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APPLICATION OF COLUMN EFFLUENT STORAGE (BUFFER STORAGE) WITH AIR SEGMENTATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Several possible uses of a little known technique, column effluent storage, are described. This general technique represents an alternative to fraction collection. It can provide for greatly extended reaction times in post-column chemical derivatization (for increased detection sensitivity), with acceptable extra-column band broadening. It also allows interfacing fast-flow columns with slow-flow detectors (*e.g.*, radioactivity counters), again for increased sensitivity. In each instance, a theoretical analysis of extra-column band broadening is given.

INTRODUCTION

In most instances, the effluent from a liquid chromatographic (LC) column flows to the detector and then to waste. Sometimes it is useful to collect all or part of the column effluent in a fraction collector for further processing and/or use. While the development of modern LC detectors has reduced the need for fraction collection, many potential uses for fraction collectors still exist.

Column effluent storage represents an alternative to fraction collection, and is more versatile and in some instances simpler. The concept of effluent storage was originated by Skeggs[1] and first applied by Karmen *et al.*[2]. The technique works as follows. As effluent leaves the column (usually prior to detection, but in some instances following the detector), air bubbles are added to the stream at regular intervals and the resulting air-segmented stream is allowed to flow into a storage coil (a length of narrow-diameter tubing). After collection in this fashion of some or all of the segmented chromatogram, the column effluent is stored for some time t before further processing. Alternatively, the column effluent can be mixed with some reagent before entering the storage coil, with reaction of the separated sample occurring during storage of the effluent–reagent mixture. In either instance, the purpose of air segmentation is to reduce longitudinal dispersion and re-mixing of the separated sample bands[3].

The analogy between fraction collection and effluent storage should be apparent: the liquid segments in the latter technique correspond to very small fractions,

just as might be collected in a fraction collector. Among the advantages of effluent storage *versus* conventional fraction collection are the following:

(1) reduced mechanical complexity of the collection–storage means;

(2) the possibility of collecting a large number of very small individual fractions (*e.g.*, fraction volumes of a few microlitres each);

(3) easy interfacing of effluent storage to later sample processing and/or detection;

(4) no loss or alteration of effluent during collection or processing.

These features of effluent storage in turn lead to the following potential applications of the technique:

(1) reaction detectors with very long incubation times, but minimal extra-column band broadening; in some applications this can mean greatly enhanced detection sensitivity;

(2) more efficient interfacing and utilization of LC systems and detectors, based on different flow-rates through the column and detector;

(3) novel recycling technique for the generation of very large column plate numbers;

(4) a general means of interfacing more complex LC systems in real time.

The use of effluent storage as an alternative to fraction collection in preparative LC seems less desirable, for several reasons. Compared with fraction collection, a disadvantage of effluent storage is that some sample dispersion occurs during effluent storage. A main purpose of this paper is to present a theoretical analysis of extra-column band broadening in typical applications of effluent storage, so that optimal conditions can be defined for the use of this technique. Various possible applications can then be evaluated in terms of their practical feasibility. The theoretical background required for this study has been described previously[3–5].

EXTENDED-TIME REACTION DETECTORS

This proposed use of effluent storage is closely analogous to the technique of air-segmented reaction detection for LC as discussed earlier[4], both in function and in theory. In normal reaction detectors based on air segmentation[6,7], the column effluent is segmented by air bubbles as it leaves the column, various reagents are added and the mixture passes through a reaction coil that provides an incubation time t_r. During the time the flowing stream spends in the incubation coil, some chromogenic (or other detection-enhancing) reaction takes place, and the reacted chromatogram then flows into the detector. One limitation of reaction detectors is the extra-column band broadening that occurs during the incubation step (see also discussion by Deelder *et al.*[8] for unsegmented and packed-bed reaction detectors). This dispersion of the sample increases for larger values of t_r; for high-performance LC systems which use air-segmented reaction detectors, a practical upper limit on t_r is about 10–20 min[4].

Some reactions of potential use in LC reaction detectors are catalysed by separated sample components. An example is the measurement of enzymes after their separation by ion-exchange chromatography, using chromogenic reactions based on catalysis by a given enzyme[9,10]. In these instances, the detection sensitivity is proportional to the time t_r available for reaction, and long reaction times may be required in order to achieve adequate detection sensitivity. For example, the MB isoenzyme of

creatine phosphokinase (CK) is of major clinical interest for the diagnosis of myocardial infarction. However, significant concentrations of this enzyme in blood or serum are low, so as to provide a major detection problem in the assay for CK–MB by LC (see discussion by Snyder *et al.*[11]).

When large values of t_r are required for the enhancement of detection sensitivity in LC, but the normal band broadening that occurs in such reaction detectors is unacceptable, effluent storage provides a practical alternative. The flow scheme for this new procedure is illustrated in Fig. 1 for the simplest possible application. Here, effluent leaves the column, air bubbles and reagents are added continuously and the reaction mixture passes through a three-way valve into a storage–incubation coil. When the total chromatogram (effluent for an entire separation) is stored in the coil, the three-way valve is switched to divert effluent to waste. After a sufficient time, t_r, for completion of the reaction of interest, the three-way valve is turned to allow flow of the stored (and now reacted) effluent to the detector. As we shall see, sample dispersion in this reaction detection scheme occurs only during filling and emptying of the storage coil, with no dispersion during actual storage (no-flow condition). Therefore, very long incubation times are possible, with relatively little extra-column band broadening.

