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REACTION COLORIMETERS AS DETECTORS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

EXTRA-COLUMN BAND BROADENING WITH SEGMENTED FLOW THROUGH THE REACTION COIL

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SUMMARY

A theoretical expression for extra-column band broadening in reaction colorimeters for liquid chromatography is presented, assuming air-segmentation of the column effluent. The effect of different variables on such band broadening is examined, and conditions for minimizing band broadening are defined.

It is shown that reaction colorimeters with holdup times of up to 20 min are compatible with high-performance liquid chromatographic systems, using, for example, 5–10 μm particles as column packings.

INTRODUCTION

Photometric detectors operating at one or more wavelengths between 200 and 700 nm are today the most popular column monitors for high-performance liquid chromatography (LC). The virtues and limitations of these devices are now well understood (*e.g.*, see ref. 1): They offer both sensitivity and specificity for a wide range of samples, they are reliable and convenient to use, and they are well adapted to the general requirements of modern LC. In some applications, however, photometric detectors fall short of the mark. Some compounds give little or no response because their absorption coefficients are quite small, particularly at wavelengths where commonly used solvents are transparent. In other cases, the determination of trace amounts of compounds of interest is desired in sample matrices that are quite complex. Here the selectivity of photometric detectors may be inadequate, even when different wavelengths are available. In such situations, other types of LC detectors may prove more applicable (*e.g.*, fluorimeters, spectrofluorimeters, electrochemical detectors).

An alternative approach, which is based on all the advantages of photometric detectors, is offered by reaction colorimetry. Column effluent is combined with suitable reagents, heated (if necessary) to effect the desired reaction, and then introduced into a conventional photometer. In this way normally non-absorbing compounds can be converted to absorbing derivatives, or the absorbance of compounds of interest can be shifted to wavelengths where other sample constituents do not absorb. Re-

action colorimeters were among the first detectors to be used in automated LC, in the form of ninhydrin systems for amino acid analyzers. The principle of reaction colorimetry for LC has also been extended to other chemical reactions (*e.g.*, refs. 2–8), using fluorimetry as well as photometry.

However, aside from the specialized use of reaction colorimeters for amino acid analysis, this LC detector has been applied to only a limited extent. In view of its apparent potential for many LC applications, particularly the repetitive assay of large numbers of samples, one may ask why this is so? One reason is that general-purpose reaction colorimeters suitable for high-performance LC are not commercially available. Another reason is that such detectors are inherently more complex than simple photometers, and are less convenient and reliable. Finally, reaction colorimeters are much more prone to extra-column band broadening than are conventional LC photometers, and this is of paramount importance when highly efficient LC columns are used. Furthermore, no work has so far been reported on the factors that determine band broadening in reaction colorimeters, particularly where use is made of segmented-flow to reduce sample dispersion within the colorimeter. It is probable that if the latter problem can be addressed, answers to the first two problems will follow.

Recently, a general model has been described[9] for the prediction of sample dispersion in segmented-flow through open tubing (as in reaction colorimetry). As a result, it is now possible to accurately describe extra-column band broadening in reaction colorimeters as a function of various experimental conditions. This in turn allows the optimum design of such colorimeters for minimum sample dispersion. It also permits us to evaluate the minimum (limiting) band spreading that must occur as the result of using these devices, which is particularly important in evaluating these detectors for use with highly efficient small-particle columns.

The present paper provides a general treatment of sample dispersion in reaction colorimeters for LC. The optimization of these detectors is discussed, and their limiting performance with high-efficiency columns is evaluated.

GENERAL PRINCIPLES OF REACTION COLORIMETRY

Several approaches to on-line reaction colorimetry for LC are illustrated in Fig. 1. In the simplest version of such devices (Fig. 1A), reagent R_1 is dispensed at some constant flow-rate into a tee-connection which combines R_1 with the total column effluent. The combined stream then enters a mixing/reaction coil and the reaction product is directed to the flowcell of a colorimeter. Four aspects of this overall process must be considered: (1) proportioning; (2) mixing; (3) reaction; and (4) dispersion.

Proportioning. Proportioning refers to the continuous blending of constant amounts of R_1 and column effluent. If the instantaneous flow-rates of R_1 and column effluent remain constant in Fig. 1A, proper proportioning results. Without proper proportioning, the ratio of reagent and effluent will vary with time, leading to noisy and/or drifting baselines, ragged looking bands, etc. Therefore devices of the type of Fig. 1A require smooth, constant delivery by both the LC pump and the pump that dispenses R_1. Many pumping systems for both LC and reagent dispensing do not give smooth, constant pumping, and such pumps may prove unsuitable for use as in Fig. 1A.

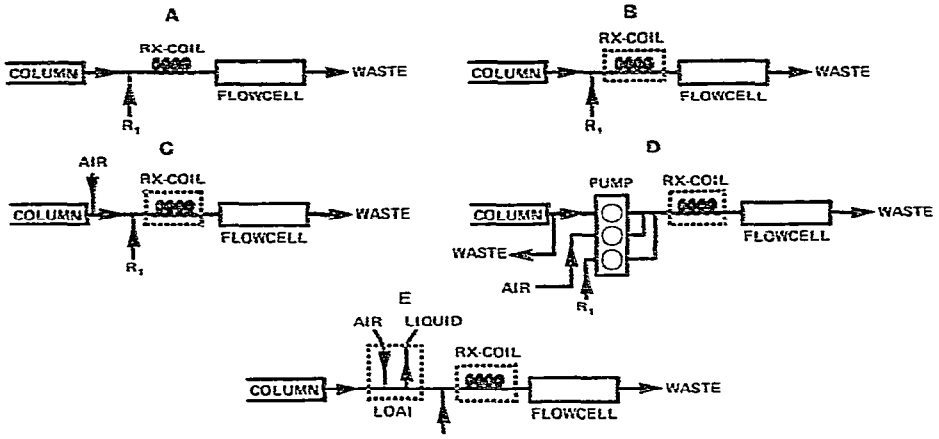

Fig. 1. Different schemes for reaction colorimetry (see text).

