SEPARATION OF COMPLEX MIXTURES OF POLYHYDROXY PHENOLS ON COLUMNS OF SEPHADEX

J. B. WOOF AND J. S. PIERCE
Research Laboratory, Arthur Guinness & Co. (Park Royal) Ltd., Park Royal Brewery, London N.W.10 (Great Britain)
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INTRODUCTION

As part of an investigation of protein-flavonoid complexes in beers, it became necessary to have a column chromatographic method by which proteins, protein-flavonoid complexes and free polyphenols present in malt and hop extracts could be fractionated and estimated under conditions which were mild enough not to cause changes in the system. The polyphenols involved ranged from phenolic acids like vanillic, protocatechuic, gallic, caffeic and chlorogenic to coumarins, catechins, anthocyanogens, flavonols and their glycosides, together with polymers of increasing molecular size.

Various column packing materials have been used successfully for fractionating phenols including cellulose[1], ion exchange resin (*e.g.* Amberlite IRC-50)[2,3], polyamide[4–7] and silica gel[8]. All these methods use more or less complex organic solvent mixtures for elution which are not suitable where proteins and protein complexes are involved.

GELOTTE[9] has shown that the cross-linked dextran gel, Sephadex, has a tendency to adsorb aromatic compounds especially phenols. BOCK[10] indicated that flavonoids and tannins were so strongly adsorbed that they could only be eluted with alkaline solution conditions, under which rapid auto-oxidation of many flavones occurs. However, by using G25 Sephadex and eluting with ammonia NILSONN[11] was able to separate a mixture of isoflavones without being able to discern any relationship between elution volume and structure. SOMERS[12] eliminated the adsorptive effects of the Sephadex by preparing the gel and eluting in aqueous alcohol media.

In this communication the behaviour of a range of phenols on Sephadex columns using aqueous eluants is described and conditions suitable for fractionation defined making use of the adsorptive properties of the gel. At the same time, the molecular sieving properties of the gel permit separation of free phenols from high molecular weight proteins and phenol polymers[13].

MATERIALS AND METHODS

Column preparation and operation

G25 Sephadex in the medium particle size grade (AB Pharmacia, Uppsala, Sweden) has been used throughout the investigation. Packing, handling and general

operation have been described in detail by FLODIN[14] and these techniques have been generally followed. Columns were 35 cm long and 2.5 cm in diameter with a total bed volume of 120 ml. Each phenol sample was dissolved in the eluting medium to give a concentration of 200 μg/ml and 1 ml of this was loaded onto the column. Even the least soluble flavonoids dissolved sufficiently to be detected. The flow rate of 25 ml/h was governed by a peristaltic pump and was constant throughout the series of experiments. The eluate from the column was either pumped to a fraction collector or to an AutoAnalyser assembly previously described[15]. In the former case the presence of the phenols in the eluate was detected by the absorption at 280 mμ whilst in the latter the phenols were coupled with diazotized *p*-aminobenzoic acid and the resultant yellow colour monitored at 420 mμ. Continuous monitoring had the advantages of convenience and accuracy in determining peak elution volumes. In addition reference to a calibration curve permitted peak area to be related to amount of the phenol present in the sample. $10^{-4}M$ concentrations of phenols could be determined in this way with a coefficient of variation of 2.7 %.

Characteristics of the column were determined in the usual manner (FLODIN[14]) and K_D values calculated from the equation $V_e = V_o + K_D V_i$. Under the conditions used V_o and V_i were found to be 40 and 55, respectively. Under standardised conditions the peak elution volume was characteristic of a particular phenol and quite reproducible whether the phenol was alone or in a mixture. Peaks were symmetrical and sharp if eluted early but very strong adsorption and consequently long elution times resulted in the spreading of the bands to some extent. This was not in fact a problem, since the strongly adsorbed compounds were usually very well separated.

Separation of flavonoids as molybdate complexes

In the case of flavonoids containing several hydroxyl functions it was necessary to weaken the adsorptive forces between them and the column material so as to achieve elution under mild aqueous conditions in a reasonable time. The formation of complexes between molybdate ions and phenols containing *ortho*-hydroxyl groups is well known. These complexes form readily in mixing aqueous solutions and are yellow in colour. They were usually formed *in situ* on the column by first loading the phenol and then adding 1 ml of 10 % sodium molybdate solution before commencing elution with water. Different groups of flavonoids show characteristic differential spectra some of which are shown in Fig. 1, which, combined with elution volume, serves to identify unknowns.