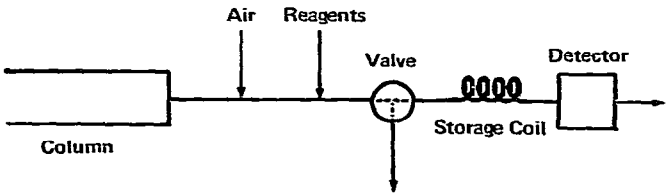

Fig. 1. Diagrammatic representation of a simple extended-time reaction detector.

Theory

Let the time required for filling the storage coil be t_1, the incubation (no-flow) time t_2 and the time for emptying the storage coil t_3. For the present, we shall assume the same flow-rates for filling and emptying, so that $t_1 = t_3$. A subsequent section discusses the case when different filling and emptying rates are used. Normally, the time $t_1 = t_3$ will be the time, t_s, required for carrying out the separation, assuming storage of the entire chromatogram. From this $t_r = t_s + t_2$.

Individual bands in the chromatogram spend different times in the storage coil during filling and emptying, but this is not significant, as the extent of dispersion during either filling or emptying will be the same for each unit of time a sample band spends in motion within the storage coil (provided that $t_1 = t_3$). Thus, the dispersion for a given band during the storage process is the sum of variances created during (i) filling–emptying plus (ii) no-flow storage.

The sample dispersion due to step (i), filling–emptying, is identical with that found in normal reaction detection without interruption of flow, as described earlier[4]. That is, if we ignore step (ii) and the flow interruption associated with that step, each sample band moves through the storage coil just as it would move through a reaction detector. The main difference for effluent storage is that the time t_1 is now the time t_s required for the separation, rather than (as in normal reaction detectors) the time t_r required for the reaction. Thus, effluent storage is a preferred technique for minimal

extra-column band broadening *versus* normal reaction detection, whenever $t_r > t_s$.

The band variance developed during step (i) was treated in detail earlier[4]. When various experimental conditions are optimized (see Table I for the conditions assumed here), the extra-column variance, σ_{ec}^2, is given approximately by

$$\sigma_{ec}^2 = 0.01\, t_s \tag{1}$$

The added variance developed during step (ii) must be extremely small, because the only mechanism for longitudinal diffusion between liquid segments is through the film surrounding the intra-segment air bubbles[3]. A rough estimate of this added variance, σ_r^2, is derived in the Appendix and is about $10^{-7}\, t_2$, *i.e.*, it is negligible compared with σ_{ec}^2 from eqn. 1.

TABLE I

OPTIMIZED CONDITIONS ASSUMED FOR EFFLUENT STORAGE AND/OR FLOW OF MOBILE PHASE THROUGH COLUMN[3,4]

Parameter	*Value*
Liquid flow-rate (F)	0.01 ml/sec
Liquid viscosity (η)	0.4 cP
Liquid surface tension (γ)	25 dyne/cm
Sample diffusion coefficient in mobile phase (D_m')	$3 \cdot 10^{-5}$ cm^2/sec
Air-segmentation rate (n)	2.5 bubbles/sec
Internal diameter of storage coil (d_t)	0.5 mm

The effect of this extra-column band broadening (σ_{ec}^2) on the overall column efficiency obtained in a given LC separation can now be derived. Let the variance developed in the column (due to separation, no extra-column effects) be σ^2 (sec^2), where the column efficiency, N, is defined as

$$N = (t_R/\sigma)^2 \tag{2}$$

t_R being the band retention time (sec). If the apparent column plate number, N', including extra-column effects is defined:

$$N' = t_R^2/(\sigma^2 + \sigma_{ec}^2) \tag{2a}$$

then

$$N' = N\,\sigma^2/(\sigma^2 + \sigma_{ec}^2)$$

We can further relate σ^2 to the primary separation parameters k' (the capacity factor of a given band) and t_0 (the column dead time[12]):

$$t_R = t_0\,(1 + k') \tag{2b}$$

Eqns. 1, 2, 2a and 2b can be used to calculate N' *versus* N, t_0, t_s and k'. Fig. 2a summarizes the results of such a calculation for representative conditions ($t_0 = 100$ sec,

t_s = 600 sec) and various values of N. Other conditions are similarly summarized in Figs. 2b and 2c and are discussed below.

In some applications of effluent storage, it is convenient to empty the storage coil by reversing the flow, *e.g.*, in Fig. 1, by emptying the coil through the three-way valve (which would now be connected directly to the detector). In this instance, t_s in eqn. 1 is replaced for each band by the time the band spends flowing within the storage coil, which will be near zero for the last-eluted band and will approach $2t_s$ for the first-eluted band. The effect of a reversed flow on effluent storage in Fig. 2a is shown by the broken curve (for N = 20,000 plates).

The use of gradient elution with effluent storage, rather than isocratic elution as in Figs. 2a–2c, is obviously more complex from a theoretical standpoint. Previous work[13–15] has laid an adequate basis for understanding the effects of effluent storage on gradient elution separations, as summarized elsewhere[16]. It can be shown that if an optimal gradient steepness is used ($b \approx 0.3$, see ref. 16), then the apparent plate number, N', after storage is related to the plate number, N, in the absence of storage by

$$N' = 2.4\,N/(2.4 + 0.01\,x\,N^{1/2}) \tag{3}$$

where x is related to t_0 and t_s by

$$t_s = x\,t_0 \tag{3a}$$

Values of x commonly vary from about 5 for fairly simple mixtures (with a small range in isocratic k' values) to 10–25 for fairly complex LC separations (wide range in sample k' values). Fig. 2d illustrates how N' varies with N and x over a range of values of common interest.