Mixing. The mixing of effluent and reagent must occur before the completion of reaction and/or entry of the reacted stream into the flowcell. Mixing in unsegmented streams is often slow, but is facilitated by the use of helical, tightly coiled tubes —as in Fig. 1A. Poor mixing leads to symptoms similar to improper proportioning, mainly noisy traces.

Reaction. The reaction of effluent and reagent ideally goes to completion before the sample enters the flowcell. This may require the use of heated reaction coils and/or long hold-up times.

Dispersion. The dispersion of sample bands after they leave the column is the main concern of this paper. In unsegmented flow, dispersion is usually quite serious, except for the case of very short mixing/reaction coils plus use of other low-dead-volume connectors and fittings. Well-designed, low-volume flowcells, such as those in general use with LC photometric detectors, do not add appreciably to overall dispersion or extra-column band broadening in most cases.

Another version of reaction colorimeters without segmented-flow is shown in Fig. 1B. Here the reaction coil is thermostatted, in order either to provide a controlled temperature or to accelerate the reaction. Control of temperature is essential if the reaction does not go to completion before reaching the flowcell; otherwise the sensitivity of the detector will vary with changes in ambient temperature. Increased temperature is advantageous for speeding up slow reactions, since the overall holdup time in the reaction coil must be kept small for minimum dispersion. Most present amino acid analyzers use the scheme shown in Fig. 1B, with ninhydrin as the reagent R_1.

Simple reaction colorimeters with air-segmentation were used in the early 1960's, as part of the first Technicon amino acid analyzers. This device (*e.g.*, Fig. 1C) is equivalent to that of Fig. 1B, except that air-bubbles are introduced near the column outlet. The dispersion and mixing in 1C is much improved over that of 1A or 1B, because the air-bubbles reduce band-spreading and promote rapid mass transfer within each liquid segment. The main disadvantage of 1C is that proportioning of reagents and column effluent can be poor. The reason is that the air-bubbles normally "pop" into the effluent stream, thereby causing momentary pressure fluctuations. Because of the compressibility of the air-bubbles, these pressure fluctuations are

translated into flow fluctuations and are propagated down the flowing stream past the flowcell. The resulting pulsating flow of the segmented effluent stream leads to similar fluctuations in the proportions of effluent and reagent combining at the R_1 tee, precluding accurate quantitative analysis in some cases.

A more elaborate reaction colorimeter is shown in Fig. 1D, where a portion of column effluent, air and reagent are pumped by a multichannel peristaltic pump with a so-called airbar (which injects air-bubbles at precisely controlled times in relation to the pump motor speed; *e.g.*, the Technicon AutoAnalyzer Pump III). By proper phasing of the airbar injection frequency, proportioning errors are in principle eliminated, as described in ref. 10. Pulsating (*e.g.*, reciprocating) LC pumps can be used with this detector, without affecting the detector signal. The primary limitation of this scheme in high-performance LC is the considerable sample dispersion that can occur prior to injection of air-bubbles. Although this approach is generally unsuitable for very fast and/or highly efficient separations, it has been used successfully in amino acid analysis[11].

Another scheme, shown in Figure 1E, overcomes all of the previously discussed problems —providing that band dispersion within the reaction coil can be limited to an acceptable level. Controlled, even pumping of column effluent and reagent(s) is assumed, and the air injection takes place by means of a socalled LOAI (Liquid Out, Air In) device[12]. The latter technique virtually eliminates pressure pulsations associated with air injection by means of a corresponding liquid withdrawal that occurs in phase with air injection.

If a segmented liquid stream is fed to the flowcell, as in Figs. 1C–E, some provision must be made for electronic debubbling of the resulting signal from the colorimeter. This can be done either by thermal conductance means[13] or (better) by computer or microprocessor (*e.g.*, ref. 12). Alternatively, the segmented stream can be debubbled just prior to entry into the flowcell. Typical debubblers are basically tee connectors, where the segmented stream is fed into one leg of the tee, air plus 30–50% of liquid is rejected (gravity separation) at another leg of the tee, and debubbled liquid leaves the third leg.

The foregoing brief discussion is not intended to be complete. Furthermore, some of the equipment described above is not commercially available at the present time. Nevertheless, this discussion provides a background to the following treatment, and illustrates the design of at least some reaction colorimeters. The remainder of this paper will focus on just one aspect of reaction colorimetry: the prediction and minimization of sample dispersion.

DISPERSION IN SEGMENTED-FLOW: GENERAL THEORY

Recently a general model has been derived and verified for the prediction of sample dispersion in segmented-flow through glass tubing[9]. This model is broadly applicable since it takes into account all of the important experimental variables. The same model should also be approximately valid for the flow of organic liquids through polymeric tubing (*e.g.*, Kel-F™, Teflon™), as long as the liquid wets the wall of the tubing (which is usually required if hydraulic problems are to be avoided).

