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COATED-OPEN-TUBULAR CHROMATOGRAPHY WITH FLOW SEGMENTATION

II. EXPERIMENTAL STUDY AND OPTIMIZATION FOR SIZE-EXCLUSION SEPARATION

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SUMMARY

Preliminary experimental results are reported on the efficiency of segmented-flow liquid chromatography (SF-LC) as a function of experimental conditions. For agarose-coated tubes (size-exclusion mode) experimental H values are significantly larger than predicted by theory. This has been traced to a combination of hydraulic instability (unless special precautions are taken) and uneven coating of the stationary phase onto the walls of the separation tube. Further work should lead to a close agreement between theoretical and experimental separation efficiencies.

The use of SF-LC as a technique for sample pretreatment prior to high-performance liquid chromatography is described. Serum samples for analysis of certain therapeutic drugs are subjected to deproteinization and drug release by protein precipitation, followed by SF-LC separation of released drugs from the particulate material comprising the protein.

INTRODUCTION

In the preceding paper[1] a new form of capillary liquid chromatography was described: segmented-flow LC (SF-LC). A theoretical treatment of column efficiency (N and H values) in SF-LC was developed, and the practical consequences of this theory were discussed. Specifically, it appears that SF-LC will not compete in most cases with conventional packed-column LC, because column efficiency is orders of magnitude higher with packed columns. It was suggested, however, that SF-LC might prove useful in certain specialized applications, as for the pre-treatment of samples prior to their further analysis by conventional LC. This would be important in the development of fully-automated systems that allow pretreatment of the sample followed by LC analysis. In this connection SF-LC offers the following advantages:

(a) particulate-containing samples can be handled without danger of plugging the SF-LC system (which consists of an open tube of relatively large diameter: 0.5–1 mm);

(b) particulates can be efficiently separated as a group from soluble sample-species, using size-exclusion SF-LC; no replaceable filter elements or phase separation are required;

(c) SF-LC requires only very simple equipment and is run at low pressures (1–3 p.s.i.);

(d) SF-LC separation can be easily combined with other continuous flow (AutoAnalyzer™) operations; thus chemical reaction, dilution, evaporation-to-dryness, etc. can be carried out in conjunction with SF-LC separation as part of an overall pretreatment procedure.

This has suggested to us that SF-LC can be an important part of fully automated systems that combine sample pretreatment with LC separation and analysis — in a fully on-line mode, without operator intervention between introduction of a raw sample (such as human serum) into the system, and the printing out of final results at the end of the analysis. We will briefly explore this possibility in the present paper.

A further (and primary) aim of the present paper is to describe preliminary results aimed at verifying the theory of part I[1]. These studies have further identified certain practical problems which prevent SF-LC from being as efficient a separation tool as predicted by theory. We will also propose certain improvements in the practical use of SF-LC, so that separation performance can approach the levels predicted by theory.

EXPERIMENTAL

Apparatus

A schematic of the equipment used in the present study for carrying out SF-LC separations is shown in Fig. 1. It is similar to an apparatus previously described[2] for measuring solute dispersion during segmented-flow through uncoated glass tubing, but there are some important differences. The peristaltic pump used to create flow of mobile phase and injection of air-bubbles was variously a variable-speed AutoAnalyzer™ Pump I (in early studies) and an AutoAnalyzer™ Pump III (in later studies). Sample was introduced into the system by manually moving the liquid inlet line from a container of mobile phase (M) to sample (S) and back again. It was also possible in this fashion to introduce additional air-bubbles ("pecking") following the sample segment, to provide reduced dispersion of the sample as it moved through the pump (prior to air injection following the pump).

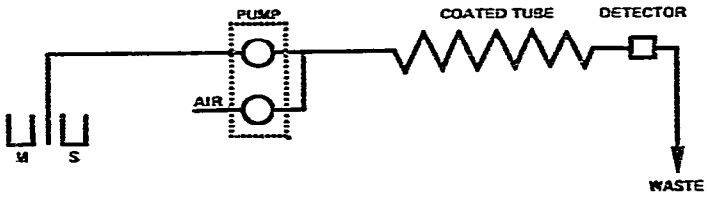


Fig. 1. Schematic of system used in present studies of band broadening in SF-SEC. M, mobile-phase container; S, sample container.

The coated tube was typically a 200 × 0.1 cm length of glass, tightly coiled with a diameter of 0.5 in. The stationary phase was porous, particulate silica in initial studies, but most of the work described here used tubes coated with agarose (3% solution in water) for size-exclusion chromatography (SEC) separation.

The detector was a transverse photodiode device constructed by us. It is similar in principle to the detector described in ref. 2, but of different construction. It was possible to monitor the tube effluent at 630 or 900 nm as it left the coated tube and passed into a straight length of 1-mm I.D. straight glass tubing. Alternatively, this device could be moved onto the inlet or exit portion of the coated tube itself, to monitor the sample as it entered or left the coated tube, without introducing any extra-column band broadening. The output of the detector is similar to that shown in Fig. 2, where both sample absorption and air-bubbles are indicated in the recorder output.