Molybdate complexes of flavonoids were adequately separated on smaller columns (0.9 cm × 30 cm, bed volume 40 ml) with a saving in time.

RESULTS AND DISCUSSION

Simple phenols

Table I shows K_D values calculated for a range of simple phenols using water, 0.05 *M* sodium chloride, 0.1 *M* ammonium hydroxide, 0.1 *M* acetic acid and, in some cases, 0.01 *M* sodium molybdate, as eluting media.

With water, elution occurred according to the number of hydroxyl groups the phenol contained. Adsorption occurred in all cases, hence fractionation was achieved not on the basis of molecular size and shape but as a result of differing degrees of

bonding between the phenol and the residual carboxyl groups present in the gel matrix. There was little or no difference between *ortho* and *meta* substituted compounds. Hydroquinone was anomalous in its elution as it is in its structure and this may be accounted for by its tendency to assume a quinone form in solution. Incorporation of neutral electrolyte into the eluting medium caused the K_D values for the phenols to

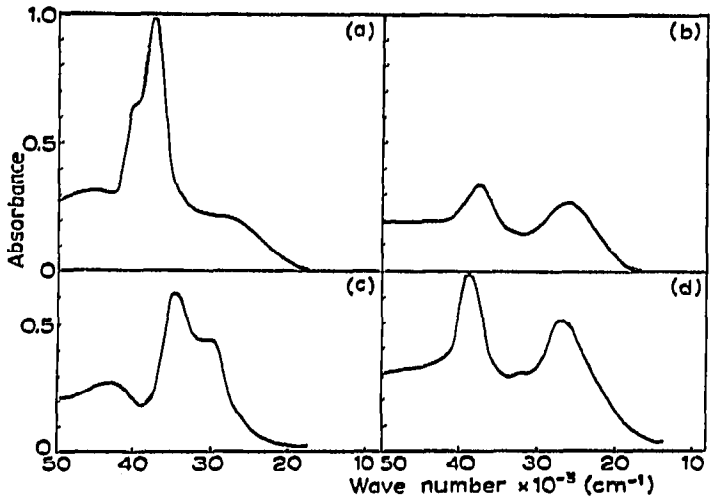

Fig. 1. Spectra of molybdate complexes of some representative flavonoids. Sample: flavonoid dissolved in 0.001 *M* sodium molybdate. Reference: 0.001 *M* sodium molybdate. (a) Catechin; (b) quercetin; (c) dihydroquercetin; (d) rutin.

fall into two series. The *ortho* substituted catechol and pyrogallol formed one group and phenol, resorcinol and phloroglucinol the other with K_D values 0.7 units greater than the corresponding *ortho* compounds. The behaviour of hydroquinone was no longer anomalous and its K_D fell between those for resorcinol and catechol. In acid medium the hydrogen bonding to the column matrix was increased, the extent depending on the number of hydroxyl groups present. ΔK_D was 0.15, 0.45 and 0.9 for one, two and three hydroxyl groups respectively. The order of elution was otherwise the same as for water. In alkaline media, adsorption was almost eliminated and any small differences in K_D may be accounted for by the shape and size of the ionic species present at these pH's.

Presence of *ortho*-hydroxyl groups permits the formation of complexes in the

TABLE I

COMPARISON OF K_D VALUES FOR SIMPLE PHENOLS IN AQUEOUS SOLUTIONS

	Eluting medium				
	Water	*0.5 M NaCl*	*0.1 M* NH_4OH	*0.1 M* CH_3COOH	*0.01 M* Na_2MoO
Phenol	1.8	2.0	1.1	1.95	—
Hydroquinone	1.05	2.4	1.7	2.4	—
Resorcinol	2.1	2.65	1.6	2.5	—
Catechol	2.05	1.9	1.05	2.5	2.2
Phloroglucinol	2.3	3.15	1.1	3.25	—
Pyrogallol	2.4	2.4	1.3	3.25	2.0

presence of molybdate ions. Under these circumstances complex formation reduced the bonding of pyrogallol, but apparently increased slightly that of catechol. This may be due to the different shape and size of the complex.