Fig. 2 shows a graphical representation of a flowing liquid stream segmented by air-bubbles. If a small amount of sample is introduced into one of these liquid

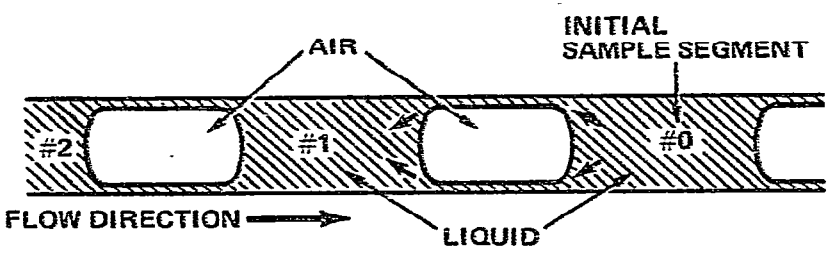

Fig. 2. Schematic of dispersion in segmented flow, showing initial dye segment (0) and flow of dye around air-bubbles.

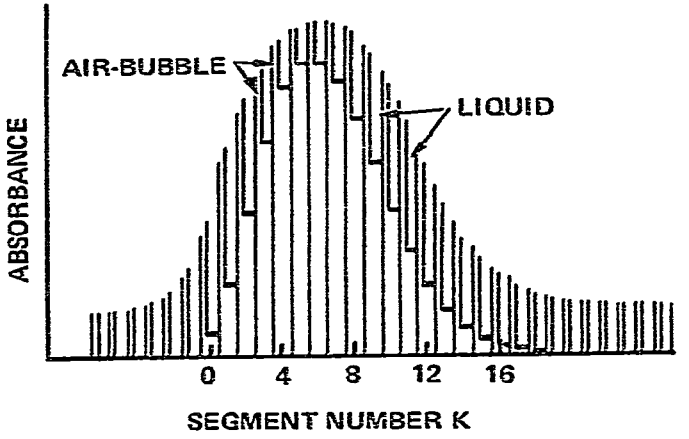

Fig. 3. Resulting dispersion from passage of dye segment through tubing coil, as in Fig. 2.

segments (*e.g.*, segment 0 in Fig. 2), and the liquid stream plus sample then flows through a length of tubing, the sample originally in segment 0 will gradually be dispersed into following segments 1, 2, etc. The reason is that the liquid wets the tubing (in the usual case), so that a thin film of liquid lies under each air-bubble. Liquid from segment 0 is deposited on the tube wall in the form of this film, and is picked up by advancing segment 1. In this way, sample is transferred from segment 0 to 1 to 2, etc. Fig. 3 shows an actual tracing of the distribution of a dye sample in a segmented stream, after a single initially dyed segment was allowed to flow through a length of tubing.

The sample dispersion in Fig. 3 is seen to be approximately Gaussian (actually Poisson), and the extent of dispersion can be expressed in terms of the variance σ^2 of this near-Gaussian curve (see ref. 9). We can measure σ in dimensionless terms, *i.e.*, the *number* of liquid segments (as in Fig. 3), or we can express σ in units more familiar to those working in LC; *e.g.*, volume units (ml) σ_V:

$$\sigma_V = \sigma V_s, \tag{1}$$

where V_s is the volume of a single liquid segment. Time units (sec) σ_t can also be used:

$$\sigma_t = \sigma_V / F_l \tag{1a}$$

where F_l is the flow-rate (ml/sec) of the liquid through the tube.

A previous model[9] gives the dispersion occurring during segmented-flow through a length of tubing as:

$$\sigma^2 = \{[(\pi^2/72)\, d_t^4\, u^{5/3}\, \eta^{2/3}/(\gamma^{2/3}\, V_s\, D'_m)] + 1\} \{0.5\, \pi\, L\, d_t^2\, (u\eta/\gamma)^{2/3}/V_s\} \tag{2}$$

Here the variance σ^2 is given as a function of tubing inside diameter d_t, liquid linear velocity u, liquid viscosity η, liquid surface tension γ, liquid-segment volume V_s, tube length L, and a mass transfer coefficient D'_m that varies with the nature of sample molecules and the viscosity of the liquid. For aqueous solutions at 25°, D'_m is related to sample (solute) diffusion coefficient D_m as shown in Table I. These values apply only for coiled tubing; straight tubing (which should be avoided) gives smaller values of D'_m (see ref. 9). For other liquids and/or temperatures, D'_m is related to the value of D_m for water and 25° ($D_{w,25}$) and liquid viscosity as:

$$D'_m = D_{w,25}\, (\eta/0.0089)^{-1.7} \approx 0.00038\, D_{w,25}\, \eta^{-5/3} \tag{3}$$

Dispersion as defined in eqn. 2 is not a function of air-bubble volume V_a (ml) as long as the air-bubbles are large enough to completely occlude the inside of the tube. This places a lower limit on air-bubble size such that its length is at least 1.5 times its diameter[14], which means that the volume of the air-bubble should be:

$$V_a \geqslant (7\pi/24)\, d_t^3 \tag{4}$$

Eqn. 2 is a theoretical expression which has been verified experimentally over a wide range in operating conditions[9]. Therefore it can be accepted as reasonably reliable for the cases we will shortly consider. While eqn. 2 is somewhat complex, and its

TABLE I

DEPENDENCE OF MASS TRANSFER COEFFICIENT D'_m ON SAMPLE DIFFUSION COEFFICIENT D_m FOR COILED TUBING*

D_m	$D'_m = D_{w,25}$**	*Sample molecular wt.***
3×10^{-5}	12×10^{-5}	27
1×10^{-5}	5×10^{-5}	167
0.3×10^{-5}	2.7×10^{-5}	1250
10^{-6}	2.2×10^{-5}	8000
10^{-7}	2×10^{-5}	400,000

* For straight tubing, $D_m \approx D'_m$ (see ref. 9).