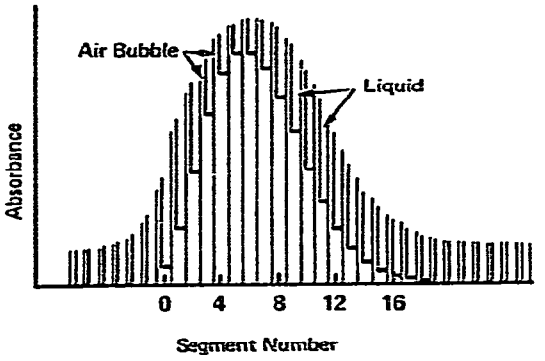


Fig. 2. Typical recorder output for sample band leaving SF-LC system.

Connections of the various elements of Fig. 1 were provided by standard Technicon SMAC™ fittings, which are compatible with 0.5–1.0-mm tubing. Injection of sample for the studies of band broadening in unsegmented-flow systems was done by direct injection after the pump, much as described in ref. 2.

The final chromatograms as illustrated in Fig. 2 were evaluated for both plate number N (and plate height H) and for retention k_{ap} (see ref. 1). Values of N were determined from bandwidths at half-height $w_{1/2}$(sec):

$$N = 5.54\,(t_R/w_{1/2})^2 \qquad (1)$$

The retention time t_R was calculated as $(1 + k_{ap})\,t_0$, where t_0 is the transit time (sec) of a bubble through the coated tube. The value of k_{ap} was determined from the position of the band center relative to the center of the original segment containing the sample (at the time of sample-injection). Thus, if the band displacement was x, k_{ap} was given as x/t_c. Values of k' were then determined from k_{ap}, as described in ref. 1, eqn. 3.

It was found that N values determined as above were essentially constant for other measurements of bandwidth (*e.g.*, at 10 or 20% of peak height). Band asym-

metry values (*e.g.*, as in ref. 3) were consistently around 1.7; *i.e.*, the bands were fairly symmetrical.

The experiments with silica or alumina-coated tubes used *n*-heptane as mobile phase, with control of the mobile-phase water content at 50% of saturation. It was also necessary, for stable column operation, to separately humidify the air used for segmentation at 50% relative humidity. Experiments with the agarose-coated tubes used 0.1% Plurafac A-39 (BASF Wyandotte) as surfactant in water for the mobile phase, and cupric chloride was used as solute unless specified otherwise. The surface tension of the resulting solution was about 28 dynes/cm. All experiments were carried out at ambient temperature (about 22°).

In the measurement of the retention of proteins (which do not absorb at 630 or 900 nm), an alternative was employed. The column effluent was combined with a larger volume of mobile phase and debubbled just before entering a conventional UV photometer set at 214 nm.

RESULTS AND DISCUSSION

Initial studies with segmented-flow liquid–solid chromatography (SF-LSC)

Our first attempts* at carrying out SF-LC separations used silica-coated 0.8-mm capillaries. A typical separation resulting from the application of these coated-tubes is shown in Fig. 3, for the separation of a synthetic sample. Not only were the resulting separations unimpressive, but the observed H values were 15–20-fold greater than predicted by theory (eqn. 19 in ref. 1):

$$H = H_t + H_r + H_s \tag{2}$$

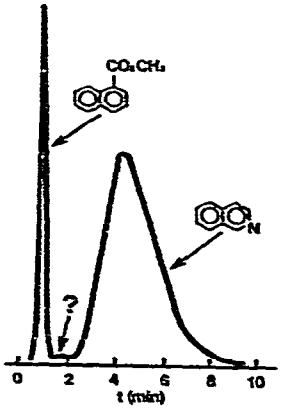


Fig. 3. Preliminary separation of a synthetic sample by SF-LSC on silica-coated capillary. Conditions as described in Experimental section.

Various modifications in the coating procedure were explored, and alumina particles were tried along with silica. None of these changes were effective in markedly lowering values of H.

* This work was carried out by R. L. Grob and E. V. Piel, using coated-capillaries provided by Cs. Horváth.

SEC studies

We next turned to the investigation of SF-LC with agarose-coated tubes. One of our reasons for looking at these SF-SEC systems was that stationary-phase mass-transfer effects should be simpler and more readily understood in an SEC system. Thus diffusion of solutes in swollen agarose is commonly observed to be similar to diffusion in water[4], so that restricted diffusion in the stationary-phase layer could be ruled out. Therefore we expected to understand more readily any discrepancies that might arise in comparing experimental H values for SF-SEC with theory (eqn. 2). A second reason for our interest in SF-SEC separation was that it appeared possible to develop an automated sample-pretreatment module around this principle.

