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NEW USES FOR MOLECULAR-SIZE EXCLUSION CHROMATOGRAPHY

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SUMMARY

The discovery of new packing materials for high-speed molecular-size exclusion has brought this mode of liquid chromatography to a level of importance equal to that of the other three modes. Substantial reductions in the time of analysis, column lengths and column diameters, coupled with improved analytical results, yield considerable improvements when applied to proven methods and make possible many new applications using molecular-size exclusion chromatography as a general analytical technique.

New applications are demonstrated by actual chromatograms and include analyses such as: (1) molecular-weight distributions on 2 m × 2.6 mm I.D. columns using 10–20 ml of solvent, giving results in 20 min with ± 5% reproducibility; (2) fractionation of blood plasma; (3) analysis of drugs in blood plasma without the need for sample clean-up; (4) analysis of alcohol in beverages; and (5) analysis of decomposition products of drugs. Other important applications are also included.

All of these applications are carried out on 0.5–2 m × 2.6 mm I.D. columns using either aqueous or organic solvents. The analyses are completed in 10–20 min using 10–20 ml of solvent and all applications are quantitative. Plate counts of 1000 plates/m are normal.

The above work demonstrates that with the advent of new high-speed molecular-size exclusion packings, molecular-size exclusion takes on a new importance as a general analytical technique and should be given equal consideration when choosing the mode of operation for solving a particular separation problem.

INTRODUCTION

Gel permeation chromatography (GPC) or, to use the more contemporary term, molecular size exclusion, has long been an extremely useful technique for analyses of any materials that differ from each other in molecular size. These materials are of many types, including biological macromolecules, polymers, organic and inorganic molecules. The problem with this seemingly useful technique is that it currently requires long analysis times, large volumes of solvents, long, wide-bore columns, and a variety of different types of column packing materials in order to handle different samples and solvents. Advances in column technology and the application of liquid

chromatographic (LC) techniques to GPC have been successful in eliminating most of the above problems concerning molecular-size exclusion and have advanced the science of molecular-size exclusion to the point where it takes on a new importance as a general analytical technique and should be given equal consideration when choosing the mode of operation for solving a particular separation problem.

The topic of this paper is a new advance in the field of molecular-size exclusion chromatography. This advance is in the form of a new range of high-efficiency column packing materials for high-speed molecular-size exclusion chromatography. These materials consist of rigid high silica glasses with a network of interconnected pores of narrow pore-size distribution. The packing materials have been selectively ground and sized and then deactivated to eliminate any irreversible adsorption. These new packing materials are commercially available from the Perkin-Elmer Corporation under the trade name Vit-X. This paper discusses the results that evolved from the research carried out on the new packing materials for molecular-size exclusion chromatography.

EXPERIMENTAL

Materials and apparatus

Polystyrene standards were obtained from $A_R R_O$ Laboratories, Inc., Joliet, Ill., U.S.A. Human blood plasma samples were supplied by Dr. M. Reidenberg of Temple University School of Medicine, U.S.A. Chemical standards were obtained from Applied Science Laboratories, State College, Pa., U.S.A. The liquid chromatograph and Vit-X columns were supplied by Perkin-Elmer Corp., Norwalk, Conn., U.S.A.

Packing the columns

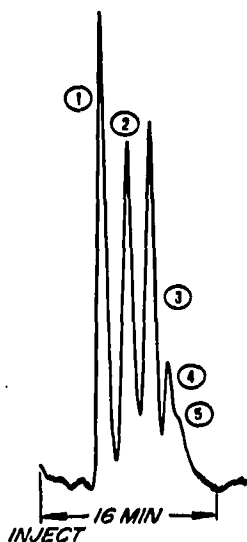
The packings were dry packed in 2.6 mm I.D. stainless-steel columns by adding 100 mg at a time to the column and tapping the column on the floor. Twenty 2-in. free-fall taps per 100 mg were used until the column would accept no more packing. The columns were then pressurized with liquid from the chromatograph and inspected. No settling was observed.

The chromatographic systems with packed columns were allowed to equilibrate when the columns were new and before every analysis that required a change of solvent, so as to allow any solvent held in the column pores to be flushed out. Equilibration was achieved when the baseline of the detector no longer drifted.