** For water as solvent, at 25°.

*** Approximate sample molecular weights corresponding to given value of D_m in water at 25°; Wilke–Chang equation[17].

significance is not in every respect immediately obvious, it can be rearranged into a form that more readily shows the relationship between sample dispersion in segmented-flow and experimental conditions. Sample dispersion can also be calculated for various experimental conditions to determine whether the dispersion expected for a given reaction colorimeter is acceptable.

DISPERSION IN SEGMENTED-FLOW REACTION COLORIMETERS: SPECIFIC CASES

Consider first the case of a reaction colorimeter with the following assumptions: (1) the dispersion is completely determined by the reaction tubing (*i.e.*, ignoring any contributions to dispersion from tee's, debubblers, flowcells, connectors, etc.); (2) the reaction tubing is in the form of a coil; (3) air-bubbles do not change size after injection into the system. Straight reaction tubes will not be considered further, since these give greater dispersion (other factors equal) and represent a poor design configuration. We will return later to the question of other contributions to sample dispersion, and the possibility of air-bubbles of changing size.

The reaction coil must be so designed that the residence time t of liquid within the coil is sufficient for the desired reaction to occur; *i.e.*, t will have some required value which is determined by the desired application of reaction colorimetry. The tubing length L will be related to this value t as:

$$L = (4/\pi)\, F\, t/d_t^2 \tag{5}$$

F is the total flow (ml/sec) of liquid plus air through the reaction coil.

The linear velocity u can also be related to the total flow-rate of liquid plus air through the reaction coil:

$$u = 4\, F/\pi\, d_t^2 \tag{6}$$

The liquid-segment volume V_s can be related to the liquid flow rate F_l and to the air-bubble rate or segmentation frequency n:

$$V_s = F_l/n \tag{7}$$

Finally, the total flow-rate F and the liquid flow-rate F_l are related in terms of the air flow-rate F_a:

$$F = F_l + F_a \tag{7a}$$

Now an increase in F_a, other variables held constant, simply increases u in eqn. 2, which in turn increases σ. Therefore the air flow-rate should be no greater than required by eqn. 4, or

$$\begin{aligned} F_a &= V_a\, n \\ &= (7\pi/24)\, d_t^3\, n \end{aligned} \tag{7b}$$

Combining eqns. 7a and 7b then gives

$$F = F_l + (7\pi/24)\, d_t^3\, n \tag{8}$$

These expressions for L (eqn. 5), u (eqn. 6), V_s (eqn. 7) and F (eqn. 8) can now be substituted into eqn. 2 to give σ as a function of experimental conditions of interest. Finally, σ_t from eqn. 1a can be substituted for σ to give dispersion in time units:

$$\sigma_t^2 = \left[\frac{538\, d_t^{2/3}(F_l + 0.92\, d_t^3 n)^{5/3}\eta^{7/3}}{\gamma^{2/3} F_l D_{w,25}} + 1/n\right]\left[\frac{2.35(F_l + 0.92\, d_t^3 n)^{5/3}\eta^{2/3} t}{\gamma^{2/3} F_l d_t^{4/3}}\right] \quad (9)$$

Eqn. 9 is the required relationship for dispersion in time units as a function of experimental variables of direct interest in the design of reaction colorimeters for LC. Given a particular reaction colorimeter system, certain variables will be more or less fixed by the requirements of the chemical reaction: η, γ, $D_{w,25}$ and t. The remaining variables (d_t, F_l and n) can then be optimized for minimum dispersion.

Optimizing d_t, F_l and n

We first assume representative conditions for the variables related to the chemical reaction: $\eta = 0.004$ P, $\gamma = 25$ dynes/cm, $D_{w,25} = 0.000035$ and $t = 100$ sec. Fig. 4a shows σ_t *vs.* n, for $F_l = 0.025$ ml/sec (1.5 ml/min), and various values of d_t (0.2, 0.1, 0.05, 0.025, and 0.01 cm). For a given value of d_t, *e.g.*, $d_t = 0.1$ cm, σ_t initially decreases with increasing n, passes through a minimum value of 0.7 sec (at $n = 7.3$ sec^{-1}), then increases with further increase in n. This minimum dispersion ($\sigma_t = 0.7$) and optimum value of n (7.3) are of primary interest. Similarly, as we decrease d_t, beginning with $d_t = 0.2$ cm, the minimum value of σ_t for each value of d_t first decreases, reaches a minimum value, and then increases with further decrease in d_t:

d_t (cm)	Optimum n (sec^{-1})	σ_t (sec)
0.2	1.6	0.92
0.1	7.3	0.70
0.05	30	0.63
0.04	46	0.62 (minimum)
0.025	116	0.64
0.010	600	0.76

Thus for every value of F_l, optimum values of d_t and n exist for which σ_t is a minimum (0.62 sec in the above example, for $F_l = 0.026$ ml/sec and optimum values of d_t and n, equal 0.04 cm and 46 sec^{-1}, respectively). Similar curves result for other assumed values of F_l (*e.g.*, Fig. 4b for $F_l = 0.25$ ml/sec). All of these curves in Fig. 4 show quite flat minima, so that d_t and n can be varied by a factor of 4 around the optimum values with only minor effect ($\pm$ 5%) on σ_t.

