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GEL FILTRATION IN LIPOPHILIC SOLVENTS USING HYDROXYALKOXYPROPYL DERIVATIVES OF SEPHADEX

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SUMMARY

Liquid-gel chromatography involving a lipophilic derivative of Sephadex has been investigated in two solvent systems. The separations studied involve gel filtration mechanisms, and elution data are quoted for eighty compounds. Analysis of column performance characteristics has indicated high efficiency, good loading capacity and quantitative recovery of sample with little 'tailing'. The potential application of these techniques to purification and group separation has been evaluated in respect of both neutral and polar lipid fractions.

INTRODUCTION

The use of gel chromatography in aqueous media has been developed extensively for routine laboratory use. Its adaptation to less polar organic solvents is less advanced: few successful applications to lipid extracts have been reported. The properties of a number of gels suitable for lipophilic solvents (in particular the modified dextran, Sephadex LH-20) are reviewed by JOUSTRA *et al.*[1] and by NYSTRÖM[2].

Gel chromatography is a general term describing all processes involving exchange of solute between a liquid mobile phase and a stationary gel phase. The distribution of a compound between the mobile and gel phase is primarily governed by its partition coefficient. A secondary mechanism also influences elution: free diffusion of solute molecules is restricted to a fraction of the gel as a result of steric interaction with the gel matrix. The behaviour of such a gel-solvent system depends on the polarity of the matrix and the choice of eluting solvent. Three cases may be distinguished:

(i) Partition chromatography, where the gel is more polar than the solvent, and samples are eluted in order of their polarity.

(ii) Gel filtration, where the gel and solvent have the same polarity characteristics and elution is in order of decreasing molecular size of the solutes.

(iii) 'Reversed-phase' partition, where the gel is less polar than the solvent, and samples are eluted in inverse order of their polarity.

Normal Sephadex and aqueous solvents are closely matched in polarity, leading to separation by gel filtration. With lipophilic gels, polarity is variable in both

gel matrix and solvent: the resultant complications have been investigated by SJÖVALL *et al.*[3]. Polarity effects may include contributions due to hydrogen bonding, hydrophobic bonding, dipolar or ionic interactions, and dispersion forces. Consequently, it is extremely difficult to devise a system in which gel filtration is unaccompanied by other separation processes. For example, Sephadex G-25 in pure water, or LH-20 in ethanol, show a retention of aromatic and polyolefinic compounds, ascribed to dispersion forces[1,4]. Similarly, a lipophilic gel–benzene system, though showing good compatibility of polarity, may exhibit powerful hydrogen-bonding effects in addition to gel filtration.

In our investigations of terpenoids in green leaves of *Petasites hybridus*, the need arose for a method of isolating the sesquiterpenoids as a class. Gel filtration appeared to be a potentially effective technique, having regard to the fact that many accompanying lipids have considerably larger molecules[5]. Consequently, we undertook the present investigation of gel filtration systems involving the novel lipophilic derivative of LH-20 described by ELLINGBOE *et al.*[6,7].

EXPERIMENTAL

Preparation of gels

In preliminary work, Sephadex LH-20 was converted to the hydroxyalkoxypropyl-Sephadex of ELLINGBOE *et al.*[7,8]: 200 g of Sephadex LH-20 yielded 399 g of derivative. The olefin oxide used, Nedox 1114 (Ashland Oil and Refining Co., Minneapolis, Minn., U.S.A.), contains chains of 11–14 carbon atoms, and the gain in weight of the gel indicates 50% content by weight of hydroxyalkyl groups. This derivative may be designated N1114-50%-LH-20. (Other derivatives having different degrees of polarity can be made by using appropriate quantities of the olefin oxide[8]. Up to 60% by weight of substituents may be incorporated by subjecting N1114-50%-LH-20 to a second reaction step under the same conditions.) After solvent washing, the gels were dried *in vacuo* for 24 h. The dried product, unlike the starting material, was hydrophobic and waxy. The bead structure was intact*. Silanisation of glassware facilitated handling Sephadex gels of all types.

Material for high resolution columns was prepared using a fraction of Sephadex LH-20 (particles $< 53\ \mu$) that passed through a 300 B.S. mesh sieve. Sieving of N1114-50%-LH-20 was not possible because of aggregation of beads. This fraction contains some very fine ($< 10\ \mu$) and colloidal materials which influence separation adversely. Derivatives with narrower particle size distributions have been prepared (*cf.* ref. 3) using the continuous flow differential sedimentation method of HAMILTON[9]. Starting material in this case was Superfine Sephadex G-25. This yielded four fractions, with bead sizes (in the dry state) of 10–16 μ, 17–23 μ, 24–30 μ, and 30–40 μ. These fractions were subsequently converted to LH-20-type derivatives using a method described by ELLINGBOE *et al.*[7].