Discussion

For a fairly typical set of conditions, Fig. 2a illustrates the general effect of effluent storage on column efficiency. For very efficient columns (large N) and small values of k', there is a pronounced decrease in apparent plate number, N'. However, for many applications this decrease in N' will not be important. Thus, compounds of interest will often have larger k' values ($\geqslant$2), and plate numbers N of 2500–5000 are common in practice. For this range of conditions, there is relatively little loss in N as a result of extended-time reaction. Therefore, it can be concluded that this technique is practical for many LC applications as a means of increasing detection sensitivity.

The decrease in column efficiency as a result of effluent storage is strongly dependent on the value of t_0, as seen in Fig. 2b in comparison with Fig. 2a. The larger is t_0, the less is the reduction in N. Conversely, for very fast separations with small values of t_0, effluent storage becomes less practical. Similarly, as can be seen in Fig. 2c, the separation of complex samples with a wide range of k' values results in lower N' values at small k', other factors being equal.

In gradient elution (Fig. 2d), there is a substantial decrease in column efficiency for most practical separations as a result of effluent storage. However, fairly large values of N' can nevertheless be maintained (1000–3000 plates), which, added to the combined advantages of gradient elution plus effluent storage, can still provide a powerful tool for certain applications.

In Fig. 2d, larger values of t_s/t_0 correspond to a wider range of solvent strengths, or more complex samples whose retention values cover a wider range. Comparison of Fig. 2d with Figs. 2a and 2c shows the same trend throughout: as the difference in isocratic k' values for the least retained *versus* most strongly retained compounds