Consider next the dependence of σ_t on F_l, when values of n and d_t are optimized for each value of F_l. A plot of these optimized values of σ_t *vs.* F_l is shown in Fig. 5. Corresponding values of d_t and n, optimized for each value of F_l, are also plotted. The minimum value of σ_t decreases continuously with F_l, and σ_t is roughly proportional to $F_l^{0.4}$. Thus there is no theoretical limit to the reduction of dispersion via reduction in liquid flow-rate. However it should be noted that d_t must be decreased and n increased as F_l is reduced (Fig. 5).

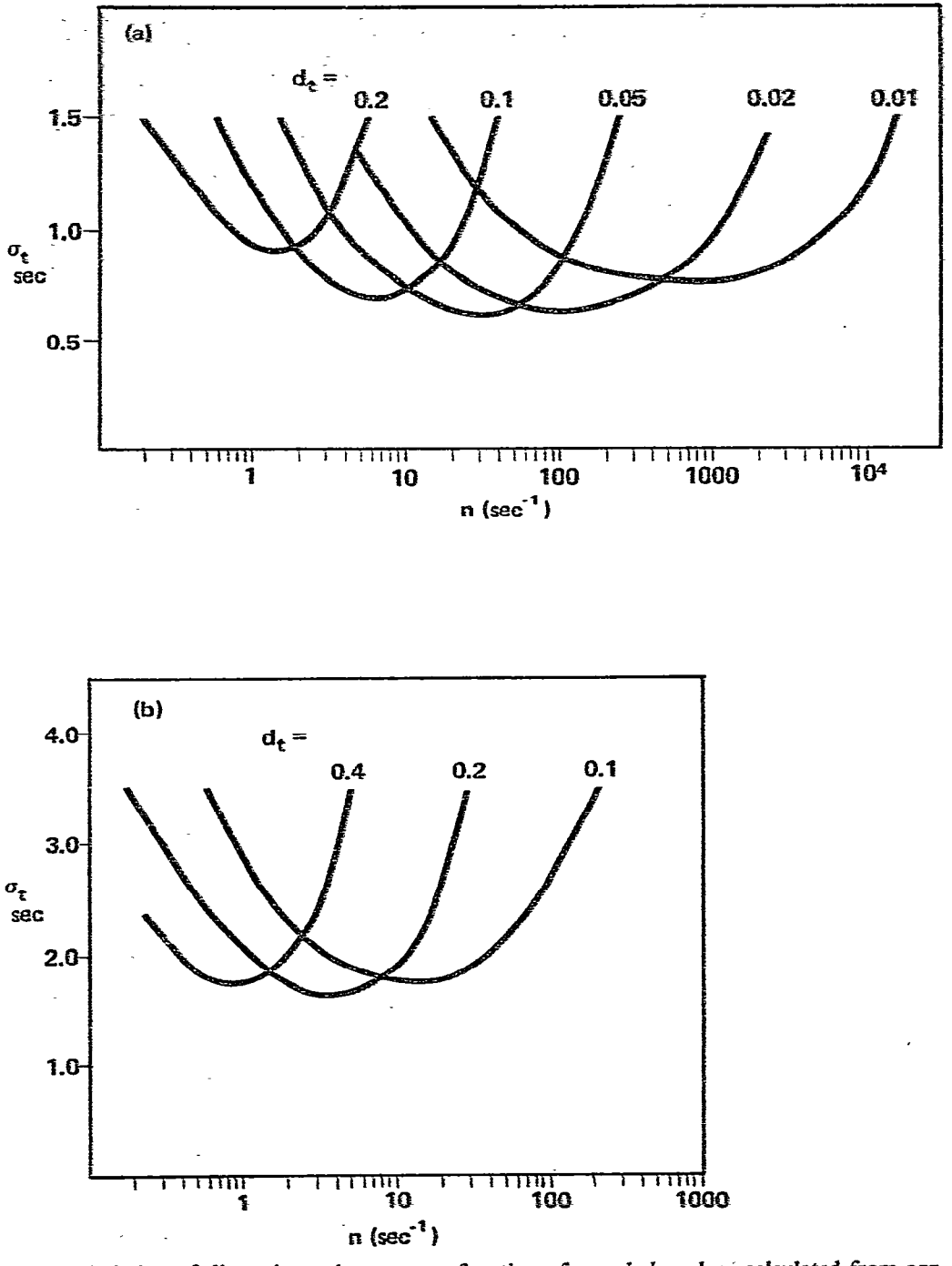

Fig. 4. Variation of dispersion values σ_t as a function of n and d_t; values calculated from eqn. 9, with $D_{w,25}$ assumed equal to 0.00035, $\eta = 0.004$ P, $\gamma = 25$ dynes/cm, and $t = 100$ sec. (a) $F_l = 0.025$ ml/sec; (b) $F_l = 0.25$ ml/sec.