Retention studies

Early experiments with nylon tubing coated with agarose showed adsorption effects for various solutes. Glass tubing as used by us seems to be free of adsorption for the solutes studied. This is illustrated in Fig. 4 by representative data from a particular 200 × 0.1 cm tube coated with agarose. Here an SEC calibration curve is plotted, in the form solute-molecular-weight *vs.* solute retention. The latter is expressed as k' for the solute divided by k' for $CuCl_2$. The solid curve through the data-point for $CuCl_2$ is that predicted for 3% agarose as stationary phase (determined by interpolation between 2 and 4% agarose, as represented by Pharmacia curves for Sepharose 2B and 4B). The solid points are for typical proteins used to calibrate SEC columns (mol.wts. of 10^4–4×10^5), Blue Dextran and a low-molecular-weight dye: Acid Blue.

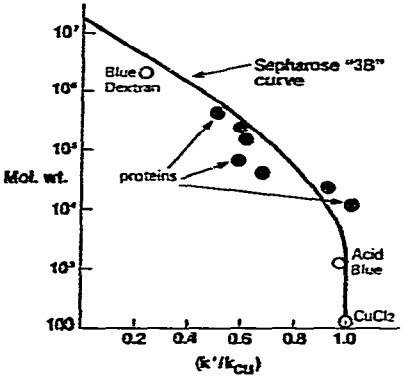


Fig. 4. Retention of samples in SF-LC with agarose-coated tubes. Tube 200 × 0.1 cm, 0.1% Plurafac in water, other conditions as in Experimental section.

On the basis of these data, it was concluded that $CuCl_2$ does not exhibit anomalous retention effects in our agarose-coated tubes, and we therefore used this solute for further band-broadening studies (it was a convenient solute for this purpose).

Band-broadening in SF-LC: general

Fig. 5 illustrates some typical data from our first experiments with SF-SEC

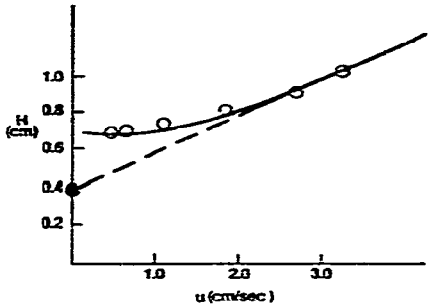


Fig. 5. Variation of plate height H with mobile phase velocity u in SF-SEC separation. Tube, 200 × 0.05 cm, d_f = 21 μm. Other conditions as in Fig. 4 (Pump I, no air-bar) ○, experimental H values; ●, calculated value of H_t.

separation. Theory predicts that plots of H *vs.* u will be linear, and intersect the H-axis ($u = 0$) at a value of H equal H_t:

$$H_t = L_s(1 - R) \tag{2a}$$

It is seen that the data points approach a linear plot at higher values of u, and this linear plot extrapolates to the correct value of H ($H = H_t$; eqn. 2a) at $u = 0$. However, values of H at low values of u are higher than predicted. Furthermore, the limiting slope of the linear plot in Fig. 5, equal to C in eqn. 22 of ref. 1, is an order of magnitude higher than given by eqn. 2 above. Data were collected as in Fig. 5 for different conditions: d_t values of 1.0 and 0.5 mm; d_f values of 10–25 μm. In every case, plots as in Fig. 5 were obtained: linear plots at large u, extrapolating to H_t at $u = 0$, but with H values falling above the linear plot at lower values of u.

Hydraulic effects

These initial H *vs.* u data as in Fig. 5 could be expressed by the equation

$$H = H_t + Cu + B/u \tag{3}$$

The term B/u is purely empirical, but does fit the deviations from linearity in Fig. 5 (and in other experiments) fairly well. We will use measured values of B here simply to assess the relative importance of these anomalously high H values at low values of u. While this B/u term is of the same form as predicted for longitudinal diffusion (see ref. 3), we have argued in ref. 1 that longitudinal diffusion is in fact completely insignificant in SF-LC systems (see also discussion in the Appendix of ref. 5. We also observed that surging/pulsing effects (see discussion in ref. 1) and segmentation irregularities were more common at low flow-rates, suggesting that these latter phenomena were related to the B/u term of eqn. 3.

Initial experiments (as in Fig. 5) were carried out on a pump (Pump I) that was not equipped with an air-bar. Furthermore, no special precautions were taken to minimize the size of the pump tubes, by using multiple small tubes rather than a single large tube for the mobile phase (as in Fig. 1). These conditions in our experience are all conductive to hydraulic problems, resulting in surging, pulsing and bubble-irregularity.