Packing of columns

Gels were allowed to swell overnight in the appropriate solvent. Before packing, the gel slurry was freed from occluded air by immersion for 5–10 min in an ultrasonic

* The beads may be damaged in the reaction if a magnetic or mechanical stirrer makes contact with the walls of the reaction vessel[8].

bath. Glass columns were fitted with an extension, and part by filled with solvent. Pre-swollen coarse gel was added, to cover the lower end of the column to a depth of a few millimetres, and the gel slurry was added in one operation. A steady flow of solvent was started, and the gel allowed to settle under continuous vibration. Under these conditions, sedimentation of gel lasted 6 h and bed volume was stabilised after 24 h. No additional pressure was applied. It was essential for even packing that the column was vertical during settling of the gel.

After 24 h, the column had passed solvent to about three times the bed volume. A filter paper was placed on top of the gel. This protected the surface against mechanical disturbance, and appeared to improve the distribution of sample on the bed. Solvent washing was continued for a further 24 h before analytical work was commenced. Gel packing factors (volumes of column occupied by 1 g of swollen gel) are given for solvents suitable for gel filtration in Table I.

Columns for high-resolution chromatography were made up with a view to minimising the effect of dead spaces on peak broadening. Glass tubing 9 mm I.D. was fitted to a standard Teflon tubing connector (Glenco No. 3020 1/2 in. to B) via a collar that held fine Teflon fabric, which acted as a bed support, and showed less

TABLE I

VOLUME OF COLUMN PACKED BY 1 g OF N1114-50 %-LH-20 AFTER SWELLING
Solvent systems suitable for gel filtration behaviour.

Solvent	*Gel packing factor (volume packed/g of dry gel)*
Benzene	3.75[a]
Tetrahydrofuran	3.71[a]
Chloroform	4.43
1,2-Dichloroethane	3.16
Benzene–isopropanol (75:25)	3.78[a]
Cyclohexane–isopropanol (75:25)	4.12

[a] Interchange of these solvents on-column is permissible.

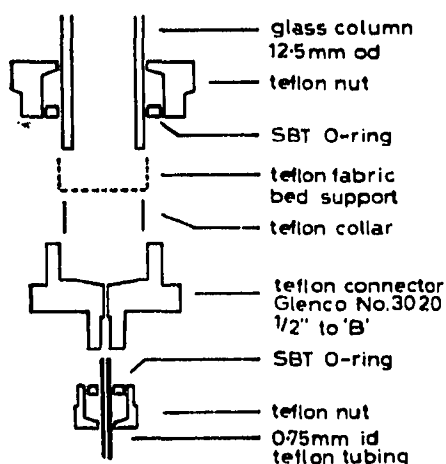

Fig. 1. Column end fitting. Teflon connector Glenco No. 3020 from Glass Engineering Co., Inc., Houston, Texas, U.S.A. Teflon fabric bed support 0659 SR25/45 from Pharmacia Fine Chemicals. O-rings cut from solvent-resistant rubber sheet type SBT, Esco (Rubber) Ltd., London.

tendency to clog than porous glass or Teflon discs. The connector was attached directly to the fraction collector by Teflon tubing (Fig. 1). As accurate determination of S.E.V. (see below) depended on accurate measurement of bed volume, such columns were calibrated volumetrically before packing. The bed volume could then be measured at any time to allow for variations arising from changes in temperature or flow rate.

Sample handling techniques

Samples of reference compounds were applied as test mixtures, of one to ten components, containing 0.05–10 mg (commonly 0.5 mg) of each component in a total of 0.1 ml of the eluting solvent: these quantities were well within the capacity of each column. Elution and collection of fractions was carried out on the BTL Chromafrac system using a peristaltic pump to deliver highly reproducible fraction volumes. Vinescol 23 fluorinated rubber tubing (Esco (Rubber) Ltd., London), used in the pump, was resistant to benzene and other solvents. All other fittings were glass or Teflon.

Samples were applied to the top of the column without mixing, by a careful procedure. Excess solvent was allowed to drain into the bed, and sample applied to the moist filter paper protecting the bed surface. The sample was washed below this surface by dropwise addition of 0.1 ml solvent before elution was started. This has been found to be the most reproducible method of sample application for analytical purposes.

The elution of samples was examined by collection of fractions (0.1–0.5 ml) and quantitative GLC, allowing separate determination of partly resolved components. Involatile compounds (plant pigments, glycerol esters and natural sterol esters) required quantitative TLC assay.

Retention data are recorded as the Standard Elution Volume, S.E.V.[5]. This is defined as the ratio between measured elution volume and total column volume, multiplied by 100. S.E.V. is a dimensionless quantity, numerically equal to 'percentage of total column volume'[3,7,8]. This serves to correlate results from different sources by reducing them to a standard form.