Table II shows the behaviour of a range of phenol derivatives in the same eluting solvents. Here the effects of solvent were much less marked. As in the case of phenol itself, both electrolyte and acid tended to increase the K_D but generally not to a very marked extent.

TABLE II

K_D VALUES OF PHENOL DERIVATIVES IN AQUEOUS MEDIA

Phenol derivative	*Eluting medium*			
	Water	*0.05 M NaCl*	*0.1 M NH_4OH*	*0.1 M CH_3COOH*
Phenol	1.8	2.0	1.1	1.95
o-Hydroxybenzoic acid	1.0	2.1	1.6	3.4
Arbutin	1.45	1.55	0.85	—
o-Nitrophenol	1.45	2.45	1.8	2.1
Saligenin	1.85	2.1	1.55	1.9
Guaiacol	2.2	1.8	1.45	2.1
o-Hydroxybenzaldehyde	2.3	2.3	1.55	2.3
o-Cresol	2.6	2.55	1.85	3.0
Orcinol	3.0	2.6	1.5	—
o-Chlorophenol	3.15	3.4	1.55	3.0
p-Hydroxydiphenyl	4.8	5.4	2.9	—

It will be noted from inspection of the quoted K_D values that separation of mixtures of these phenols should be possible and water or acid are probably the most suitable media. Fig. 2 shows two tracings of the elution pattern obtained automati-

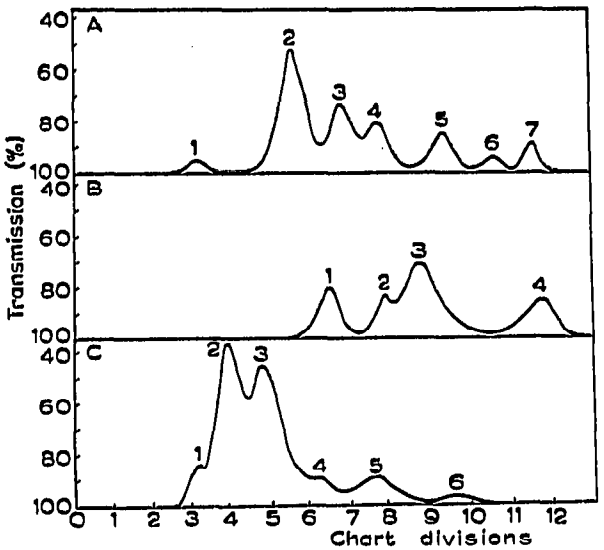

Fig. 2. Separation of phenol mixtures on columns of G25 Sephadex. (A) 35 cm × 2.5 cm column eluted with water. 1 = Hydroquinone; 2 = phenol; 3 = catechol + resorcinol; 4 = guaiacol; 5 = pyrogallol + phloroglucinol; 6 = cresol; 7 = orcinol. (B) Same column eluted with 0.1 *M* acetic acid. 1 = Phenol; 2 = hydroquinone; 3 = resorcinol + catechol; 4 = pyrogallol + phloroglucinol. (C) 0.9 × 25 cm column eluted with 0.1 *M* acetic acid containing 0.05 *M* sodium chloride. 1 = Saligenin; 2 = nitrophenol + guaiacol; 3 = salicylaldehyde; 4 = catechol; 5 = cresol + chlorophenol; 6 = salicylic acid.

cally with the AutoAnalyser. The upper one (Fig. 2A) was obtained with water as eluant, the middle one (Fig. 2B) with dilute acetic acid. Fig. 2C shows the separation of various phenol derivatives using acetic acid as eluant. In this case acid was used because of the anomalous behaviour of hydroxy acids in water.

The behaviour of even simple phenols on Sephadex columns is complex and it is difficult to predict K_D from the structure. The factors determining elution volume include both number and position of hydroxyl groups, substituent groups which effect strength of bonding and medium. In addition steric factors may govern penetration of gel grains. Neither K_D or change in K_D on passing from neutral to alkaline medium seemed to be related directly to the pKs of the phenol. Only nitro and carboxylic acid groups decreased bonding. However, it will be seen from Table III that the groups shown causing increased bonding do so in the order expected from their electron donating properties.