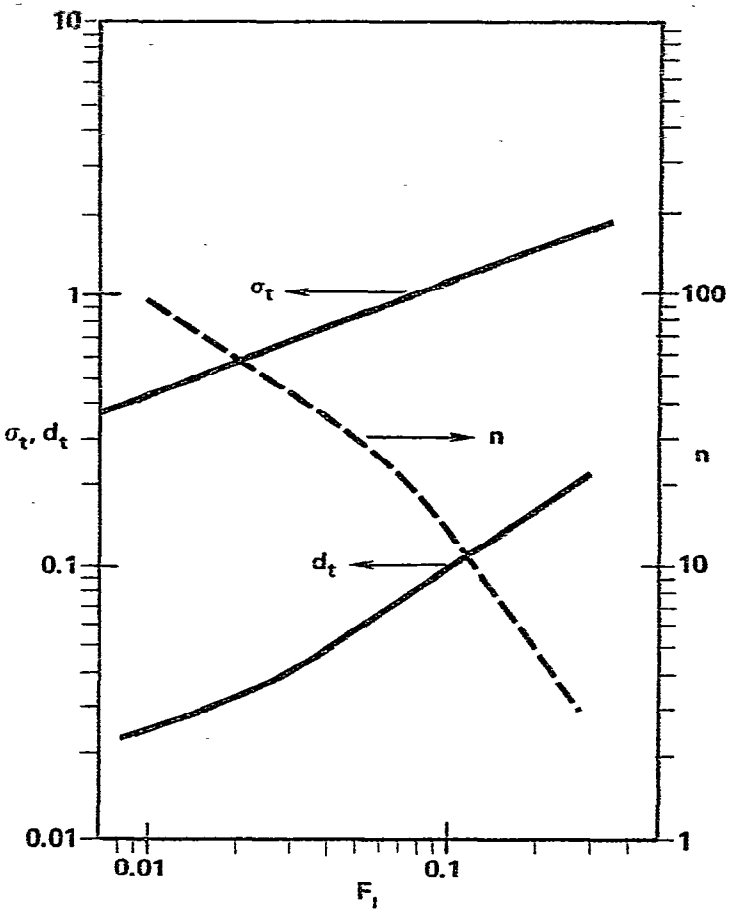

Fig. 5. Variation of optimized values of σ_t, d_t and n with F_t; other conditions as in Fig. 4.

There is a limit to how far tube diameter d_t can be reduced, as it has been found in practice that very small-diameter lines in an LC system are readily plugged by sample particulates, column packing fines, etc. Most workers accept that it is difficult to work with inside diameters smaller than 0.01 in. (0.025 cm). Problems with plugging the reaction coil in LC reaction colorimeters will probably be accentuated by reagent coating effects, sample reactions leading to precipitates, etc., so that d_t will probably have to exceed 0.025 cm. On the other hand, plugging is not a problem in continuous-flow analysis with 1-mm-I.D. tubing. Therefore we will assume a practical lower limit of d_t in the present application, equal to about 0.05 cm. For $d_t = 0.05$ cm, Fig. 5 suggests an optimum value of $F_t = 0.03$ ml/sec, and a minimum value of $\sigma_t = 0.7$ sec.

The preceding analysis overlooks the fact that further reduction in F_t (with d_t constant at 0.05 cm) can allow further reduction in σ_t, because of the broad minima shown in Fig. 4. This is illustrated in Fig. 6. Here conditions are held the same as in Figs. 4 and 5, except that d_t is now fixed at 0.05 cm. Fig. 6 shows that further reduction in F_t is possible (below the above optimum value of 0.03 ml/sec), with a simul-