The instrument was a Perkin-Elmer Model 1210 liquid chromatograph. The features of the instrument that are significant for the molecular-size exclusion separations are: constant delivery pumps of 500-ml capacity (constant delivery is extremely useful in molecular-size exclusion work involving polymers where build-up of viscous polymers and residues on the column makes it difficult to obtain reproducible results when using constant pressure pumps); on-column septum injection as well as valve-loop injection; and ultraviolet (UV) and refractive index (RI) detectors.

RESULTS AND DISCUSSION

Initial work carried out in order to develop controlled mono-energetic adsorbents revealed that body porous adsorbents could be used as molecular-size ex-



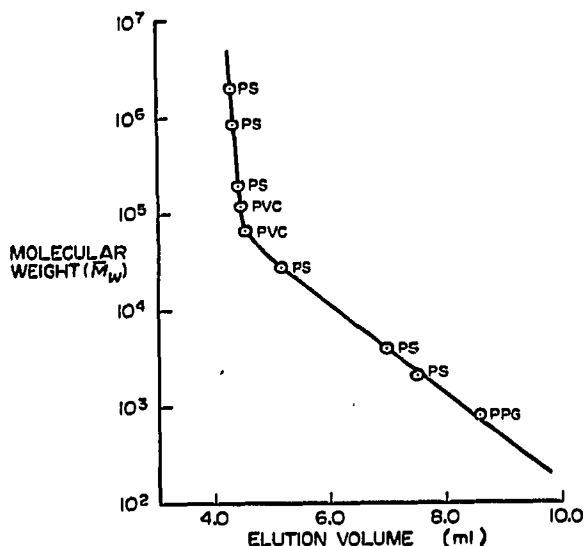


Fig. 1. Size exclusion calibration run on silica gel. Sample size, 200 μl, 1 μg/μl; column, 2 m × 2.6 mm; packing, silica gel, 36–44 μm; solvent, tetrahydrofuran; flow-rate, 0.82 ml/min; detector, RI; sensitivity, ×4. Peaks: (1) M_w = 20,000; (2) M_w = 4000; (3) M_w = 790; (4) acrylamide; (5) unknown.

Fig. 2. Calibration plot for silica gel in the size exclusion mode. Column: 2 m × 2.6 mm packed with silica gel. PS = polystyrene; PVC = poly(vinyl chloride); PPG = poly(propylene glycol).

clusion packings that exhibit improvements in efficiencies and resolution in comparison with currently used conventional methods. Figs. 1 and 2 demonstrate the molecular-size exclusion calibration performed on processed silica gel. It is interesting that while using the same column and merely changing the solvent, one can successfully change from one mode of LC (adsorption) to another (size exclusion). There is, however, a fundamental drawback in using silica materials, namely the great increase in back pressure due to a hydrophilic effect when using aqueous solutions in narrow-bore columns. These high back pressures are inconvenient.

We therefore investigated porous glass as an alternative to silica as a packing material. Previous work on porous glass suggested that it has many advantages over cross-linked polystyrene gels and agarose gels. Haller[1–4] reported on the development of porous glasses for use in molecular-size exclusion. The characterization and chromatographic properties of Corning porous glasses have been reported by Cooper *et al.*[5]. The basic advantages of porous glass over cross-linked polystyrenes are the capability of running with all types of solvents and the ability to operate at pressures above 7000 p.s.i., but a problem is that the plate heights reported for cross-linked polystyrene gels are much better than those for porous glasses. The main problem thus appeared to be the development of a series of porous glass packings that had the advantages of rigid glasses coupled with the increased efficiencies reported for polystyrene gels. A series of such packings has been developed (Table I) that are solvent resistant, capable of handling acidic and basic solutions and capable of being used at high pressures (above 7000 p.s.i.) without collapse of the packing.