TABLE III

EFFECTS OF NUCLEAR SUBSTITUTION IN THE STRENGTH OF HYDROGEN BONDING AND HENCE ON THE ELUTION VOLUME FROM SEPHADEX COLUMNS

Water is used as eluant.

Increased bonding	ΔK_D	*Parent phenol*	*Decreased bonding*	ΔK_D
o-Cresol (2.6)	+ 0.8		Salicylic acid (1.0)	−0.8
Guaiacol (2.2)	+ 0.4	Phenol (1.8)		
Catechol (2.05)	+ 0.25		*o*-Nitrophenol	−0.35
Orcinol	+ 0.9		3,5-Dihydroxybenzoic acid (1.5)	
Phloroglucinol (2.3)	+ 0.2	Resorcinol (2.1)		−0.6

Phenolic acids

Monocarboxylic acids were not separated on Sephadex when eluted with water. As reported by GELOTTE[9], the carboxylic acid group has a "negative sorption effect" and in most cases elution occurred much earlier than would be imagined from the parent phenol. Intra-molecular hydrogen bonding is not always responsible since there is no difference between *o*- and *p*-hydroxybenzoic acid in water and the 2,4-dihydroxy acid was eluted early while the 2,3-substituted one was not. In electrolyte solution this "negative sorption" effect disappeared in all the acids and there were stronger absorptive forces. The rate of elution seemed to depend on the number, and orientation of free hydroxyl groups, (*i.e.* those not involved in internal hydrogen bonding) and the extent to which these groups are accessible to the carboxyl group of the gel. Most of the acids were eluted very early with ammonia since they also carry a negative charge and will only be repelled by similar acid groups in the gel. Formation of a complex with molybdate resulted in earlier elution as would be expected except in the case of the 2,6-dihydroxy acid which, although both groups can complex, was very strongly adsorbed. Methylation, as in vanillic and syringic acid, resulted in behaviour very much like a monohydroxy acid. They were not differentiated on Sephadex columns.

From Table IV it is thus apparent that electrolyte is required if separation of mixtures of phenolic acids is to be achieved. Fig. 3 shows the separation of a mixture of acids already reported in barley. Resolution in the first stage is not complete but fractions can be collected as shown and completely resolved by re-running the samples

TABLE IV

K_D VALUES OF PHENOLIC ACIDS IN AQUEOUS ELUTING MEDIA

Phenolic acid	*Eluting medium*			
	Water	*NaCl*	NH_4OH	Na_2MoO
o-Hydroxybenzoic acid	1.0	2.1	1.6	1.2
m-Hydroxybenzoic acid	1.0	1.8	0.85	—
p-Hydroxybenzoic acid	1.0	1.45	0.8	—
2,3-Dihydroxybenzoic acid	2.6	1.6	0.4	2.0
2,4-Dihydroxybenzoic acid	0.85	2.7	1.1	1.4
2,5-Dihydroxybenzoic acid	1.0	3.0	1.7	1.4
2,6-Dihydroxybenzoic acid	0.7	1.45	0.4	2.95
3,4-Dihydroxybenzoic acid	1.7	2.1	0.7	—
3,5-Dihydroxybenzoic acid	2.2	1.9	0.7	—
3,4,5-Trihydroxybenzoic acid	1.05	2.5	1.05	1.9
2,3,4-Trihydroxybenzoic acid	2.05	2.65	1.1	2.0
Vanillic acid	0.85	1.95	0.85	—
Syringic acid	0.8	1.95	0.85	—
Chlorogenic acid	1.6	3.9	1.35	—

using water as solvent. Fig. 4 shows the separation on a small analytical column of alkaline degradation products of a flavonoid mixture.

Finally, in Tables V and VI K_D values are given for cinnamic acid derivatives and larger phenols with two benzene rings. In caffeic acid, the two hydroxyl groups imparted quite strong binding properties. These were reduced by methylating one of them and reduced still further by the introduction of an additional methoxyl group, probably because of steric hindrance. Diphenyl and naphthalene derivatives were generally strongly adsorbed, especially in water.