At this point we modified the experimental procedure somewhat in order to confirm that the B/u term indeed arises because of hydraulic irregularities. With a particular coated tube (200 × 0.1 cm, $d_f = 10\,\mu$m), we determined values of B for the following changes (all with Pump I, no air-bar):

Change	*B*
Regular conditions (no change)	0.008
Large bubbles	0.04
Pulse-damper	0.00

The increase in air-bubble size (bubble length 0.5 cm, *vs.* 0.15 cm originally) is seen to increase B. This was accompanied by increased and more violent surging of the mobile phase during these separations. The explanation is that with increased total air volume in the coated tube, the liquid-plus-air within the tube is more compressible, and can store more energy to be released as a surge in liquid flow at some point during the separation (see discussion of ref. 1).

The addition of a pulse damper immediately after the pump was observed to result in decreased surging, and a lower value of B as observed above.

We next changed the pump in our SF-LC system from the Pump I (no air-bar) to the Pump III (with air-bar). A dramatic improvement in hydraulic stability and segmentation regularity was apparent. Furthermore, in typical experiments the B term was now near zero. This is illustrated in Fig. 6a for a 400 × 0.1 cm coated tube ($d_f = 10\,\mu$m). The longer tube length here would be expected to increase hydraulic instability, because of the higher pressure drop along the tube. Nevertheless, because of the addition of the air-bar, the resulting plots of $(H - H_t)$ *vs.* u are now seen to be linear over the full range in u ($0.2 < u < 2.0$ cm/sec).

With an air-bar and a limited choice of pump speeds (our Pump III was modified to pump at half-normal speed and 3 × normal), data such as that of Fig. 6 require simultaneous variation in L_s, and therefore in H_t. Values of H (rather than

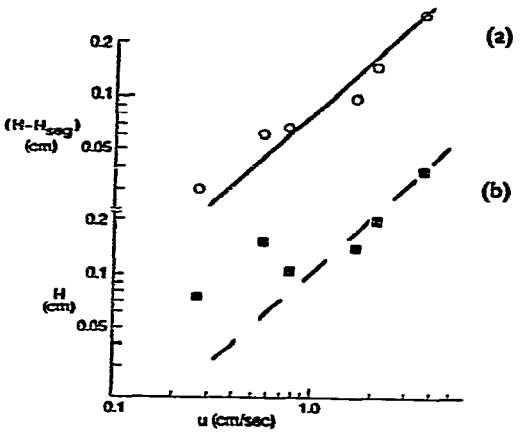


Fig. 6. Variation of plate height H with mobile phase velocity u in SF-SEC separation with improved hydraulics (Pump III with air-bar, multiple small pump tubes). Tube, 400 × 0.1 cm, $d_f = 12\,\mu$m, other conditions as in Fig. 4.

$H - H_t$) are plotted in Fig. 6b. The greater scatter of the data here, *versus* in Fig. 6a, illustrates the importance of the H_t term, and confirms the validity of our theoretical expression for H_t (eqn. 2a).

Despite the use of an air-bar as in Fig. 6, hydraulic instability in an SF-LC system is not totally eliminated. This therefore remains an area for further study and improvement. We have looked briefly at the use of more effective surfactants as a further means of increasing hydraulic stability. Some such commercial products allow surface tension to be reduced to values as low as 17 dyne/cm. Brief experiments with such compounds show a dramatic further improvement in hydraulic stability, but with a simultaneous increase in SF-LC H values. The reason for the latter is still speculative, but it appears from visual examination of such systems that the air bubbles may no longer be "strong" enough to occlude effectively the inside of the coated tube and to provide separation from adjacent liquid segments. Possibly this problem is aggravated by uneven coating of the stationary phase film (see following section).

The C term in SF-SEC: comparison with unsegmented flow

An initial speculation as to the cause of the larger-than-predicted C term in plots such as that of Figs. 5 or 6 was that some unexpected effect might be operative; *i.e.*, the form of eqn. 2 might be incorrect, or incomplete. A possible check on such a hypothesis is to study band-broadening in analogous separation systems where segmentation of the mobile phase is eliminated. Fig. 7 shows such a comparison. The experimental points at the top of Fig. 7 correspond to H *vs.* u values for unsegmented flow through a 200 × 0.1 cm column with $d_f = 25\,\mu$m. The solid line which intersects this plot at lower values of u is that calculated from the Golay equation (eqn. 24 in ref. 1). The deviation of experimental values from this plot for $u > 1$ cm/sec is expected, since secondary flow in these tightly coiled tubes leads to interference with laminar flow and to lower predicted values of H (see discussion of ref. 6). The lower dashed line is the plot of $H - H_t$ *vs.* u for segmented-flow through this same tube (all other conditions the same). The bottom dotted line is the plot of $H - H_t$ *vs.* u predicted by eqn. 2.