TABLE I

CHARACTERISTICS OF VIT-X POROUS GLASS PACKINGS

Vit-X packing	*Particle size (μm)*	*Mean pore diameter (Å)*	*Pore volume (cm^3/g)*	*Pore distribution (± %)*	*Operating range in terms of molecular weight*
328	36–44	84	0.53	8.7	18–28,000
648	36–44	101	0.65	6.93	650–48,000
1068	36–44	171	0.78	5.6	1050–68,000
1195	36–44	210	0.82	5.7	1150–95,000
5150	36–44	321	1.21	9.0	5000–150,000
15300	36–44	660	0.82	5.7	15,000–300,000
40550	36–44	1206	0.8	7.4	40,000–550,000
120120	36–44	1933	0.87	6.8	120,000–1,200,000

Porous glass packings have been known to cause tailing of polar compounds owing to absorption by the free hydroxyl sites of the packing material. Cooper and Johnson[6] have shown that the absorption effect of porous glass packings can be eliminated by deactivation with hexamethyldisilazane. A slightly different technique was used, which yielded similar results, with our porous glass substrate. The deactivated packing did not show any chemical decomposition when it was treated with organic solvents and acidic and basic solutions.

The results with silica gel indicated that a particle size of *ca.* 35 μm with a narrow particle-size range (15 μm) would be useful. This range of particles can easily be dry packed by the free-fall tap method, thus making the packing of columns a quick and easy procedure that can be carried out by the chromatographer in the laboratory without any special equipment. The packing characteristics of this range of particles reduces the band spreading effects incurred by using larger particle sizes. The efficiencies obtained when using this particle-size range were found to be better than those previously reported for porous glass packings. This particle-size range also permits operation at lower pressures. For example, with a 2 m × 2.6 mm column, which is all that is necessary for a molecular-weight distribution, a solvent flow-rate of 1 ml/min generates a pressure of about 1000 p.s.i. It is felt that the increase in efficiency is due to the nature of the material used. The criterion of DiMarzio and Guttman[7] was applied in order to determine whether or not the pores in these glass particles were continuous or terminal. According to their criteria, the pores were found to be continuous. Better resolution was observed when using packings of large pore diameters than packings with small pore diameters. Comparisons were made of packings with small differences in pore diameters and the improvement in resolution was marginal and in most instances negligible. Only when comparisons were made of materials with significant differences in pore diameters measurable differences could be found. It is considered that the limited pore volume of packings with small pore diameters was responsible for the marginal differences in resolution.

Mass transfer is much more efficient on the porous glass material with continuous pores than it is on terminal-pore cross-linked polystyrene. This effect helps to account for the reduction in the time of analysis.

These new packing materials have a measured capacity factor, k', of 0.5 and

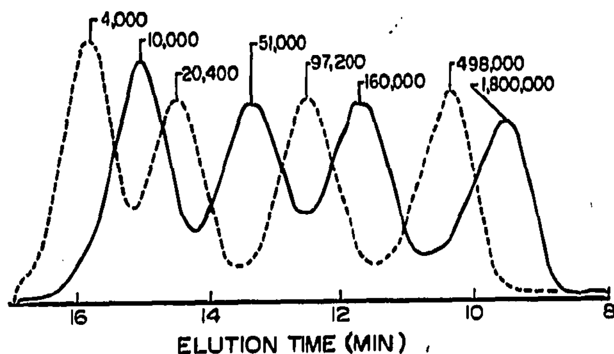


Fig. 3. Calibration curve on Corning porous glass. Sample size, 0.062 mg; column, 6.4 m × 2 mm; packing, 75, 175, 240, 370, 700, 1250 and 2000 Å; solvent, tetrahydrofuran; flow-rate, 1.39 ml/min; detector, DRI; sensitivity, ×2. (From *J. Chromatogr. Sci.*, 9 (1971) 65).

Fig. 4. Molecular-weight-distribution calibration run on Vit-X. Sample size, 3 μl, 10 μg/μl; column, 2 m × 2.6 mm; packing, 1 m Vit-X-1195, 1 m Vit-X-15300; solvent, tetrahydrofuran; flow-rate, 0.5 ml/min; detector, UV; sensitivity, 0.05 × 1. Peaks (polystyrene): (1) M_w = 2,610,000; (2) M_w = 498,000; (3) M_w = 200,000; (4) M_w = 20,800; (5) M_w = 4000 and 2100.