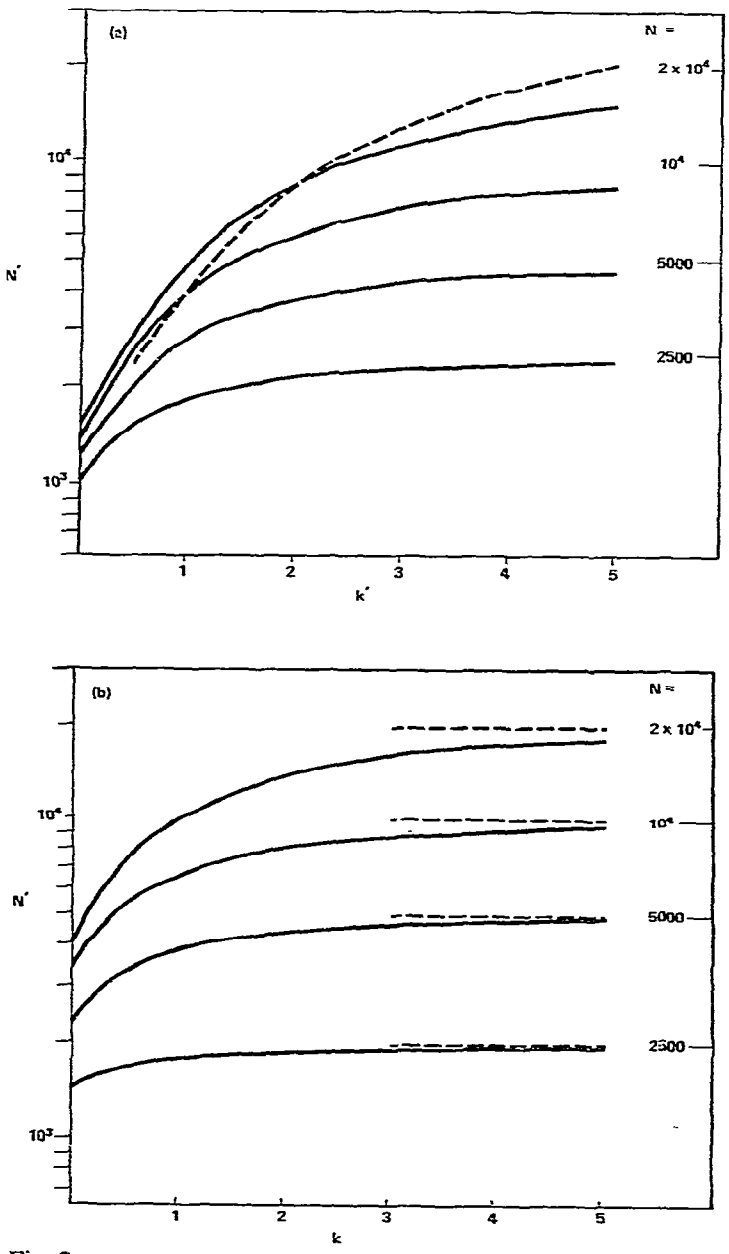

Fig. 2

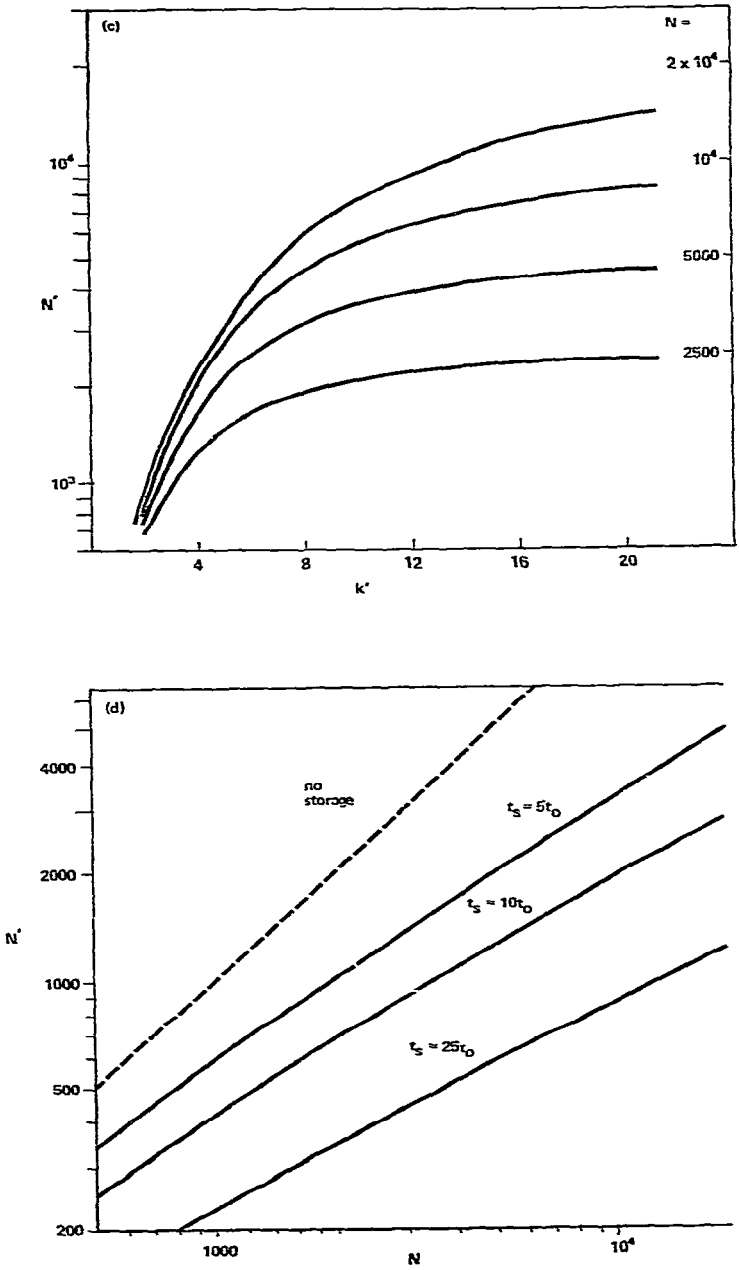

Fig. 2. Decrease in column efficiency due to effluent storage for extended-time reaction detection. Curves calculated (a–c) from eqns. 1, 2, 2a and 2b, isocratic elution: (a) $t_0 = 100$ sec, $t_s = 10$ min; (b) $t_0 = 300$ sec, $t_s = 30$ min; (c) $t_0 = 25$ sec, $t_s = 10$ min. Curves for (d), gradient elution, calculated from eqns. 3 and 3a.

in a sample increases (*i.e.*, more for complex samples), the N' values possible with effluent storage tend to decrease.

The use of normal reaction detectors with gradient elution (no effluent storage) has not been discussed previously; only isocratic elution was treated earlier[4]. Fig. 2d can be used to predict the decrease in column efficiency for such applications. Thus, the time of reaction (t_r) of the effluent between the column and the detector can be set equal to t_s in Fig. 2d. For example, with $t_0 = 25$ sec and $t_r = 250$ sec, the curve for $t_s = 10\ t_0$ (Fig. 2d) is applicable. For this condition, N' is substantially less than N for $N > 500$ plates. The reason is simply that bands are normally much narrower in gradient elution, and are therefore much more susceptible to extra-column band broadening. Hence the technique of reaction detection in combination with gradient elution results in a serious decrease in the effective column efficiency. However, in some applications the advantage of reaction detection may overcome this limitation.

INTERFACING FAST SEPARATION WITH SLOW DETECTION, AND *VICE VERSA*

Normally, the flow of effluent from the column passes directly to the detector, so that separation and detection occur in serial fashion. In this instance, the flow-rates of the mobile phase through the column and detector are equal (for a given separation). However, in some instances there are reasons for desiring either a faster or a slower flow of mobile phase through the detector, relative to the column. Karmen *et al.*[2] proposed collecting the effluent from a series of LC separations, followed by the rapid flow of stored effluent through a detector. They referred to this technique as "buffer storage". The purpose of buffer storage was essentially "detector multiplexing", whereby a single detector could thus be used to serve several columns simultaneously. Alternatively, the part-time use of a detector could provide full-time service to a single column. In the detector multiplexing mode, as illustrated in Fig. 3, several columns are connected through effluent storage coils to a single detector. By suitable valving and alternate pumping devices, the effluent from column 1 is first stored in coil 1 (flow a in Fig. 3). Assuming n columns for a single detector, at the completion of the first separation by column 1, the contents of coil 1 are emptied through the detector at a flow-rate n times that through the column (flow b in Fig. 3), using the discharge pump. At this time, the first separation in column 2 is complete (by suitable timing of separations on columns 1 and 2), and the contents of coil 2 are now emptied into the detector at a rate n-fold greater than the column flow-rate. This process continues for each of the n columns in turn, after which the cycle begins again with column 1.