RESULTS AND DISCUSSION

The lipophilic dextran N1114-50%-LH-20 has been investigated in two solvent systems, *viz.* benzene, and benzene–isopropanol (75:25). Elution data are recorded in Tables II–V, and certain regularities are illustrated in Fig. 2 (*cf.* also Fig. 5). Molecular shape has a very marked influence on the effective molecular size for gel filtration: linear compounds tend to be eluted earlier than branched or cyclic molecules. Members of a homologous series show a progression in S.E.V. values that is related to the logarithm of the molecular weight: this is more clearly exposed in Fig. 3 for the *n*-alkane series.

A detailed examination of the benzene–gel system (Tables II and III and Fig. 2) shows that compounds having a free hydroxyl group are retarded on the gel. Elution of such compounds occurs later than would be allowed by the purely steric mechanism of gel filtration, for which the limiting value is about 80 S.E.V. Other polar groupings, such as the carbonyl groups in progesterone, do not affect elution in the same way.

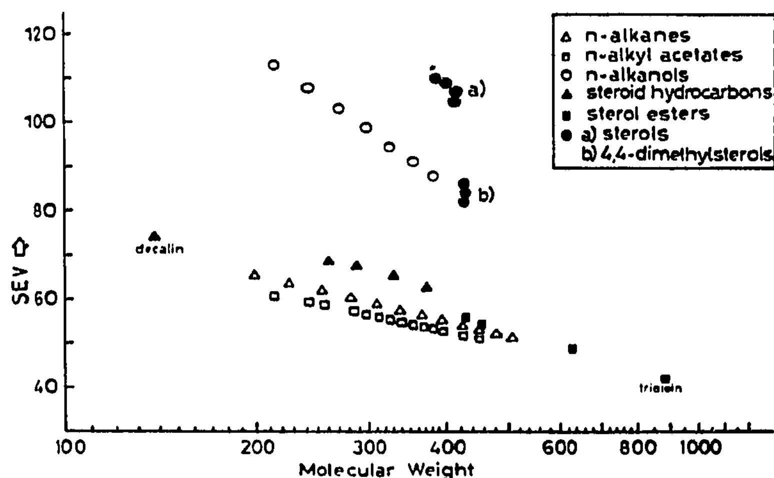

Fig. 2. Relationship between Standard Elution Volume (S.E.V.) and molecular weight for the N1114-50%-LH-20/benzene system. Cyclised molecules (solid markings) tend to have higher S.E.V. values for a given molecular weight than straight-chain types (open symbols) except where steric hindrance of hydroxyl groups influences retention, *e.g.* the 4,4-dimethylsterols.

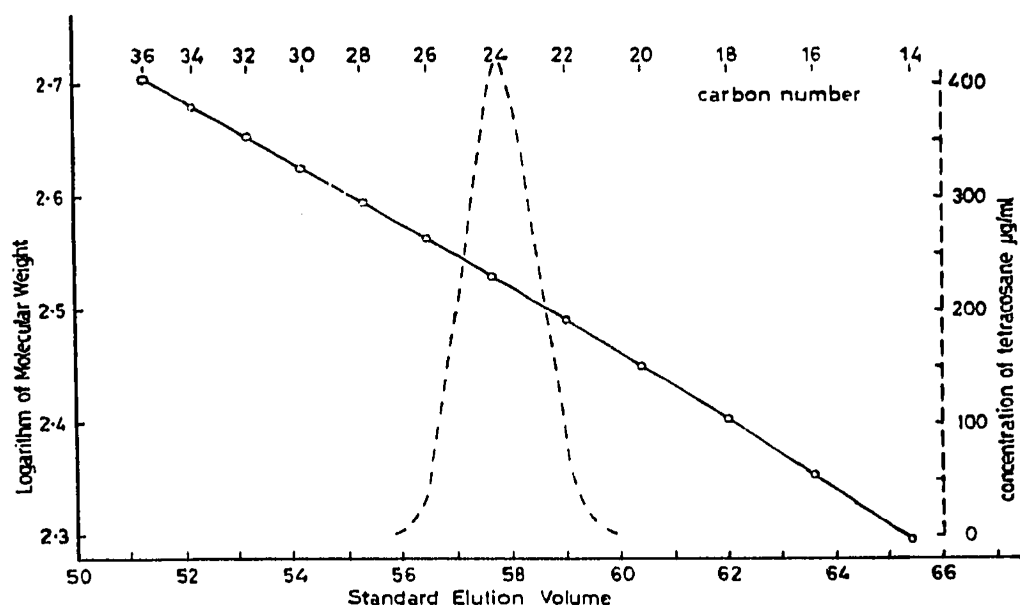

Fig. 3. Elutions of homologous series from N1114-50%-LH-20/benzene columns. *n*-Alkanes display an almost linear relationship between S.E.V. and the logarithm of the molecular weight. The slope in the region from C_{24}–C_{36} is such that 10% change in molecular weight (Δ log mol. wt. = 0.041) corresponds to a difference of 1.5 units in S.E.V. The broken line indicates the elution profile of tetracosane from the 100 cm column, showing the degree of overlap with neighbouring alkanes.