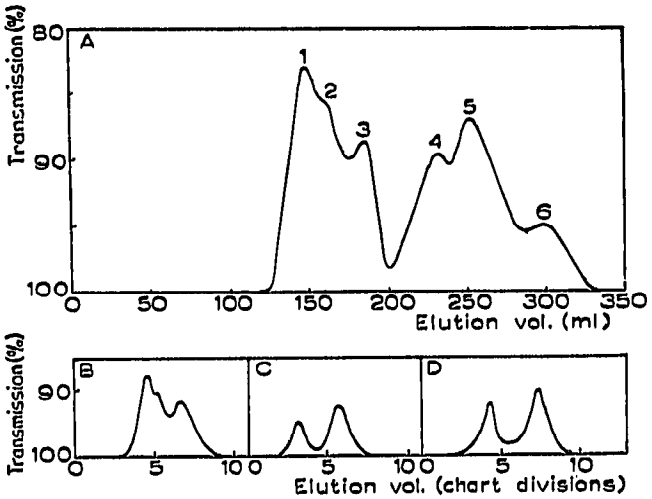

Fig. 3. Separation of phenolic acid mixtures on G25 Sephadex columns. (A) Fractionation of the phenolic acids reported to be present in worts on a 2.5 × 35 cm column eluted with 0.1 *M* sodium chloride. 1 = Vanillic + syringic; 2 = 3,4-dihydroxybenzoic; 3 = gallic; 4 = ferulic; 5 = sinapic; 6 = chlorogenic + caffeic. (B) Rechromatography of fractions 1 + 2 on a 0.9 × 25 cm column of Sephadex using water as eluant. (C) Separation of a ferulic–sinapic acid mixture under the same conditions. (D) Resolution of caffeic and chlorogenic acids from fraction 6 under the same conditions.

Flavonoids

Behaviour of the flavonoids in the solvents previously used for simple phenols was investigated first. 1 ml of the appropriate solvent containing approximately 200 μg of each flavonoid (or a saturated solution if the solubility was very low) was loaded on to the column and elution carried out with either water, 0.5 *M* sodium chloride or 0.1 *M* ammonia. It can be seen from Table VII that apart from the parent chalcone and the single hydroxylated 3-hydroxyflavone, only the glycosides rutin and quercitrin could be eluted with water. Even these were relatively very strongly adsorbed. Binding was even stronger in dilute electrolyte solution. In ammonia, all the compounds could be eluted as sharp peaks with very different K_D values. Naringenin and hesperetin were still strongly adsorbed as was rutin. Quercitrin, however, with

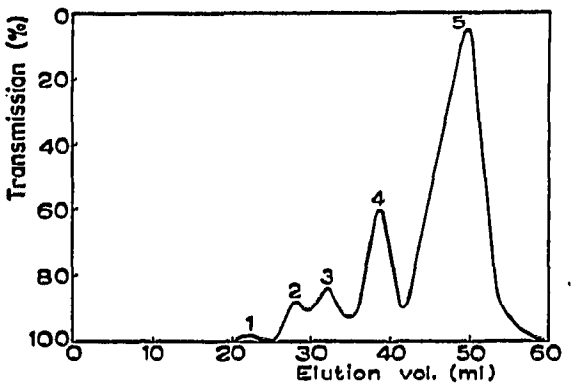

Fig. 4. Separation of alkaline degradation products of a barley flavonoid extract on a 0.9 × 25 cm column of G25 Sephadex using 0.1 *M* sodium chloride as eluant. 1 = *p*-Hydroxybenzoic acid; 2 = vanillic acid; 3 = 3,4-dihydroxybenzoic acid; 4 = 2,4-dihydroxybenzoic acid; 5 = phloroglucinol.