Fig. 5. Broad molecular-weight distribution. Sample size, 3 μl, 10 μl/μg; column, 2 m × 2.6 mm; packing, 1 m Vit-X-1195, 1 m Vit-X-15300; solvent, tetrahydrofuran; flow-rate, 0.5 ml/min; detector, UV; sensitivity, 0.05 × 1. Peak: polystyrene, M_w = 252,500.

an HETP of 1.5 mm measured on a monodisperse polystyrene of molecular weight 4000. An HETP of 0.1 mm for small molecules was measured using benzene.

In order to illustrate the improvements that are obtained, one can compare the results obtained by Gudzinowicz and Alden[8], as shown in Fig. 3, using their version of high-speed gel permeation chromatography on Corning porous glass, with a similar calibration run, shown in Fig. 4, using a Vit-X column system.

Fig. 5 shows the results for a polystyrene with a broad molecular-weight distribution. Calculations were based on the method described by Ezrin[9] and the results

TABLE II

BROAD MOLECULAR-WEIGHT DISTRIBUTION OF SAMPLE

$A_n = \frac{M_n}{Q} = 1679$; $A_nQ = M_n = 68{,}839$; $A_w = \frac{M_i^2N_i}{Q} = 6157$. $A_wQ = M_w = 252{,}455$; reported value of 252,500.

Counts	(Hi) height M_iN_i	Cumulative height	Cumulative weight percent	Chain-length (Å) $A_i = \frac{M_i}{Q}$	Number of particles $\frac{H_i}{A_i} = N_iQ$	$H_iA_i = \frac{M_i^2N_i}{Q}$
14	26	801	100	26,000	0.0010	676,000
15	64	775	96.75	14,000	0.0045	896,000
16	102	711	88.76	9700	0.0105	989,400
17	132	609	76.02	6900	0.0191	910,800
18	143	477	59.55	5000	0.0286	715,000
19	112	334	41.69	3600	0.0311	403,200
20	66	222	27.71	2600	0.0253	171,600
21	45	156	19.47	1850	0.0243	83,250
22	31	111	13.85	1350	0.0229	41,850
23	22	80	9.98	960	0.0229	21,120
24	16	58	7.24	690	0.0231	11,040
25	13	42	5.24	500	0.0260	6500
26	10	29	3.62	360	0.0277	3600
27	8	19	2.37	230	0.0347	1840
28	6	11	1.37	120	0.0500	720
29	5	5	0.62	40	0.1250	200
Total	801				0.4767	4,932,120

are shown in Table II. The important factor is that the molecular-weight distribution can be carried out on 2 m × 2.6 mm I.D. columns in approximately 20 min with approximately 15 ml of solvent. A more thorough study of the application of LC techniques to high-speed GPC has been made by Otacka[10]. The two advantages that are evident are: (1) the ability to use smaller amounts of materials and shorter analysis times gives an economic justification for using this method; and (2) the short times necessary to obtain results coupled with the possibility of even shorter times for obtaining mathematical results using data reduction equipment makes it possible for the first time to obtain data in sufficient time to permit the conditions applied in polymerization reactions to be changed before the reactions overrun the desired end-point.

Fig. 6 shows the results for two different samples of epoxy resins. The relative differences in the amount of high-molecular-weight polymer present can be seen by the increased shoulder present in curve 1, and the earlier elution time.

Fig. 7 shows similar runs for alkyd resins. Other polymers which have been run on these columns are polyurethanes, formaldehyde–urea resins, polyvinylpyrrolidone and many others, which suggests a broad applicability for these types of applications.