It appears that a hydrogen bonding effect is operating, with the gel acting exclusively as the basic component. In accord with this, retention of hydroxylic compounds is related to the environment of the hydroxyl groups (Table III). The *n*-alkanol series shows progressive gel filtration behaviour superimposed on the retention due to the hydroxyl group. Phenols are more strongly retained than aliphatic alcohols, and carboxylic acids are eluted too slowly for practical use under these conditions. There is no retention effect associated with aromaticity. Esters and ketones are eluted

TABLE II

S.E.V. VALUES OF NON-HYDROXYLIC COMPOUNDS ON THE Ni114-50%-LH-20/BENZENE SYSTEM
Column: 100 × 0.9 cm I.D. Flow: 6 ml/h. HETP: 0.14 mm. Operating temperature: 18°.

Compound	*S.E.V.*	*Mol. wt.*	*Compound*	*S.E.V.*	*Mol. wt.*
n-Hexatriacontane	51.3	506	α-Amyrin acetate	57.1	468
n-Tetradecane	65.4	198	Stigmasteryl acetate	55.2	454
Methyl lignocerate	53.4	382	Cholesteryl TMSi	55.9	458
Methyl behenate	54.5	354	Cholestan-3-one	60.0	386
Methyl arachidate	55.6	326	Cholest-4-ene-3,6-dione	58.6	398
Methyl stearate	56.8	298	Cholesteryl chloride	59.7	404.5
Methyl myristate	59.5	242	Progesterone	61.3	314
Methyl laurate	60.8	214			
			Isopetasin	56.9	316
Cholesteryl palmitate	49.0	624	Isopetasyl acetate	59.1	276
Cholesteryl butyrate	54.5	456	Isopetasone	61.3	232
Cholesteryl acetate	56.3	428	S-Petasin	54.2	334
Cholesteryl benzoate	54.0	490			
Cholestane	62.7	372	Tetralin	71.0	132
Coprostane	62.6	372	Decalin	73.8	138
5α-Cholane	65.3	330	Naphthalene	73.2	128
5α-Pregnane	67.7	288			
5α-Androstane	68.5	260			

TABLE III

S.E.V. VALUES OF HYDROXYLIC COMPOUNDS ON THE Ni114-50%-LH-20/BENZENE SYSTEM
Column: 100 × 0.9 cm I.D. Flow: 6 ml/h. HETP: 0.14 mm. Operating temperature: 18°.

Compound	*S.E.V.*	*Mol. wt.*	*Compound*	*S.E.V.*	*Mol. wt.*
Ceryl alcohol	88.1	382	Phytol	93.5	296
Lignoceryl alcohol	91.3	354	*trans,trans*-Farnesol	97.7	222
Behenyl alcohol	94.6	326	*cis,trans*-Farnesol	93.5	222
Arachidyl alcohol	99.0	298	Nerolidol	77.7	222
Stearyl alcohol	103.3	270	Isopetasol	119.6	234
Cetyl alcohol	108.2	242			
Myristyl alcohol	113.2	214	Methyl deoxycholate	156	406
			Methyl hyodeoxycholate	236	406
Cholesterol	110.2	386	Methyl ursodeoxycholate	134.5	406
Lanosterol	82.3	426	Methyl chenodeoxycholate	156	406
Dihydrolanosterol	84.5	428			
Cycloartenol	86.5	426	Estrone	360	270
Stigmasterol	105.1	412			
β-Sitosterol	107.4	414			
Campesterol	109.2	400			

earlier than might be expected on the basis of molecular weight. This effect was first observed by ENEROTH AND NYSTRÖM[10] for ketonic steroids on other lipophilic gels, in chloroform or methylene chloride, and has been illustrated further by SJÖVALL *et al.*[3].