TABLE V

	Eluting medium			
	Water	*NaCl*	NH_4OH	MoO_4
p-Coumaric acid	1.45	1.3	1.2	—
Caffeic acid	1.4	4.0	0.95	2.8
Ferulic acid	1.0	3.1	1.1	—
Sinapic acid	0.8	3.25	1.15	—

TABLE VI

	Eluting medium			
	Water	*NaCl*	NH_4OH	MoO_4
2,2'-Dihydroxydiphenyl	2.85	3.4	2.35	—
4,4'-Dihydroxydiphenyl	0.8	—	2.4	—
2-Naphthol	6.45	—	1.7	—
2,7-Dihydroxynaphthalene	—	—	2.2	—
2,3-Dihydroxynaphthalene	7.4	—	2.9	7.2
1,3-Dihydroxynaphthalene	9.1	—	3.05	—

TABLE VII

K_D VALUES OF SOME FLAVONOIDS AND RELATED COMPOUNDS ON G25 SEPHADEX COLUMNS

Flavonoid	*Eluting medium*		
	Water	*0.05 M NaCl*	*NH_4OH*
Chalcone	3.1	3.1	—
3-Hydroxyflavone	4.2	5.0	4.0
Rutin	5.6	6.5	3.9
Quercitrin	11.4	*	1.85
Robinetin	*	*	0.60
Quercetin	*	*	0.65
Hesperidin	*	*	0.8
Morin	*	*	1.2
Catechin	*	*	1.3
Dihydroquercetin	*	*	1.6
Naringenin	*	*	5.6
Hesperetin	*	*	6.25
Fisetin	*	*	—

* Irreversibly adsorbed.

similar structure, was eluted much earlier and in a similar position to dihydroquercetin. Quercetin and robinetin were apparently not adsorbed at all and washed from the column very early.

On this evidence elution and fractionation can only be achieved with ammonia. Fig. 5 shows the elution pattern for a synthetic mixture where resolution is obtained except for the group of compounds with K_D 1.2–1.8.

Although separation in ammonia is useful it has the drawback already mentioned, namely that some compounds are not stable under these conditions. Accordingly, the behaviour of molybdate complexes was studied. A column of Sephadex was equilibrated with 0.001 *M* sodium molybdate and elution carried out with this

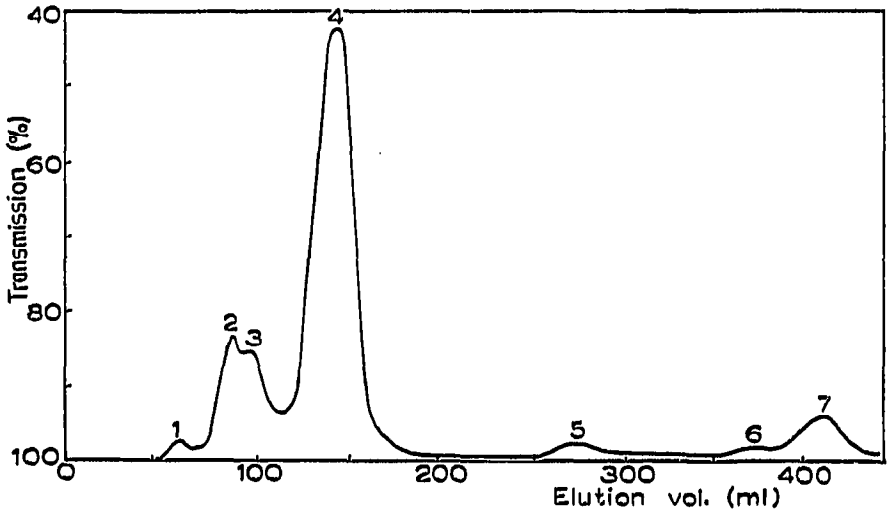

Fig. 5. Separation of a synthetic flavonoid mixture on a 2.5 × 35 cm column of G25 Sephadex using 0.1 *M* ammonium hydroxide as eluant. 1 = High molecular weight impurity; 2 = robinetin + quercetin; 3 = hesperetin; 4 = dihydroquercetin + morin + quercitrin + catechin; 5 = 3-hydroxyflavone + rutin; 6 = naringenin; 7 = hesperidin.

