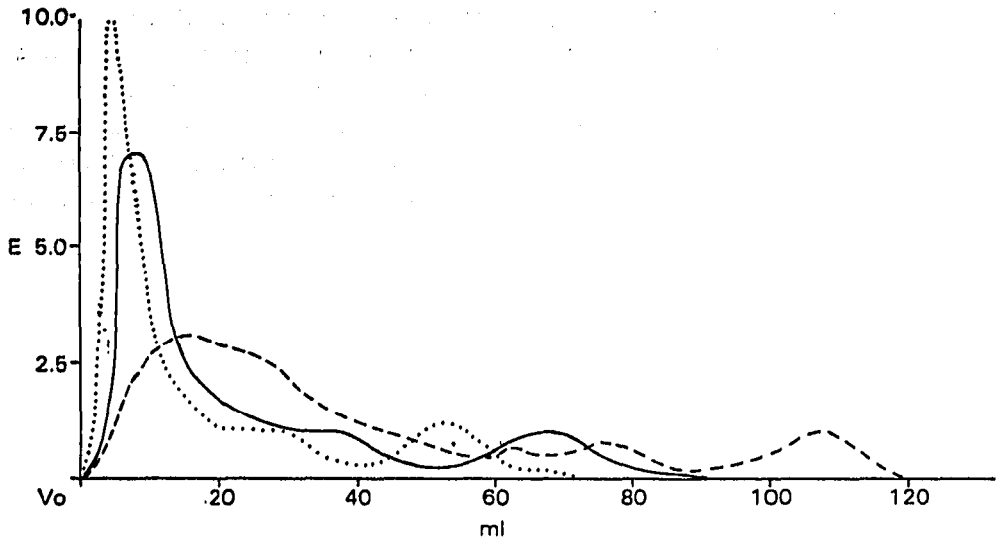


Fig. 2. Elution of the phenolic components of tannic acid with 40, 50 and 60% acetone. Optical densities shown are of the colours produced by the reaction of the Folin–Denis reagent with individual fractions. – – –, 40% acetone; —, 50% acetone; . . . . . , 60% acetone.

against log(molecular weight) to be approximately 900, 500 and 300, respectively. The values are probably within ± 50 of the true molecular weights.

As expected, an increase in the proportion of acetone in the eluent has a large effect in decreasing the limit of molecular weight for a compound to be excluded. When the eluent is 50% acetone the molecular weight limit is much less than the suggested value 2000 referred to earlier. It seems that compounds having a wide range of molecular weights greater than the limiting values might be separated on Sephadex G-25 by altering the relative proportions of acetone and water in the

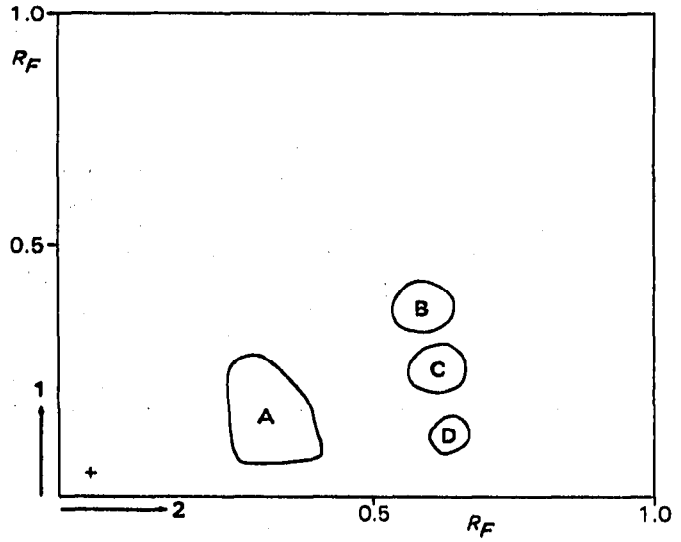


Fig. 3. Diagram of a typical two-dimensional chromatogram of tannic acid on Whatman No. 2 paper. Solvents: first way, 6% acetic acid; second way, *sec.*-butanol–acetic acid–water (14 : 1 : 5). Components were revealed by ferric chloride and potassium ferricyanide. A = gallotannin; B = gallic acid; C = *m*-digallic acid; D = trigallic acid.

eluent. Pharmacia Fine Chemicals (Uppsala, Sweden), manufacturers of Sephadex gels, give a fractionation range of 1000–5000 for peptides and globular proteins, and 100–5000 for dextrans separated on Sephadex G-25 (Fine) with water as eluent. Thus, by varying the proportions of water and acetone in the eluents, compounds of molecular weights between 900 and 5000 should be excluded. However, it is clear that the extent of adsorption increases as the proportion of acetone is decreased, particularly with polyphenols; the separation of the phenolic components of tannic acid with 40% acetone is favoured by adsorption. Paper chromatography of the separate peaks shows that gallotannin trails back slightly into trigallic acid, but a re-run of the almost pure trigallic acid fraction would separate the components completely. By choosing a suitably large column, and by a moderate increase of the quantity of tannic acid applied, the individual components can be isolated on a preparative scale.

The limits of molecular weights excluded with 50 and 60% acetone seem to be about 500 and 300, respectively, because the components of tannic acid are not separated by these eluents. Trigallic acid (mol. wt. 504) is masked by gallotannin in 50% acetone, and as shown by paper chromatography both trigallic acid and *m*-digallic acid (mol. wt. 342) are eluted with gallotannin by 60% acetone. Gallic acid was separated from the other components, with the eluents studied due not only to its small molecular weight, but chiefly to its adsorption by the gel.

Although these results indicate that Sephadex G-25 eluted with aqueous acetone behaves as a molecular sieve, the possibility was investigated that the compounds might also have been separated by partition chromatography, where the stationary phase could be considered as a water-rich phase, and the mobile eluent as an acetone-rich phase.

The model compounds, singly and in mixtures, were thus chromatographed on Whatman No. 1 paper, and on columns of cellulose, with 40, 50 and 60% acetone as eluents. The $R_F$ values of all of the compounds on paper were between 0.9 and 1.0, and it was not possible to separate from one another the components of any mixture.

Chromatographing tannic acid with the above solvents again showed that no separation into its constituents had taken place. After column chromatography, all of the effluent fractions showed the presence of gallotannin, trigallic acid, *m*-digallic acid and gallic acid. In no case did the attempted separations take the same course as those of the experiments with Sephadex, and it would seem, therefore, that it is unnecessary to attempt to correct for a partition effect when assessing the results.

By using Sephadex G-25 with the eluents 40, 50 and 60% acetone, compounds can be separated into groups covering increasing molecular weight ranges, *i.e.* (a) up to 300, (b) from 300 to 500, (c) from 500 to 900, and (d) greater than 900. Having removed unwanted substances from one group, it is possible to separate the components of that group by re-running them on Sephadex G-25.

The method would seem to have useful possibilities in the separation of groups of components of plant extracts containing much polyphenolic material, *e.g.* vegetable tannins, in which many of the constituents, particularly those immobile in organic chromatographic solvents, and unresolved on paper chromatograms, are usually referred to as of high molecular weight. Our experience with Sephadex G-25 and

50% acetone indicates that a considerable amount of paper chromatographically unresolved polyphenolic material is not excluded from the gel, suggesting that the molecular weights of the components of such material may be less than 500.

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