On addition of an alcohol to the eluting solvent, hydroxylic compounds are selectively displaced from the gel (presumably through competitive hydrogen bonding): other compounds are not affected to any great extent (Fig. 4). At a level of 25 % isopropanol in benzene, there is no observable polarity effect for cholesterol, nor for any

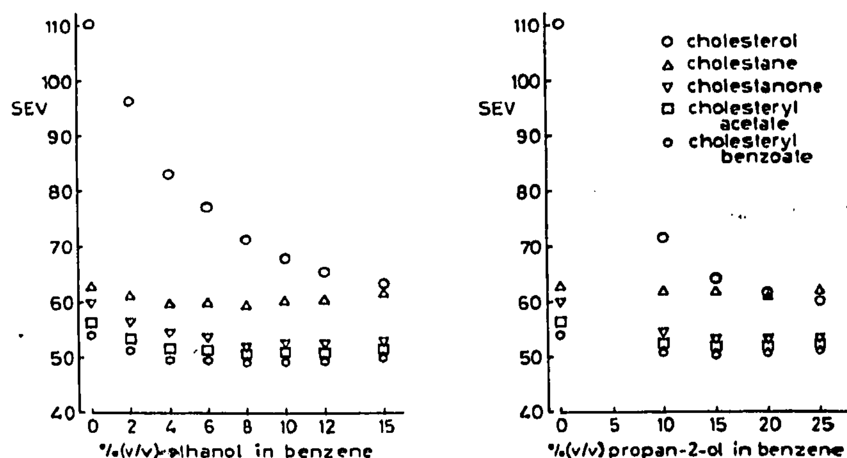

Fig. 4. Influence of alcohols in eluting solvents on the elution of cholestane derivatives.

TABLE IV

S.E.V. DATA FOR THE N1114-50%-LH-20/BENZENE–ISOPROPANOL (75:25) SYSTEM

Δ (S.E.V.) refers to [S.E.V. for benzene (Tables II and III) — S.E.V. for benzene–isopropanol]. Column: 100 × 0.9 cm I.D. Flow: 6 ml/h. HETP: 0.17 mm. Operating temperature: 18°.

Compound	*S.E.V.*	Δ *(S.E.V.)*	*Mol. wt.*	*Compound*	*S.E.V.*	*Mol. wt.*
n-Hexatriacontane	50.3	1.0	506	5α-Cholan-24-ol	63.2	346
n-Tetradecane	64.0	1.4	198	5α-Cholane-3α,24-diol	59.3	362
Octacosyl acetate	48.1	3.1	452	5α-Cholane-3α,12α,24-triol	59.9	378
Myristyl acetate	55.2	3.7	256	5α-Cholane-3α,7α,12α,24-tetrol	59.2	394
Ceryl alcohol	53.9	24.2	382			
Myristyl alcohol	62.2	51.0	214	Lithocholic acid (3α-OH)	58.6	376
Lignoceric acid	54.2	—	368			
Lauric acid	63.0	—	200	Deoxycholic acid (3α,12α-OH)	59.9	392
				Hyodeoxycholic acid (3α,6α-OH)	56.0	392
				Ursodeoxycholic acid (3α,7β-OH)	56.0	392
Cholesteryl acetate	53.3	3.0	428	Chenodeoxycholic acid (3α,7α-OH)	59.6	392
Cholesteryl benzoate	52.2	1.8	490			
Cholestan-3-one	54.5	5.5	386	Cholic acid (3α,7α,12α-OH)	59.4	408
Cholesterol	60.8	49.4	386			
Cholestane	62.8	−0.1	372	Gibberellin A_1	64.5	348
Coprostane	62.6	0	372	Gibberellin A_3	64.5	346
5α-Cholane	65.9	−0.4	330	Gibberellin A_4	61.6	332
5α-Pregnane	68.2	−0.5	288	Gibberellin A_5	59.1	330
5α-Androstane	69.3	−0.8	260	Gibberellin A_7	61.6	330
				Gibberellin A_9	56.8	316
Stigmasteryl acetate	52.1	3.1	454			
Stigmasterol	59.2	45.9	412			
Lanosterol	58.5	23.8	426			
Isopetasin	50.3	6.6	316			
Isopetasyl acetate	51.1	8.0	276			
Isopetasone	53.1	8.2	232			
Isopetasol	58.2	61.4	234			

of the more polar compounds studied. Free acids are not separated from their methyl esters. Higher levels of alcohol give rise to reversed-phase partition[7]. Data for the system benzene–isopropanol (75:25) are cited in Table IV. Non-hydroxylic compounds behave substantially as in the pure benzene system, but polar compounds no longer show distinctive behaviour (Fig. 5). No compound has been found to be retained longer than 80 S.E.V., and this is the nearest to ideal gel filtration encountered in the present study. The most apparent abnormality is the early elution of carbonyl compounds. A carbonyl group is equivalent to about eight methylene units in this system.