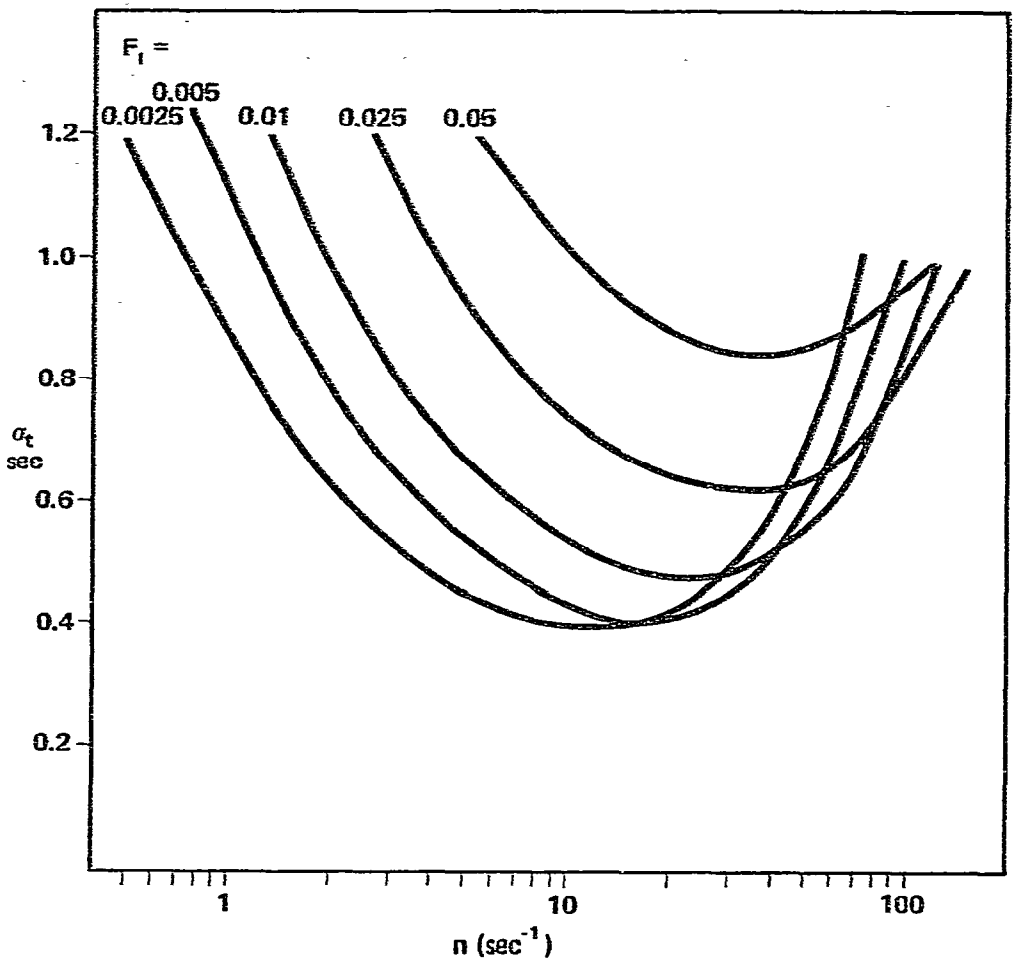

Fig. 6. Variation of σ_t with F_t and n; $d_t = 0.05$ cm and other conditions as in Fig. 4.

taneous lowering of σ_t. A limiting dispersion of $\sigma_t = 0.4$ sec is indicated by Fig. 6, at flow-rates of 0.0025–0.005 ml/sec (0.15–0.3 ml/min). This is also accompanied by lower n values, which has practical advantages (see below).

Constraints set by the flowcell

If the air-bubbles pass through the flowcell (no debubbling), the cell must be completely filled by each liquid segment at some time during passage of liquid through the flowcell. Additionally, a finite time is required to monitor each liquid segment as it passes through the flowcell, so that V_s must significantly exceed the flowcell volume V_c. Since small values of V_s generally favor minimum dispersion, it is clear that a compromise in the value of V_s is required. We arbitrarily assume that $V_s = 2\,V_c$ represents a reasonable trade-off between adequate monitoring time for each segment and minimum dispersion.

The use of eqn. 7 allows V_s to be calculated for various values of F_t and n,

and $d_t = 0.05$ as in Fig. 6. The usual flowcells available for LC photometers have $V_c = 0.008$ ml (1 × 10 mm), but flowcells with V_c as small as 0.001 ml have been used[15]. We can now calculate from eqn. 9 the σ_t values possible for flowcell volumes of 0.002 and 0.008 ml ($V_s = 2\ V_c = 0.004$ and 0.016 ml) for minimum d_t (0.05 cm) and other variables as in Figs. 4–6.

F_l	$n = F_l/V_s$ (sec^{-1})		σ_t (sec)	
	$V_c = 0.002$	$V_c = 0.008$	$V_c = 0.002$	$V_c = 0.008$
0.10	25	6.2	1.24	1.56
0.05	12.5	3.1	0.95	1.45
0.025	6.3	1.5	0.85	1.51
0.01	2.5	0.6	0.89	1.70
0.005	1.3	0.3	0.97	1.90

From the above we see that with a 0.002-ml flowcell, $F_l = 0.025$ (1.5 ml/min) is about optimum, and a σ_t value of 0.85 is achievable. For a 0.008-ml flowcell, $F_l = 0.05$ (3 ml/min) is optimum, and $\sigma_t = 1.45$ is possible. For an infinitely small volume flowcell, Fig. 6 suggests a limiting dispersion of 0.4 sec, which is appreciably smaller than for the above cases involving 0.002–0.008-ml flowcells.

Another constraint of the flowcell is invoked if its diameter is larger than that of the reaction coil tubing. This is the case, for example, in the above optimized system ($d_t = 0.05$) when a 1-mm-diameter flowcell is used. In this case, the size of the air-bubbles must be increased so as to satisfy eqn. 4 for this flowcell. If the ratio of the flowcell diameter to that of the reaction coil tubing is r, and if $r > 1$, then the value of V_a assumed in eqn. 9 must be increased by the factor r^3. This has the effect of increasing the constant 0.92 (which appears in both terms of eqn. 9) by r^3. Repetition of the calculations above for $V_c = 0.008$ (*i.e.*, increasing V_a by 8-fold) shows that larger values of V_a have only a minor effect on σ_t. The optimized value of σ_t is how 1.52, *vs.* the original value of 1.45 sec.

If bubbles are removed prior to the flowcell, we must consider possible problems and additional dispersion created by this operational mode. For a detailed discussion see Appendix II.

Effect of liquid viscosity

Eqn. 9 shows that σ_t increases with liquid viscosity η. Depending on the value of n, σ_t will vary with viscosity as $\eta^{1/3}$ to $\eta^{7/6}$. In the region of optimum n (see above), the dependence of σ_t on viscosity approaches $\eta^{7/6}$. Therefore, dispersion is significantly greater for more viscous liquids. For example, with $F_l = 0.025$ ml/sec, $D_{w,25} = 0.00035$ and $\gamma = 25$ dynes/cm, and optimum values of d_t and n in each case, the following results for two different values of η are calculated from eqn. 9:

	$\eta = 0.004$	$\eta = 0.008$
d_t (cm)	0.04	0.075
n (sec^{-1})	50	6.5
σ_t (sec)	0.63	1.4

The viscosity of the final reaction mixture should be as low as possible, which means that the viscosities of column effluent and of reagent R_t should both be low. The viscosity of the column effluent will have been minimized (*e.g.*, 0.3–0.5 cP) in order to optimize the LC separation (*e.g.*, see ref. 1, p. 491). It is desirable to maintain the reagent solvent viscosity in the same range as that of the column effluent, although somewhat higher reagent viscosities can be tolerated when the volume of reagent added to the system is much less than the volume effluent. It is desirable to have the reaction temperature close to the LC separation temperature. When the reaction temperature is substantially higher than the column temperature, an unnecessarily-high-viscosity solvent must be used in the LC separation to avoid boiling of the solvent when it enters the high-temperature reaction coil. Other problems also arise when these two temperatures are quite different (see Appendix I, dealing with change in bubble size after injection).