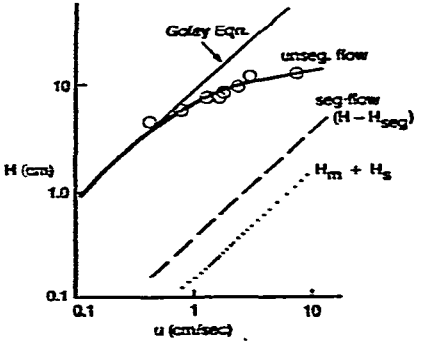


Fig. 7. Variation of plate height H with mobile phase velocity u in SEC separations without segmentation. Tube, 200 × 0.1 cm, d_f 25 μm. ○ and ——— are for unsegmented mobile phase, – – – is same conditions but with segmentation, ... is calculated curve for segmented-flow separation in this system.

From the data of Fig. 7, it appears unlikely to us that some unexpected effect is responsible for the larger-than-expected $H - H_t$ values found (segmented flow): *i.e.*, the form of eqn. 2 seems to be correct. Other workers[7] have commented on the fact that the Golay equation has yet to be stringently verified. While in one sense this is correct, the discrepancy between SF-LC H values and eqn. 2 seems to be much larger, and to demand an alternative explanation.

During our studies of unsegmented flow, as in Fig. 7 we encountered another effect that was initially puzzling, but which was of little significance so far as our interest in band broadening was concerned. Measured k' values for segmented and unsegmented flow through a 200 × 0.1 cm agarose tube ($d_f = 25\,\mu$m) were found to differ markedly for the same solute ($CuCl_2$): $k' = 0.11$ (segmented), $k' = 0.26$ (unsegmented). Reduction of the segmentation frequency n caused no increase in k'. As long as any segmentation at all was involved, k' was constant and equal to 0.11. Further experiments with uncoated tubes showed that the effect persisted, and was therefore not related to the agarose coating. We have previously shown that retention in segmented-flow through uncoated tubes is "normal"[2], so the anomaly is present in the unsegmented-flow cases. Further study of this effect in unsegmented flow through uncoated glass tubes (200 × 0.05 cm) showed that retention varied with flow-rate:

u (cm/sec)	*k'*
2.0	0.05
3.2	0.07
5.4	0.10
10.3	0.13

Unpublished findings from another laboratory[8] suggest that this effect is a general one. That is, in unsegmented flow through narrow tubing, lower-molecular-weight solutes tend to be retained as a result of flow itself (no sorption on the tube walls), and can in fact be separated from larger molecules. No further work was carried out to better understand this phenomenon.

The C term in SF-SEC: dependence on d_t, k' and solvent viscosity

We next attempted to determine if the large values of C we observed arose from the mobile or stationary phase. The value of C from eqn. 22 of ref. 1 can be expressed as:

$$C = C_r + C_s \tag{4}$$

where these mobile phase (r) and stationary phase (s) coefficients are given from eqns. 18 and 20 of ref. 1 as

$$C_r = (1 - R)^2\, d_t^2/36\, D_m' \tag{4a}$$

and

$$C_s = (2/3)\, R\,(1 - \mathrm{R})\, d_f^2/D_s \tag{4b}$$

In eqn. 4b we assume $\gamma = 1$ for agarose layers. For SEC separations as in the present case, with thin layers of agarose, $R \approx 1$, and $(1 - R) \approx k'$. From eqns. 8 and 9 of ref. 1, k' is seen to be further related to d_f and d_t ($\approx d_t^0$) as

$$k' = 4d_f/d_t \tag{4c}$$

With these latter approximations, eqns. 4a and 4b above can be reexpressed as

$$C_r = (4/9)\, d_f^2/D_m' \tag{5}$$

and

$$C_s = (8/3)\, d_f^2/d_t D_s \tag{5a}$$

The features that differentiate these two terms, and offer potential insight into the reason for C being too large in our SF-SEC experiments to date, are as follows:

(a) a higher dependence on d_f of C_s *versus* C_r; however, we should keep in mind that values of d_f are inferred from values of k', and a uniform coating (d_f = constant) is assumed;

(b) the C_r term is independent of d_t, whereas C_s increases as d_t decreases

(c) C_r depends on D_m', whereas C_s varies with D_s, which for agarose as stationary phase $\approx D_m$; D_m and D_m' vary differently as solute molecular weight or mobile phase viscosity is varied.

Table I summarizes experimental data on the variation of C with d_f, d_t and mobile phase viscosity η.

TABLE I

VARIATION OF C (EQN. I-22) WITH EXPERIMENTAL CONDITIONS

Agarose-coated tubes (L = 200 cm), $CuCl_2$ solute, 0.1% Plurafac in water except 0.1% Plurafac in glycerol–water (30:70) where noted, Pump I without air-bar.

d_t^0 (cm)	k'	C (sec)	d_f* (μm)	C_r**	C_s***	$C/(C_r + C_s)$	C_g/C§
0.100	0.041	0.05	10.0	0.0052	0.0014	7.6	1.5
0.100	0.105	0.16	24.9	0.028	0.020	3.3	1.2
0.050	0.086	0.29	10.3	0.0050	0.0029	37	1.5
0.050	0.182	0.42	20.9	0.017	0.022	11	2.1

* From eqns. 8 and 9 in ref. 1.