The original aim of detector multiplexing was to minimize the need for expensive LC detectors. Today, the cost of LC detectors is not excessive in comparison with the remainder of the LC system and there therefore seems to be less need for such schemes. Also, by suitable valving and intermittent measurement of small increments of each column effluent at frequent intervals[16], detector sharing can be accomplished by other means. More important, as can be seen in Fig. 4, buffer storage results in a significantly reduced column efficiency. As dispersion in effluent storage is greater at higher flow-rates[3,4], the decrease in column efficiency is greater in detector multiplexing. As can be seen in Figs. 4a ($N = 2500$) and 4b ($N = 10{,}000$), there is a severe reduction in apparent column efficiency, N', for $n \geqslant 5$. This decrease in efficiency is

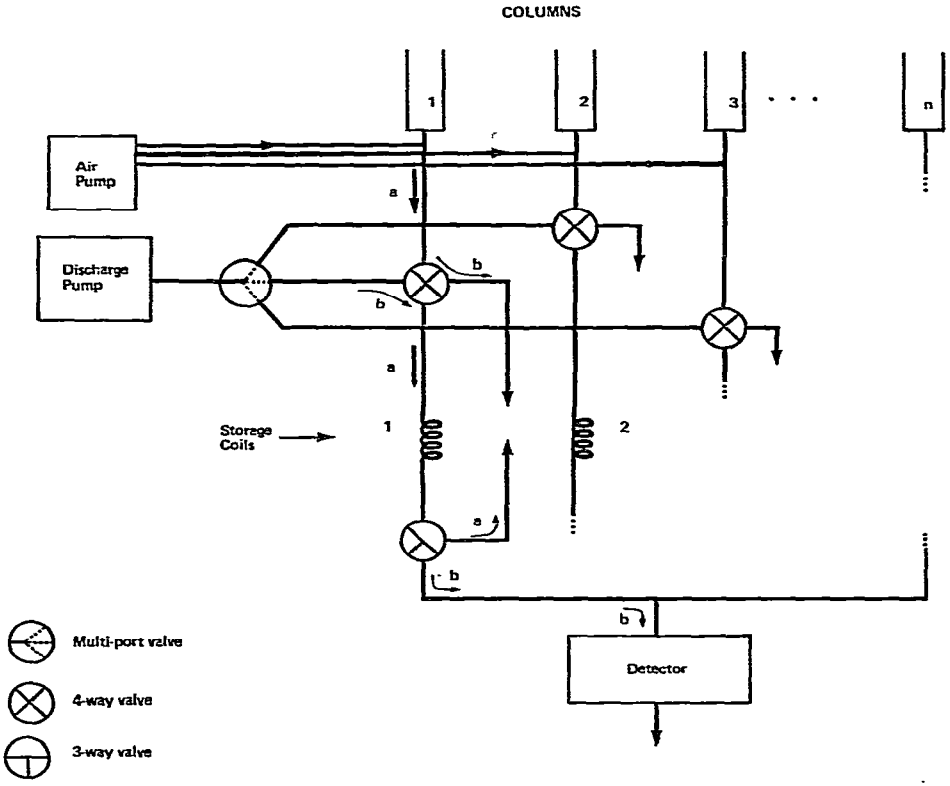

Fig. 3. Diagrammatic representation of a buffer storage system for detector multiplexing.

greater as the number of columns (n) for a single detector increases, and is greater as the original column plate number, N, increases. As buffer storage would appear to be worthwhile only for relatively large values of n, there is little incentive to consider its use in conjunction with modern high-performance LC applications.

It is also possible to foresee advantages in decreasing the flow-rate through the detector relative to the column. For example, with radioactivity detectors, sensitivity is increased at lower flow-rates of effluent through the counter. Thus, for maximal sensitivity in given applications, it might prove useful to collect the effluent from a given separation, then flow the effluent very slowly through the detector. As can be seen in Fig. 4, a 5- or 20-fold reduction of the flow-rate through the detector in this fashion leads to a reduction in the decrease of column efficiency, *versus* effluent storage with $t_1 = t_3$. From the standpoint of column efficiency, therefore, fast separation with slow detection seems to be a more practical technique, than *vice versa*.

Why would one want to slow down the flow of effluent through the detector, as opposed to simply decreasing the flow of mobile phase through both the column and the detector? There are two reasons that might be applicable in a particular instance. Firstly, the flow of mobile phase through the column is generally optimized for maximal resolution. The best (*i.e.*, slow) flow of mobile phase through the detector might be different from the best flow of mobile phase through the column for maximal