Other applications of this material are in the clinical field. Fig. 8 shows the fractionation of the UV-absorbing components of horse plasma, which provides a simple method of isolating and collecting the various components in plasma. It is also a method of determining the amounts of various components present, or the

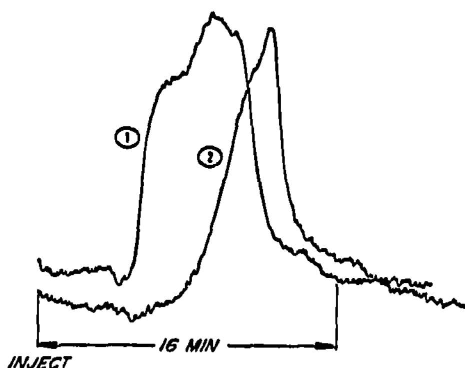


Fig. 6. Epoxy resins, samples 1 and 2. Sample size, 100 μl, 2 μg/μl; column, 1 m × 2.6 mm; packing, Vit-X-648; solvent, methyl ethyl ketone; flow-rate, 0.21 ml/min; detector, RI; sensitivity, ×2.

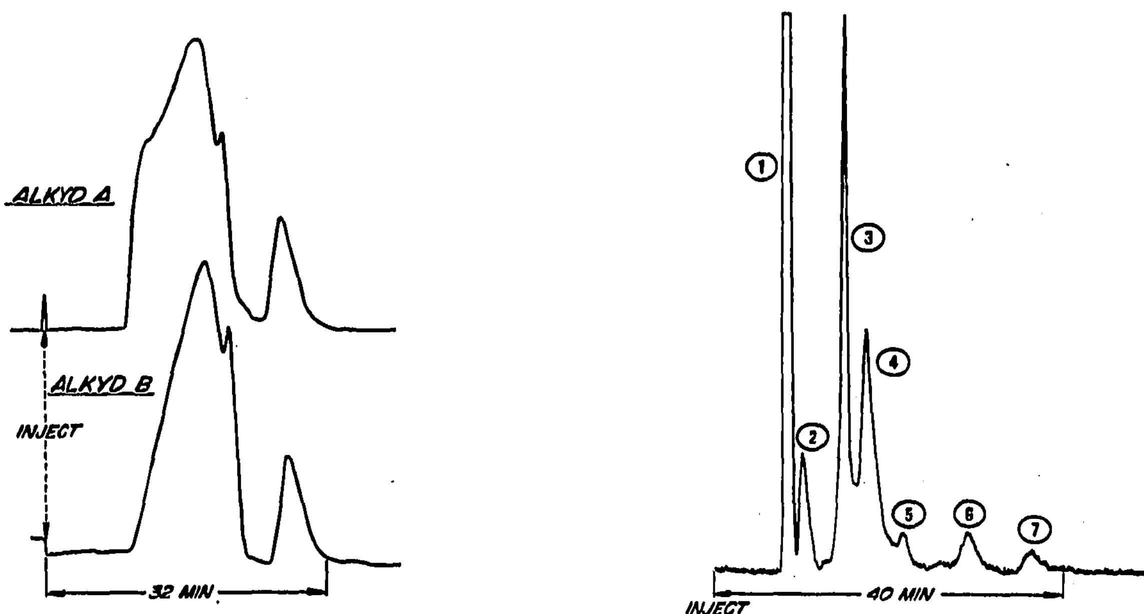
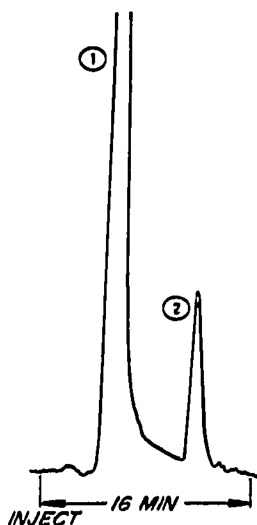


Fig. 7. Alkyd resins A and B. Sample size: 200 μl, 0.5 μg/μl; column: 2 m × 2.6 mm; packing: Vit-X-328; solvent: tetrahydrofuran; flow-rate, 0.42 ml/min; detector: UV; sensitivity, 0.1 × 1.