** From eqn. 4a.

*** From eqn. 4b.

§ Ratio of C_g (30% glycerol) to C (water).

In the next-to-last column of Table I are shown values of $C/(C_r + C_s)$; *i.e.*, the ratio of experimental to calculated C values. Thus experimental columns are anywhere from 3- to almost 40-fold less efficient than predicted, at higher values of u where the Cu term dominates. The most important comparisons are those involving tube diameter d_t (or d_t^0). If C_m were the dominant term, values of C should be independent of d_t when d_f is held roughly constant (eqn. 5). If C_s is more important, values of C should increase with decrease in d_t. The data of Table I show a clear

trend in the latter direction. Thus, C values are greater by a factor of 3–6 for $d_t^0 = 0.05$ versus $d_t^0 = 0.1$ in Table I. This suggests that the C_s term is for some reason unexpectedly large.

The data in the last column of Table I show the increase in C for an increase in mobile phase viscosity. Here, C_g refers to the value of C for 30% glycerol–water as mobile phase, for which $\eta = 2.16$ cP, and which is 2.4-fold more viscous than water without added glycerol (the normal mobile phase for which C values were measured). A change in η of the mobile phase would be expected to affect D_m and D_s in eqns. 5 and 5a. Thus, for diffusion in agarose, D_s should be approximately the same as for D_m of the solute ($CuCl_2$) in water. According to the Wilke–Change equation (*e.g.*, ref. 9), D_m (and therefore D_s) should decrease in proportion to η, so that C_s should be proportional to η. D_m, on the other hand, is proportional to $\eta^{-5/3}$ (ref. 10), so that C_r should increase as $\eta^{5/3}$. Thus, if η increases by 2.4-fold (30% glycerol versus pure water) in Table I, C_r should increase by $2.4^{5/3} = 4.4$-fold, and C_s should increase by 2.4-fold. The observed increase in C, equal to C_g/C in Table I, is seen to average about 1.6-fold, which is much closer to the value predicted if C_s is the dominant term. Again, our experimental data suggest that C_s (not C_r) is anomalously large. The somewhat lower value of C_g/C in Table I *vs. sus* theory (1.6 *vs.* 2.4) may reflect preferential exclusion of glycerol from the agarose phase, but this is speculative.

The dependence of C on d_f, with d_t^0 held constant, varies from 0.5 to 1.2-power, *versus* 2nd-power (C_r, eqn. 5) and 3rd-power (C_s, eqn. 5a), other variables constant. However, we believe that the values of d_f inferred as in Table I are probably incorrect. Thus, if we argue that C_s is larger than predicted by eqn. 4b, the question is: why? The most likely answer is that the coating of agarose within the tube is not uniform, but is "puddled", "sagged", or otherwise distributed in uneven fashion. Attempts to confirm an uneven coating of the agarose layer are complicated by the coiling of the tube and the thinness of the agarose layer. Nevertheless, staining of the agarose in typical coated-tubes indicates that the coating was indeed uneven. The low-power dependence of C on d_f can then be explained in terms of patches of agarose being present on the walls of thinly-coated tubes, with increase in agarose content (and apparent increase in d_f) serving to fill in the bars spots between patches, without really increasing the true value of d_f.

Our tentative conclusion, therefore, is that lower values of C can be obtained when we succeed in coating these tubes more evenly with stationary phase. Preliminary attempts in this direction have so far proven unsuccessful, but work along these lines is continuing.

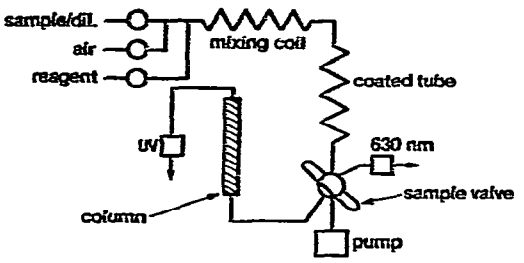
Preliminary application of SF-SEC to sample pretreatment for LC