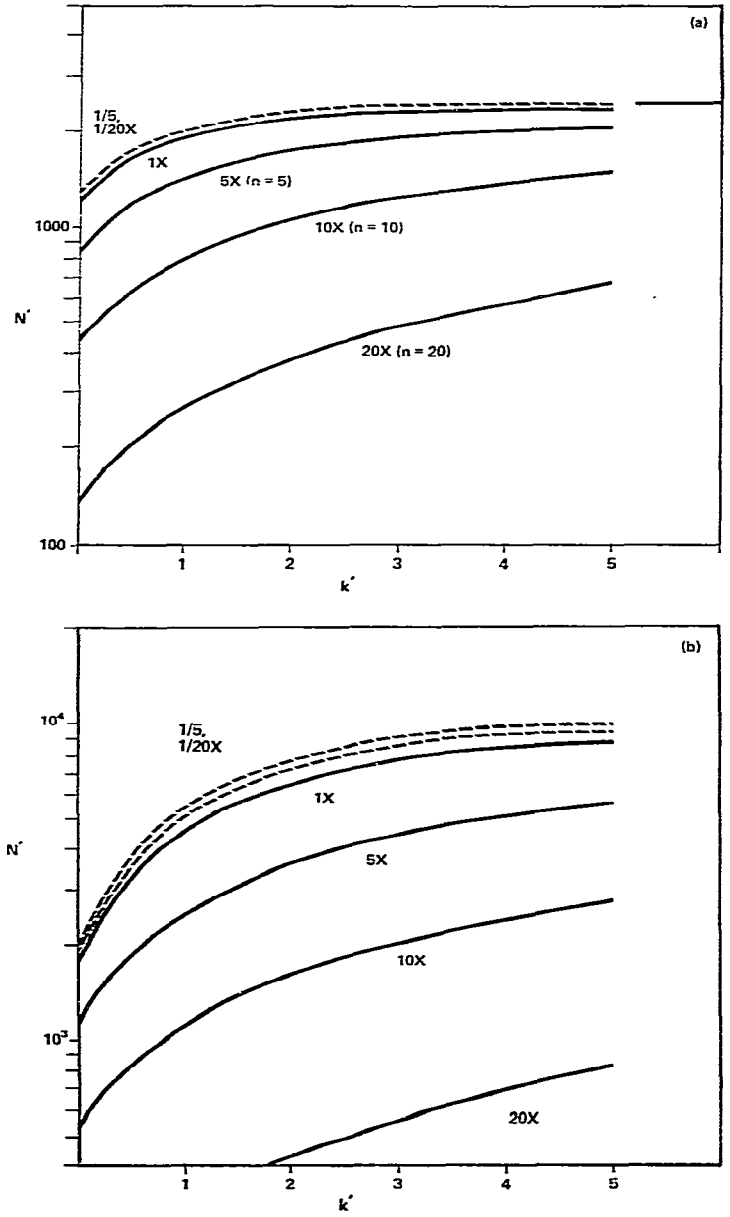

Fig. 4. Effect on column efficiency of effluent storage with indicated change in flow-rate through detector, relative to column. Calculated as in Figs. 2a–2c (isocratic elution), with σ_{cc} obtained as described earlier[4], using conditions of Table I for effluent leaving column. (a) $N = 2500$; (b) $N = 10{,}000$.

resolution. Secondly, one might choose to monitor only occasional LC runs with slow detection. By separating the column from the detector, slow detection does not needlessly slow down the utilization of the column (and associated LC unit) for other applications.

"FULL-CHROMATOGRAM" RECYCLE LIQUID CHROMATOGRAPHY

Recycle LC is now a well established technique[12] for obtaining increased values of N from a given column, for any of several practical reasons. The principle of conventional recycle LC, in one experimental mode, is shown in Fig. 5a. After injection