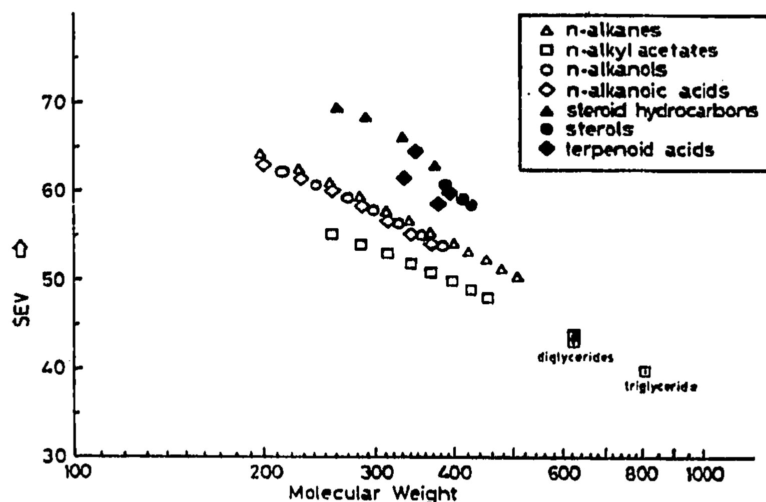

Fig. 5. Relationship between S.E.V. and molecular weight for the system N1114-50%-LH-20/benzene–isopropanol (75:25).

Application of gel chromatography to biological separations

While deviations from ideality are substantial in the systems described, molecular size is clearly a major factor determining elution. Group separations, *e.g.* of lipids from lipoproteins, or glycerol esters from simple terpenoids, are thus potentially feasible. Gibberellins, for example, have closely related structures, and in gel filtration systems this results in similar S.E.V. values. The system benzene–isopropanol (75:25) elutes these compounds as a composite peak (Fig. 6). A correlation of elution data for

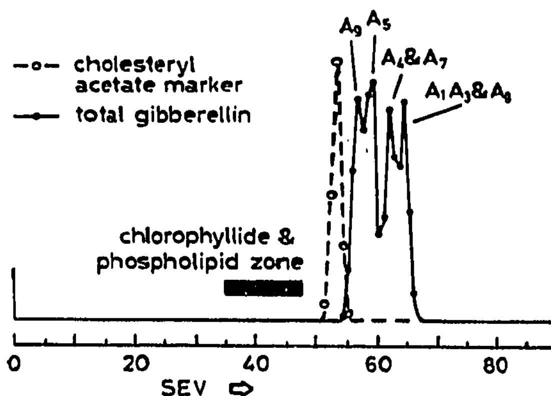

Fig. 6. Elution pattern of gibberellin mixtures in N1114-50%-LH-20/benzene–isopropanol (75:25).

TABLE V

CORRELATION OF S.E.V. DATA FOR COMMON LIPIDS AND NATURAL PRODUCTS

Systems: A = N1114-50 %-LH-20/benzene. B = N1114-50 %-LH-20/benzene–isopropanol. (75:25) C = LH-20/benzene–ethanol (1:9).

Compound	*System*		
	A	*B*	*C*
Triolein	42.2	—	52.0
Tripalmitin	43.2	39.8	—
Lecithin[a]	—	36.8	—
Phosphatidic acid[a]	—	43.8	—
Distearin (2-OH)	—	43.2	—
Distearin (3-OH)	—	44.0	—
Monostearin (1,2-diOH)	280	55.4	—
Chlorophyll *a*	45.0	—	73.5
Chlorophyll *b*	44.8	—	70.0
β-Carotene	47.4	—	70.0
Squalene	52.0	51.0	58.5
β-Sitosterol	107.4	59.4	69.0
Cholesterol	110.2	60.8	70.0
Lanosterol	82.3	58.5	68.5
Petasin	56.9	50.3	76.5
S-Petasin	54.2	—	80.0

[a] These samples were not homogeneous.

compounds of biological origin is given in Table V. On unmodified Sephadex LH-20 (ref. 4), retention of aromatic compounds does not allow the separation of involatile compounds from those suitable for gas chromatography. This is best achieved by the N1114-50 %-LH-20/benzene system, which eliminates compounds of high molecular weight by elution in early fractions, and retains compounds of excessive polarity. A polar fraction so retained may be eluted subsequently by washing through with solvent containing alcohol. This effectively purges the column, which may then be re-used after equilibration with benzene.

Column performance

Columns were prepared with particular attention to high efficiency. The sieving of Sephadex LH-20 was most important in this respect, together with the design of the actual chromatographic system. Theoretical plate heights have been calculated from the elution of cholesteryl acetate[5] (Fig. 7). For standard columns (100 × 0.9 cm I.D.) 7000 plates have been obtained, corresponding to a HETP of 0.14 mm. Commercial Sephadex LH-20, unsieved, gives a HETP of 0.5 mm, and material fractionated by sedimentation has been reported[3] to give a HETP in the region of 0.1 mm. The elution of cholesteryl acetate was monitored by the collection of fractions (0.1 ml) and quantitative GLC: 25 determinations were required to construct each peak in Fig. 7.