When high-performance liquid chromatography (HPLC) was first introduced a decade ago, one of its attractions was the fact that most samples could be injected directly onto the column, without prior sample processing as was often required in gas chromatography. This is still true for HPLC today, although with the extension of the technique to virtually every class of compounds and to a wide variety of sample matrices (*e.g.*, blood, soil, pharmaceuticals, etc.), it is more and more common to see some sample pretreatment required before injection onto a small-particle HPLC column. As in the case of gas chromatography (GC), where sample pretreatment is required in LC, it is usually the most time-consuming, least well-controlled step in

the overall analysis. This has led to the need for automating sample preparation in certain analyses by HPLC, those where large numbers of difficult samples must be analyzed routinely and reliably. Examples of such applications or potential areas for such sample-pretreatment automation include: clinical laboratories and the analysis of human sera for therapeutic drugs, various metabolites, etc.; quality control laboratories in which solid samples such as tablets must be assayed for drugs, vitamins, etc.; environmental laboratories where large numbers of samples of soil, foodstuff, etc. must be analyzed for residues, potentially hazardous waste-products, etc. A general, reliable and simple approach to the automation of sample pretreatment for HPLC analysis is already provided by continuous-flow technology, as discussed by Burns[11] and reduced to practice by Dolan *et al.*[12] for the assay of fat-soluble vitamins tablets (FAST-LC™; Technicon Corp., for Fully-Automated-Sample-Treatment). A variety of common chemical operations used in sample pretreatment for LC have been adapted to this AutoAnalyzer™ approach. For example, proportioning of sample and reagents, incubation at various temperatures for any reasonable time, solvent extraction, etc. A recent addition to the capability of the AutoAnalyzer™ in this regard is the development by Burns[11] of the evaporation-to-dryness module (EDM) which allows removal of volatile solvents from sample mixtures, exchange of one solvent for another, and concentration of the sample[11,13]. One operation, however, that until now has not been well executed in an AutoAnalyzer™ system is the simple filtration of particulates from a sample mixture, or the physical separation of the sample as by chromatography. For example, a common procedure for preparing serum samples prior to their assay for therapeutic drugs is to precipitate sample-protein by addition of organic solvent and/or strong acid, centrifuge to remove protein, filter through a 0.5-μm filter, and then inject onto the LC column. The use of mechanical, renewable paper filters in AutoAnalyzer™ systems is possible (but awkward), and complete removal of very fine particles as for use with small-particle LC columns is not possible.

The present technique of SF-SEC suggests itself as a solution to the latter problem. Thus with agarose columns that provide total permeation of low-molecular-weight solutes (see Fig 4), it is possible to readily separate particulate-containing samples into fractions of particulates and of low-molecular-weight solutes, the latter than being ready for injection onto the LC column. The specific advantages of this approach have already been addressed in the Introduction.

Although the agarose-coated tubes we have investigated so far appear to be less efficient than theory predicts will be possible, it appears that these tubes are nevertheless suited for sample-pretreatment in an automated mode. The experimental scheme we have used in preliminary studies is outlined in Fig. 8. An automatic sampler (Technicon Sampler III) is used to pick up small volumes of sample from each vial, with aqueous diluent separating adjacent samples in time. The sample–diluent stream is combined with precipitating reagent and with air, then sent to a mixing coil where precipitation of protein is completed. From the latter coil the sample proceeds to an agarose-coated tube where separation of soluble solutes of interest from precipitated protein takes place. The separated mixture (see Fig. 9) passes through the sample-loop of an automatic sample-injection-valve, to a waste-line where the effluent from the valve is monitored with a photodiode detector (for particulates). The system is first timed for injection of sample after particulates have cleared the valve loop, then updated by monitoring the particulate band from subsequent samples. A typical



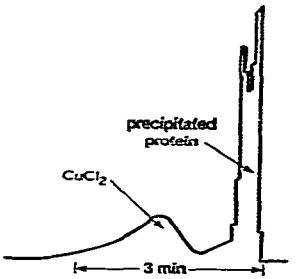


Fig. 8. Schematic of scheme used for automated sample deproteinization–filtration by SF-SEC prior to LC analysis. See text.

Fig. 9. Separation of denatured protein (particulates) from low-molecular-weight Solute ($CuCl_2$) in SF-SEC module of Fig. 8. Redrawn to eliminate bubble noise as in Fig. 2.

separation is shown in Fig. 9, where $CuCl_2$ is used to simulate the low-molecular-weight solutes of interest.

In the above fashion it is possible to separate samples for subsequent LC analysis at rates of up to 20 samples/h. Of course, this requires LC separation times of 3 min or less per sample. We have applied the scheme of Fig. 8 to the fully-automated analysis of serum samples for therapeutic drugs of interest, at rates of analysis of 10–20 samples/h. While this work is preliminary, and certain practical problems await final resolution, it nevertheless seems an attractive approach to simple and reliable assay of samples requiring pretreatment before LC analysis.

CONCLUSION

Our general theory for separation efficiency in SF-LC has been tested experimentally, using agarose-coated tubes in SEC separations. The results agree with theory in terms of the segment-length term H_i, but the velocity-dependence of H on velocity (C term) is 3–40 times too large. Preliminary data suggest that this is due to uneven coating of the tube-walls with the agarose layer, and improvements in this coating process are expected to lower experimental H values so as to approach the separation power predicted by theory.