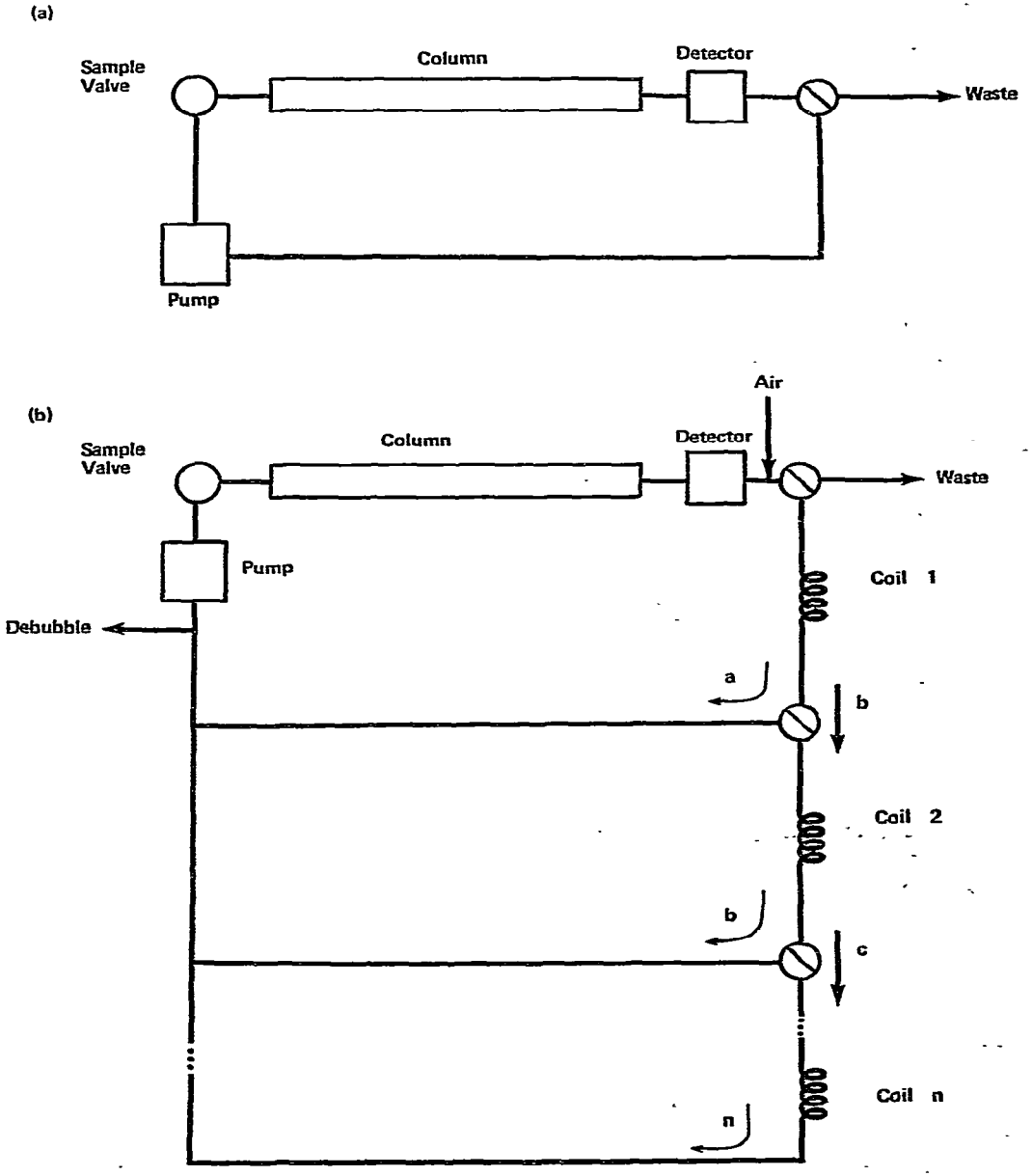

Fig. 5. Diagrammatic representations of (a) normal recycle LC and (b) full-chromatogram recycle (FCR) LC.

of the sample, separation proceeds in the usual manner with effluent from the detector being diverted to waste. The purpose of conventional recycle is to increase further the resolution of a pair of overlapping bands in the chromatogram, by diverting this pair of bands through the three-way valve back to the pump and column. In this fashion, the overlapping bands can be further resolved by two (or more) passes through the column. If extra-column band broadening can be made insignificant (which is never the case with this approach), and if the efficiency of the column (single pass) is N, then for n passes through the column (n recycles), the effective column efficiency is increased to $n \cdot N$.

Conventional recycle has several possible aims in practice. Firstly, for preparative separation, a column can be greatly overloaded, to the point where the resolution of two bands of interest is much degraded. By continued recycling (plus removal of purified material during recycling), a given column can provide a considerably increased throughput rate with minimal attention by the operator. Secondly, if only a single column length of given type is available for a particular application, recycle provides a means of increasing N without having to add additional lengths of column to the system. Finally, in principle, recycle allows a greater value of N to be developed for a particular column type (and particle size) than can be achieved by conventional LC with a specified maximal column pressure.

A limitation of recycle LC is that only a single pair of bands can normally be recycled, because the full chromatogram leaving the column would overlap on itself if it were fed back to the column inlet. This is the result of the broadening of the initial sample volume into a total volume (for the final chromatogram) which can considerably exceed the column internal volume, V_m. In principle, intermediate effluent storage provides a solution to this problem, and allows the possibility of "full-chromatogram" recycle (FCR) chromatography. The procedure for FCR is shown schematically in Fig. 5b. In the first separation cycle, the chromatogram (column effluent) is run into storage coil 1, which is just large enough to hold the entire chromatogram. At this point, the three-way valve at the end of coil 1 is set to allow return of the column effluent to the pump and column (step a, with flow indicated by the arrows in Fig. 5b). Following the second separation cycle (step b), with doubling of the total chromatogram volume, the three-way valves are set to allow filling of coils 1 and 2, with diversion of flow from coil 2 back to the pump and column. The process can be continued for any number of n cycles, provided that sufficient coils and valves are included in the system. Initially, all coils and lines are filled with the solvent used as mobile phase in the separation.

When would FCR seem to be applicable for a practical LC problem? Certainly not for preparative separations, which we have already noted are not suited to effluent storage techniques. This then leaves the possibility of generating very large N values from a single column, for an entire chromatogram (not just two adjacent bands). While FCR is capable in principle of increasing column N values for this case, an alternative in many instances is simply to reduce the flow of mobile phase through the column. The latter approach with currently available columns easily allows plate numbers of 10,000 and greater to be achieved. However, FCR might be advanced as a technique for increasing N to very much larger values (but with a proportional increase in separation time).

We can assess the practicality of FCR for the latter application from eqns. 1–

2b. The results of such a calculation for a typical set of initial separation conditions ($t_0 = 100$ sec, $t_s = 600$ sec and $N = 10^4$) are shown in Fig. 6, where N' is plotted against n for different values of k' (differing cycle numbers in FCR). While FCR can in principle provide larger values of N', the increase in N' with further recycling tends to level off, particularly at lower values of k'. In fact, after five cycles ($n = 5$), bands with $k' < 1$ are not as well resolved as for simple isocratic elution without effluent storage and recycle.