TABLE VIII

ELUTION CHARACTERISTICS OF THE MOLYBDATE COMPLEXES OF SOME FLAVONOIDS ON G25 SEPHADEX COLUMNS

Flavonoid	K_D *(calculated)*	V_t/V_0
Quercitrin	0.24	1.31
Rutin	0.26	1.35
Catechin	0.34[a]	1.45
3-Hydroxyflavone	0.5	1.66
Dihydroquercetin	1.03	2.37
Hesperetin	1.3	2.72
Hesperidin	1.95	3.6
Morin	2.16	3.95
Fisetin	3.33	5.42
Robinetin	4.62	7.14
Naringenin	4.9	7.55
Quercetin	—	10

medium. Table VIII show how some representative flavonoids were eluted. In this case the smaller column was used and values of V/V_0 (V = elution volume, V_0 = void volume) calculated. This is equally characteristic of the phenol under the standard conditions and is open to fewer errors since the value of V_t, the inner volume, need not be determined.

There is, however, a simple relationship between the V/V_0 ratio and the K_D value,

$$V/V_0 = 1 + \alpha K_D \text{ where } \alpha = V_t/V_0$$

and this has been used to calculate the K_D values for comparison with those obtained on a larger column of G25 fine crushed Sephadex.

V/V_0 ratios were very reproducible and were different for many of the flavonoids

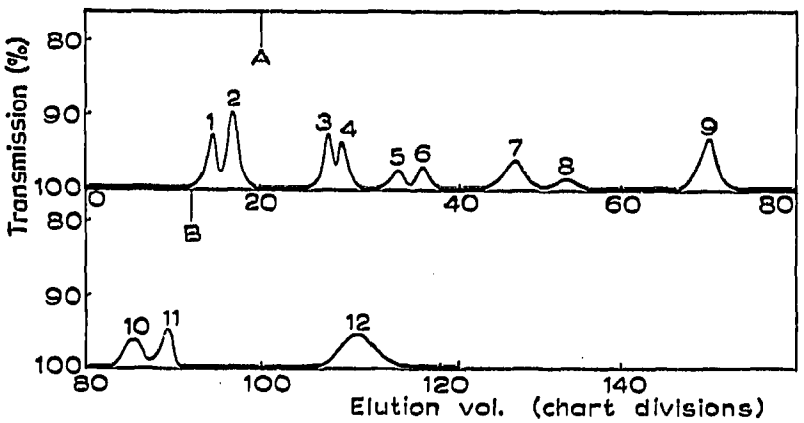

Fig. 6. Fractionation of a complex flavonoid mixture on a 0.9 × 30 cm column of G25 (fine bead) Sephadex. Phenol content of the eluate continuously recorded by the diazonium coupling reaction on an AutoAnalyser assembly. Elution with water proceeded up to point A when 1 ml of 1 *M* sodium molybdate was layered on top of the column. Elution of complex was continued with water to point B where 1 ml 0.1 *M* sodium hydroxide was placed on top of the column. Quercetin was then eluted with water. 1 = 3-Hydroxyflavone; 2 = rutin; 3 = quercitrin; 4 = catechin; 5 = dihydroquercetin; 6 = hesperitin; 7 = hesperidin; 8 = morin; 9 = fisetin; 10 = robinetin; 11 = naringenin; 12 = quercetin.

studied. Flavone glycosides and catechin were relatively loosely bound to the gel matrix but aglycones were strongly adsorbed.

Inspection of the values of V/V_0 again indicates that separation of flavonoids in a mixture could be achieved. Whilst quercetin, rutin, catechin and 3-hydroxyflavone have similar elution volumes as molybdate complexes, in water the K_D values are well separated. A complete elution scheme is shown in Fig. 6 in which preliminary elution is carried out with water and the remaining flavonoids are eluted as their molybdate complexes. Quercetin differs from the others in that it requires alkaline conditions for desorption.

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SUMMARY

It has been demonstrated that mixtures of a wide range of phenols and flavonoids can be fractionated on Sephadex columns by making use of the adsorptive properties. K_D values for a number of phenols and derivatives have been determined, using several aqueous elution solvents which have permitted the selection of the optimum conditions for any particular separation. Flavonoids are generally more strongly adsorbed but quite complex mixtures may be fractionated by first eluting with water, then converting the remaining flavonoids to molybdate complexes. This decreases the strength of binding to the column but allows fractionation.

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