Peaks eluted from dextran gels in general appear to be Gaussian in form, with little tailing. Tailing has been investigated by application of a high loading (Fig. 7). GLC analysis of the effluent was able to detect 10 p.p.m. of the applied sample:

30 p.p.m. was found in a fraction 3 S.E.V. units from the peak centre, and none was detectable at 3.2 S.E.V. units. These results have been substantiated using 30 μCi [24-^{14}C]cholic acid, which demonstrated little long-term retention of radioactivity, and minimal radioactive bleed after the sample had passed (Fig. 8). This capability is essential for efficient fractionation of radioactive extracts obtained in lipid metabolic studies.

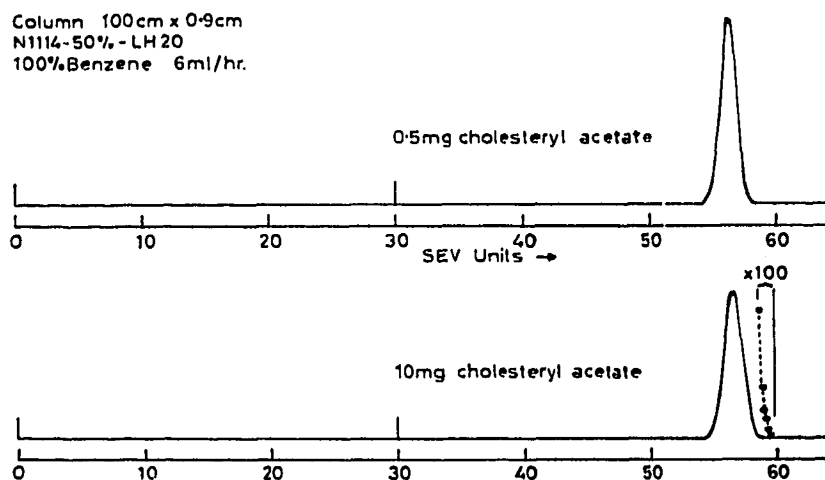

Fig. 7. Elution profile of cholesteryl acetate from N1114-50%-LH-20/benzene. The upper profile has a peak width at baseline of 2.7 S.E.V. units from which the HETP of 0.14 mm may be derived[11]. The lower profile shows the effect of a higher loading and a 100-fold scale expansion (broken line) illustrates the absence of serious 'tailing'.

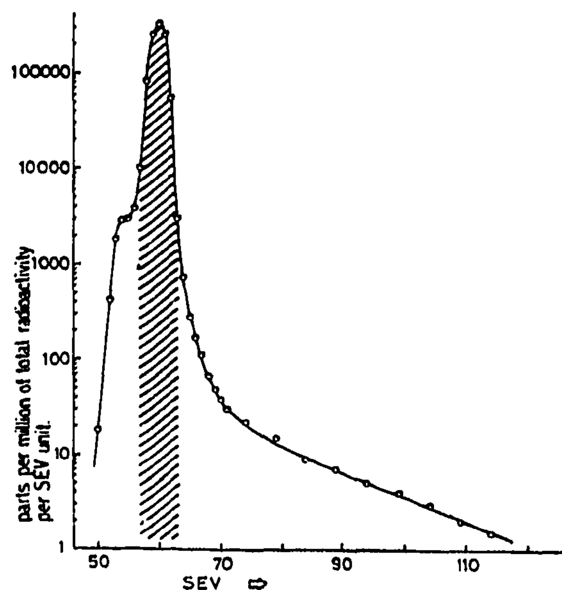

Fig. 8. Elution of 30 μCi [24-^{14}C]cholic acid (200 μg sample) from N1114-50%-LH-20/benzene-isopropanol (75:25). No inactive carrier was added to the sample. Recovery was measured as 102% of total activity administered, 62.28 × 10^6 d.p.m., 101% falling in the shaded region. The logarithmic scale permits an unbroken curve to be plotted to 1 p.p.m. of total activity. The hump at S.E.V. 54 represents an impurity not separable by TLC (0.7% of total activity).

Theoretical aspects

The theoretical aspects of gel chromatography have been discussed by GIDDINGS AND MALLIK[11], and zone dispersion in particular by BILLMEYER *et al.*[12]. HEITZ AND ČOUPEK have investigated experimental factors describing zone dispersion[13]. Factors determining separation have been considered by FLODIN[14] and SJÖVALL *et al.*[3]. It is observed that the HETP varies with the nature of the solute[12] as well as with the expected operating factors such as flow rate, particle diameter[3], and temperature[8]. In this investigation it has been found that for gel filtration processes, peak width is independent of elution volume (Table VI) and this result has also been reported by BOMBAUGH *et al.*[15] using polystyrene gels. Polarity partition processes follow the normal behaviour associated with chromatography.