Fig. 8. Fractionation of horse plasma. Mode, molecular-size exclusion; column: 1 m × 2.6 mm; packing, 1 m Vit-X-1195, 1 m Vit-X-15300; sample size, 5 μl of plasma; solvent, distilled water; flow-rate, 0.5 ml/min; detector: UV; sensitivity, 0.1 × 1; chart speed, 7.5 in./h. Peaks: (1) γ-Globulin; (2) fibrinogin; (3) albumin; (4) unknown; (5) unknown; (6) unknown; (7) salts.

absence of such components. An example is the change in the ratio of component peaks due to presence of phenylbutazone metabolites. This technique can also be used to isolate various drugs from plasma or to analyze plasma to determine various drugs without the need for sample clean-up. Examples of this type of analysis are shown in Fig. 9 for the isolation of xanthenes from plasma and Fig. 10 for the isolation of bar-



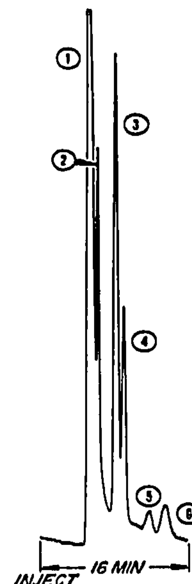


Fig. 9. Isolation of xanthines from blood plasma. Sample size, 100 μl, 10 ng/μl; column, 0.5 m × 2.6 mm; packing, Vit-X-328; solvent, distilled water; flow-rate, 0.42 ml/min; detector, UV; sensitivity, 0.05 × 1. Peaks: (1) Plasma components; (2) caffeine, theobromine and theophylline.

Fig. 10. Barbiturates in blood plasma. Sample size, 1 μl, 100 ng/μl; column, 1 m × 2.6 mm; packing, 1 m Vit-X-328; solvent, distilled water; flow-rate, 0.5 ml/min; detector, UV; sensitivity: 0.02 × 1. Peaks: (1) and (2) Plasma components; (3) phenobarbitol; (4) sodium diethylbarbiturate; (5) unknown; (6) caffeine.

bitol and phenobarbitol from plasma. An interesting analysis can be seen in Fig. 11, which demonstrates the ability to isolate and determine diphenylhydantoin (Dilantin; Parke, Davis & Co.).

An application of this technique to the pharmaceutical field is the analysis of antibiotic complexes, as depicted in Fig. 12. Many antibiotics are complexed with sugars to improve their absorption into biological systems, and once these drugs have been complexed, it is desirable to know their shelf-life and decomposition characteristics. This information can be obtained by size exclusion. The complex is a large molecule, and when it decomposes into the free drug and the sugar, the molecules of both of these components will be smaller than those of the original complex and hence the degree of decomposition can be readily determined.

Another novel application of molecular-size exclusion is in the analysis of components of beverages. Fig. 13 shows the separation of the various components in beer. The early eluting peaks are flavor components, the large broad peak is a distribution of sugar and the narrow, late-eluting peak is ethanol. Other beverage analyses have been carried out that demonstrate the ability of the method to determine caffeine in coffee, tea, cocoa, and soft drinks.