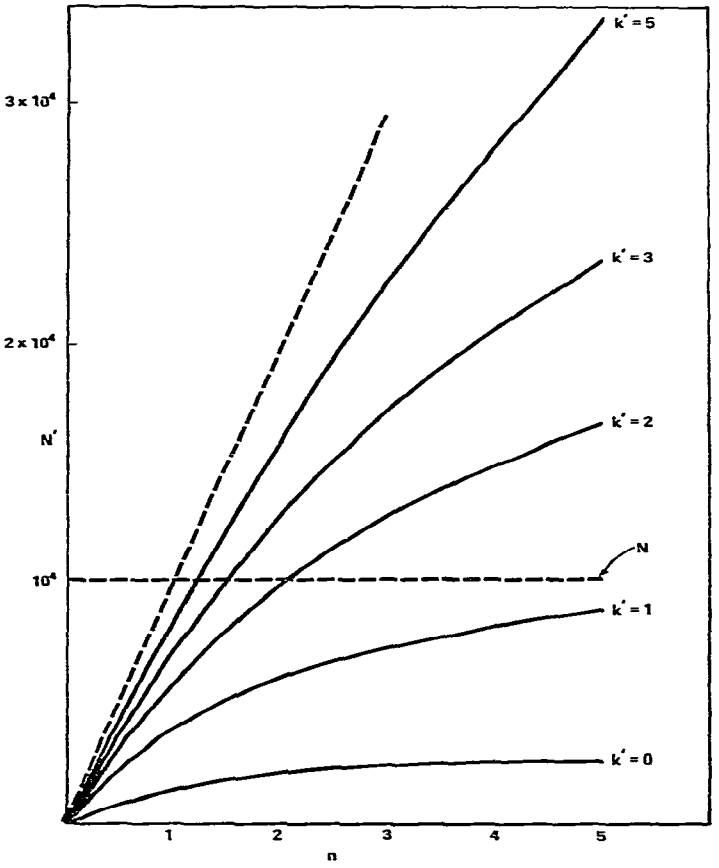

Fig. 6. Increase in effective plate number, N', in full-chromatogram recycle (FCR) LC as a function of cycle number (n) and k'. Calculated from eqns. 1–2b, with $t_0 = 100$ sec, $t_s = 600$ sec and $N = 10{,}000$.

As the calculations in Fig. 6 assume that no other extra-column effects occur during recycle (except those due to effluent storage *per se*), and as extra-column effects due to recycle are normally significant, the N' values in Fig. 6 are probably over-optimistic. Further, we have ignored the mechanical complexity of the system in Fig. 5b, with the problems of repeated bubbling and de-bubbling[8]. For these reasons, as well as the data in Fig. 6, FCR chromatography does not appear attractive for high-performance LC.

COMPLEX LIQUID CHROMATOGRAPHIC SYSTEMS

As the complexity of an LC system increases, other applications of effluent storage become possible. For example, two-column systems with valve switching allow the use of an initial column to prepare fractions for further separation by a final column of different type[17,18]. Effluent storage of the intermediate fractions from the first column would allow more flexibility in the separate operation of the two columns. Thus, flow and separation times for the two columns need no longer be matched. Also, intermediate effluent storage in this instance allows further processing of effluent from column 1 prior to introduction into column 2. As one example, the collected fractions might be concentrated into a smaller total volume, or the solvent (mobile phase) from the first separation might be exchanged for some other solvent (*e.g.*, mobile phase for the second separation).

The theoretical analysis of dispersion from effluent storage in such instances follows directly from the previous discussion, and is therefore not repeated here.

CONCLUSIONS

The present analysis suggests that effluent storage is potentially useful in two situations: extended-time reaction detection, and decreased flow of effluent through the detector for increased sensitivity (as in radioactivity detection). Some decrease in column efficiency results in these applications, particularly in extended-time reaction detection with gradient elution. The increased complexity of any application that makes use of effluent storage will probably restrict its application to special problems, where the additional effort involved can be justified. Detector multiplexing (buffer storage) and full-chromatogram recycle do not appear to be worthwhile.

The successful use of effluent storage will require the usual optimization of continuous flow conditions[19,20]. Typical conditions, including addition of surfactant to the stored effluent, are given in Table I.

SYMBOLS

a, b, c = successive steps and related flow-paths in schemes of Figs. 3 and 5;
FCR = full-chromatogram recycle;
k' = capacity factor for a given band[12];
n = cycle number in FCR, or column number in detector multiplexing (Fig. 3);
N = column plate number[12];
N' = apparent column plate number after effluent storage, due to extra-column band broadening;
t = effluent storage time (sec);
t_0 = column dead-time (sec)[12];
t_r = reaction time in extended-time reaction detection; $t_r = t_s + t_2$ (sec);
t_R = retention time for a given band (sec)[12];
t_s = separation time (sec); time between injection of sample and elution of last band of interest;

t_1, t_2, t_3 = time a band spends in extended-time reaction detection in entering storage coil, incubating within coil and leaving coil (sec);
x = t_s/t_0 for gradient elution;
σ^2 = variance of sample band as it leaves column $(sec^2)^{12}$;
σ_{ec}^2 = variance developed during effluent storage (sec^2).

APPENDIX

Dispersion in effluent storage while flow is discontinued

The thickness of the film surrounding the air bubbles can be calculated as described earlier[3], using the conditions of Table I: $d_f = 7 \cdot 10^{-5}$ cm. This value corresponds to the film thickness during filling or emptying the storage coil; theoretically, d_f approaches zero as flow through the coil decreases to zero, so $d_f = 7 \cdot 10^{-5}$ cm is a maximal value. The variance σ_{ii}^2 developed during storage (without flow, step ii) can be calculated from the Einstein equation:

$$\sigma_{ii}^2 = 2Dt \qquad \text{(i)}$$

where D is the effective diffusion coefficient of a sample compound in the segmented system and t is storage time ($= t_2$). To apply eqn. i, we must convert length units in this equation into time units, allow for diffusion only through the film (in regions occluded by air bubbles) and assume that diffusion through liquid segments is relatively rapid (non-rate-limiting). As this calculation is of limited interest, details will be omitted. The predicted result, which probably overstates dispersion during actual storage, is

$$\sigma_{ii}^2 = 2 \cdot 10^{-7}\, t \qquad \text{(ii)}$$

This variance is so much smaller than the value for step i (eqn. 1) as to justify the intuitive conclusion that no extra-column band broadening occurs in storage *per se* (step ii).

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