TABLE VI

ZONE BROADENING EFFECTS IN GEL FILTRATION

Peak widths[10] (4σ) of *n*-alkanes on the N1114-50%-LH-20/benzene system.

n-Alkane	*S.E.V.*	4σ	*n-Alkane*	*S.E.V.*	4σ
C_{34}	52.2	2.6	C_{22}	59.0	2.6
C_{32}	53.2	2.6	C_{20}	60.4	2.9
C_{28}	55.3	2.7	C_{18}	62.0	2.8
C_{26}	56.5	2.9	C_{16}	63.6	2.7
C_{24}	57.7	2.8	C_{14}	65.4	2.6

Compared with any process dependent on polarity, gel filtration is a weak method of separation. High column efficiencies are essential, and must be coupled with a collection or detection system appropriate to the small separation volumes frequently obtained. In some respects, dextran derivatives are considerably more effective than polystyrene media. They are also well suited to the technique of 'recycling chromatography'[16] as demonstrated by NYSTRÖM AND SJÖVALL[17], who obtained, in this way, efficiencies of over 50000 theoretical plates using methylated Sephadex on 60×2.5 cm columns. N1114-50%-LH-20 separates over a molecular weight range from 100 to 2000, and 30000 plates would allow separation of compounds differing by 10% in molecular weight. A similar separation on a polystyrene gel required 180000 plates in a column of 160 ft.[15].

Practical aspects of gel chromatography

The 80 S.E.V. limit for gel filtration has many advantages in the application of gel chromatography to analytical liquid chromatography. Compounds are eluted more rapidly than in other techniques, for a given flow rate. Secondly, the column has no tendency for prolonged retention of samples, and may be used repeatedly. Re-use of a column is one of the major factors in the development of analytical chromatography. Reproducibility in analytical work is more attainable when there is no variation in column parameters from one trial to another. The results listed in Tables II–V were determined on a single column. S.E.V. values could be measured directly to a standard error of ± 2 %, and by correlation[5] with cholesteryl acetate (S.E.V. 56.3), to ± 0.5 %. As a corollary to reproducibility, predictability of results is also extremely good, and S.E.V. values may be taken from a qualitative analysis and used to estimate

actual elution volumes in a subsequent preparative separation. Gel chromatography is well adapted to preparative work, and separation is not affected by high sample loadings. Fig. 7 shows two peaks, at loadings of 0.5 mg and 10 mg respectively, without increase of peak width for the larger sample. The limit for a 9 mm column is about 30 mg, when peaks may be 10 % broader. Higher loadings may be tolerated if separation factors are sufficiently high. Fig. 9 compares loadings of 6 and 250 mg/cm^2; peak broadening is due mainly to the large sample volume required. The inert chemical nature of the modified dextran is another asset in preparative work. Recovery of compounds has been found quantitative: the most satisfactory evidence has been derived from studies of [^{14}C]cholic acid (Fig. 8), and of [^{14}C]cholesterol, which yielded similar results. It appears that gel chromatography may be generally preferable in the separation of labile substances, and biological extracts may be applied directly, without conversion to derivatives.

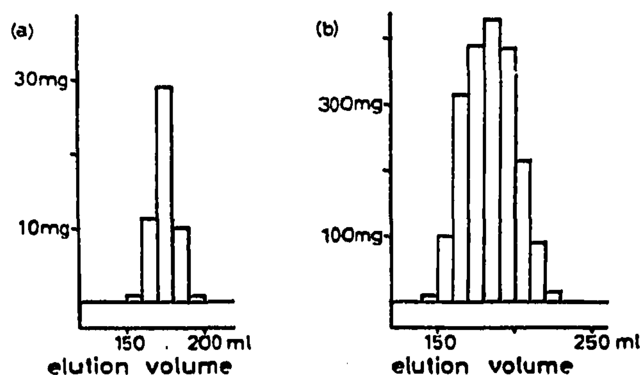

Fig. 9. Effect of high loading in preparative gel chromatography. Column 37 × 3.2 cm Sephadex LH-20 in chloroform; sample diphenyldiselenide (supplied by Dr. D. D. MACNICOL). (a) 50 mg in 1 ml chloroform; (b) 2 g in 25 ml chloroform.

CONCLUSION

While it is apparent that the technique of gel filtration in lipophilic solvents does not give the same separating power as GLC, the method may be developed as a means of group separation for biological extracts. Gel chromatography in general has advantages of versatility, high efficiency and reproducibility. The inert chemical nature of the gel means that there is little or no irreversible adsorption of sample, allowing sensitive samples to be analysed, and permitting repeated use of columns. Conversion of polar samples to derivatives is not necessary, as solvent systems may be employed that are independent of sample polarity. Liquid chromatography is convenient for preparative work, and gel chromatography has in addition to the above advantages a high sample loading capacity.

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