CONCLUSION

In conclusion, we can say that the advent of new high-efficiency molecular

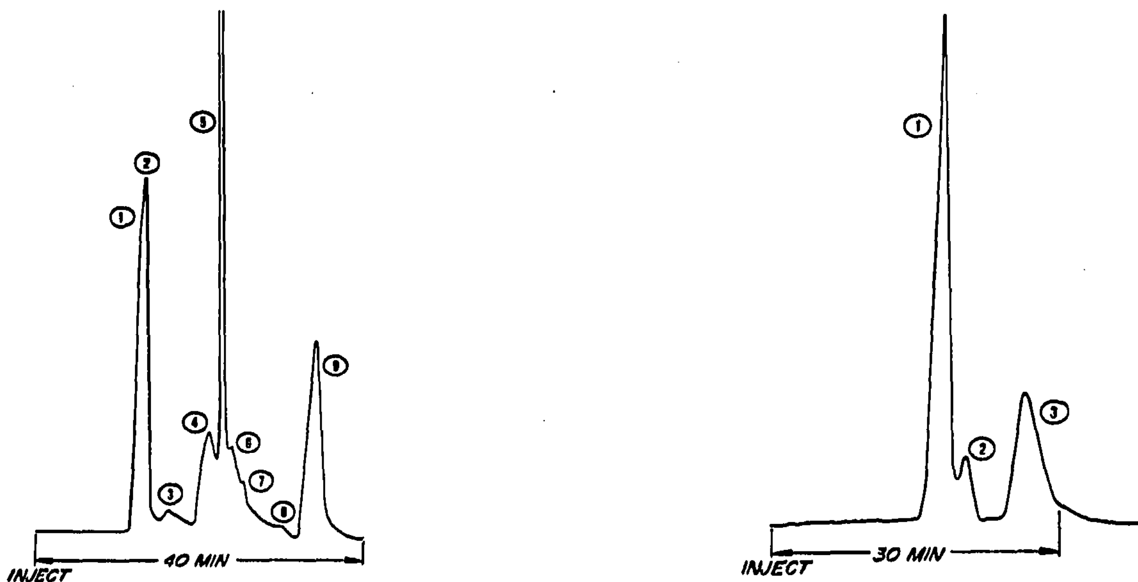


Fig. 11. Diphenylhydantoin in blood plasma. Sample size, 7 μl, 100 ng/μl; column, 3 m × 2.6 mm; packing, 1 m Vit-X-328, 1 m Vit-X-1195, 1 m Vit-X-15300; solvent, distilled water; flow-rate, 0.5 ml/min; detector, UV; sensitivity: 0.05 × 1. Peaks: (1)–(8) Normal plasma components; (9) diphenylhydantoin.

Fig. 12. Decomposition of antibiotic complexes. Sample size, 200 μl, 0.5 μg/μl; column, 2 m × 2.6 mm; packing, Vit-X-328; solvent, tetrahydrofuran; flow-rate, 0.42 ml/min; detector, UV; sensitivity, 0.1 × 1. Peaks: (1) Antibiotic sugar complex; (2) antibiotic decomposition fragment; (3) sugar decomposition fragment.

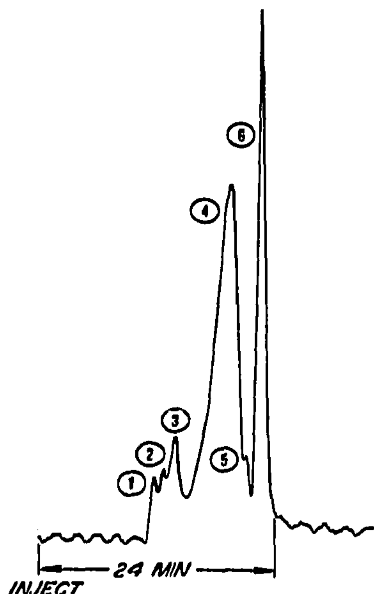


Fig. 13. Fractionation of beer. Sample size, 1.5 μl; column, 2 m × 2.6 mm; packing, Vit-X-328; solvent, distilled water; flow-rate, 0.48 ml/min; detector, RI; sensitivity, ×2. Peaks: (1)–(3) Flavor components; (4) sugars; (5) unknown; (6) ethanol.

size exclusion chromatography and the application of LC techniques to it has brought molecular-size exclusion to the foreground as a valuable analytical technique which reduces analysis times, column lengths and elution volumes. Molecular-size exclusion should be given equal consideration when choosing the mode of LC for solving a particular separation problem.

REFERENCES

1 W. Haller, *J. Chem. Phys.*, 42 (1965) 686.
2 W. Haller, *J. Chromatogr.*, 32 (1968) 676.
3 W. Haller, *Nature (London)*, 206 (1965) 693.
4 W. Haller, *Virology*, 33 (1967) 740.
5 A. R. Cooper, A. R. Bruzzone, J. H. Cain and E. M. Barrall, II, *J. Appl. Polym. Sci.*, 15 (1971) 571.
6 A. R. Cooper and J. F. Johnson, *J. Appl. Polym. Sci.*, 13 (1969) 1487.
7 E. A. DiMarzio and C. M. Guttman, *Macromolecules*, 3 (1970) 681.
8 B. J. Gudzinowicz and K. Alden, *J. Chromatogr. Sci.*, 9 (1971) 65.
9 M. Ezrin (DeBell and Richardson), personal communication.
10 E. Otacka, *J. Chromatogr. Sci.*, 76 (1973) 149.