An application of SF-SEC for the automated sample-pretreatment of serum samples prior to their LC analysis for therapeutic drugs is presented. Although this work is at a preliminary stage, it appears possible to duplicate classical deproteinization–centrifugation–filtration procedures by a much simpler and fully automatable process. Other applications of SF-LC for the pretreatment of LC samples can readily be visualized, and we are further exploring these options.

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SYMBOLS (Parts I[1] and II)*

A	Constant term in eqn. I-22; equal to H_t (cm).
B	Empirical coefficient in eqn. II-3; a measure of hydraulic instability and its effect on H (cm²/sec).
C	Coefficient in eqn. I-22; the dependence of H on u (sec).
C_g	The value of C for glycerol–water (30:70, v/v) as mobile phase in Table I of Part II.
C_m	Concentration of solute in mobile phase.
C_r	Contribution to C of mobile-phase mass-transfer effects; eqn. eqn. II-4a (cm).
C_m	Contribution to C of stationary-phase mass-transfer effects; eqn. II-4b.
d_f	Thickness of stationary phase film (cm); see eqn. I-9 for SF SEC.
d_t	Diameter of mobile-phase stream in capillary LC (cm); see eqn. I-5a.
d_t^0	Internal diameter of tube or capillary (cm).
D_m	Solute diffusion coefficient in mobile phase (Table I-I) (cm²/sec).
D_m'	Solute mass transfer coefficient in mobile phase in SF systems (Table I-I) (cm²/sec).
D_s	Solute diffusion coefficient in stationary phase (cm²/sec).
$f(D_m)$, $f(R)$	Terms in eqn. I-24 for unsegmented capillary LC; equal to $(1/D_m)$ and $(6R^2 - 16R + 11)/96$, respectively.
F, F_a, F_m	Mobile-phase flow-rates (ml/sec) of air-plus-liquid, air and liquid, respectively.
$g(D_m)$, $g(R)$	Terms in eqns. I-25 and I-25a for segmented capillary LC; equal to $1/D_m'$ and $(1 - R)^2/36$, respectively.
H	Height equivalent of a theoretical plate (cm).
H_f	Contribution to H in SF-LC from segment length; eqns. I-16 and I-19.
H_m	Contribution to H in unsegmented capillary LC from mobile-phase mass-transfer; eqn. I-24.
H_r	Contribution to H in SF-LC from mobile-phase mass transfer; eqns. I-18 and I-19.
H_s	Contribution to H in capillary LC (either segmented or unsegmented) from stationary-phase mass transfer.
H_{us}	H in unsegmented capillary LC; eqn. I-23 (cm).
k'	Capacity factor in either segmented or unsegmented LC; eqn. I-1.
k_{ap}	The apparent capacity factor in SF-LC; eqns. I-2 and I-3.
k_{Cu}	k' value for $CuCl_2$ as solute
L	Length of capillary or tube (cm).
L_s	Length of liquid segment in SF-LC (cm).

* Reference to eqn. I-3 or II-2 indicates eqn. 3 of Part I and eqn. 2 of Part II, respectively.

m_m, m_s	Total amount of mobile phase (m) or stationary phase (s) within tube or capillary.
M	Mobile phase (Fig. II-1).
n	Segmentation frequency (bubbles/sec) in SF-LC (sec^{-1}).
n'	Segmentation frequency (bubbles/cm) expressed as bubbles per unit length of capillary (cm^{-1}); see Fig. I-3.
N	Theoretical plate number for tube or capillary (or column).
N_{eff}	Effective plate number, equal to $N(1 - R)^2$.
P	Pressure drop along tube or capillary (c.g.s. units in eqn. I-21)
q	Retention of a solute in an SF-LC system, measured in units of liquid segments; see discussion of eqns. I-11, I-12 and I-13.
R	Retention parameter, equal to $k'/(1 + k')$.
S	Sample (Fig. 1 of Part II).
sf	Denotes segmented-flow.
SD	Standard deviation.
SF-SEC	Segmented-flow size-exclusion chromatography
t_0	In SF-LC the time for a bubble to pass through the tube; otherwise, the time for an unretained solute to pass through an LC system (sec).
t_R	Solute retention time (sec).
u	Velocity of mobile phase (cm/sec).
us	Denotes unsegmented flow.
V_m	Volume of mobile phase contained within the tube or capillary, exclusive of mobile phase within the stationary phase in SEC systems (ml).
V_s	Volume of a liquid segment in SF-LC (ml).
V_R	Solute retention volume (ml).
$w_{1/2}$	Width of a solute band at half-height (sec).
x	Time separating t_0 and the solute band-center (sec).
σ^2	Variance of band, measured in units of liquid segments (SF-LC).
σ_i, σ_r	Contributions to σ from segment-length (i) and mobile-phase mass transfer (r); eqn. I-14.
η	Mobile phase viscosity (Poise).

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