Effect of liquid surface tension

Eqn. 9 shows that σ_t varies with liquid surface tension as $\gamma^{-1/3}$ to $\gamma^{-2/3}$. Normally the liquid surface tension will not vary over wide limits (*e.g.*, 20–30 dynes/cm). In the case of higher-surface-tension liquids such as water, surfactants must be added to lower the surface tension and avoid hydraulic problems. Therefore the maximum variation in σ_t as a result of varying γ is usually about $1.2^{2/3}$, or about $\pm$ 15%. The liquid will normally be chosen for other characteristics than its surface tension.

Effect of $D_{w,25}$

$D_{w,25}$ is determined entirely by the type of sample being measured in the colorimeter, and is a variable over which we have no control. In LC, sample compounds commonly have molecular weights varying from about 200 to 1500, and in Table I it is seen that $D_{w,25}$ will then vary from 2.5 to 4 $\times$ 10^{-5}. The average value assumed in Figs. 4–6 (3.5 $\times$ 10^{-5}) will be close to actual values for most systems.

The dependence of σ_t on $D_{w,25}$ (eqn. 9) is small: $D_{w,25}^{0}$ to $D_{w,25}^{0.5}$. An example is given below, for variation of $D_{w,25}$ from 2 to 5 $\times$ 10^{-5}, and corresponding to a change in sample molecular weight (Table I) from 167 to above 4 $\times$ 10^{5}.

	$D_{w,25} = 2 \times 10^{-5}$	$D_{w,25} = 5 \times 10^{-5}$
d_t (cm)	0.2	0.2
n (sec^{-1})	2.7	4.2
σ_t (sec)	2.1	1.5

Here $F_t = 0.25$ ml/min, $\eta = 0.004$ and $\gamma = 25$, with n optimized.

Effect of reaction time t

The dispersion σ_t varies as $t^{1/2}$ (eqn. 9). Thus t should be kept as small as possible. For example, if the reaction of interest is complete in 5 min, there is little reason to make t much greater than 5 min. We can usually reduce t by increasing reaction temperature, but this should then be matched by increasing column temperature. The use of elevated temperatures for the reaction coil must also consider the bubble-size-change problem (see Appendix I).

Other effects

Other effects can increase the dispersion actually observed in segmented-flow reaction colorimeters. For example, where two sections of tubing are connected together (*e.g.*, at a tee) the connections should be perfectly butted to avoid pools of stagnant liquid. Similarly, if the tubing becomes coated by sample or reagents, the sample dispersion can increase greatly over that predicted by our basic model. Finally, the time during which column effluent is unsegmented must be held to an absolute minimum. Delay in segmenting the effluent, the pumping of unsegmented effluent prior to segmentation (*e.g.*, Fig. 1D), etc., can increase sample dispersion many times over that calculated for simple segmented-flow through a reaction coil.

Comparison of band dispersion with segmented vs. unsegmented flow

The dispersion of sample for unsegmented flow through a straight length of tubing is given (*e.g.*, ref. 16) as:

$$\sigma_t^2 = \pi d_t^4 L/384\, D_m F_t \tag{10}$$

Combining this relationship with eqn. 5 (and noting that $F = F_t$ for unsegmented flow) gives:

$$\sigma_t^2 = d_t^2\, t/96\, D_m \tag{10a}$$

Dispersion is independent of F for unsegmented flow. For a reaction time of 100 sec, a minimum practical value of $d_t = 0.05$ cm, and a typical value of D_m equal 2×10^{-5} cm^2/sec, eqn. 11a predicts a dispersion σ_t equal to 11.4 sec. This compares with a value of 0.4 sec previously calculated for *segmented*-flow (similar conditions). We thus see that dispersion (σ_t) with segmented flow is about 30-fold less than with unsegmented flow.

EXTRA-COLUMN BAND BROADENING

We are now in a position to assess the increased dispersion that will occur in typical LC systems, as a result of band broadening in the reaction colorimeter. Let the variance of a sample band as it leaves the column be given as $\sigma_{s,t}^2$ (time units), and let the allowable increase in $\sigma_{s,t}$ be some small fraction x, so that the final dispersion observed in the flowcell is given as $(1 + x)\,\sigma_{s,t}$. Then:

$$[(1 + x)\,\sigma_{s,t}]^2 = \sigma_{s,t}^2 + \sigma_t^2$$

which for small values of x reduces to:

$$\sigma_t = (2x)^{1/2}\,\sigma_{s,t} \tag{11}$$

The dispersion σ_t can be related to quantities of more direct interest in the design of LC separations: the column plate number N and the time of separation t_s. Beginning with the definition of N,

$$N = (t_R/\sigma_{s,t})^2 \tag{12}$$

and the dependence of retention time t_R on band capacity factor k' and breakthrough time t_0 (see ref. 1),

$$t_R = t_0 (1 + k') \tag{12a}$$

combination of eqns. 12 and 12a yields

$$\sigma_{s,t} = t_0 (1 + k')/N^{1/2}$$

which with eqn. 11 gives

$$\sigma_t = (2x)^{1/2} t_0 (1 + k')/N^{1/2} \tag{13}$$

Now the analysis of the preceding section suggests that for our standard conditions (Fig. 6), which are near-optimum, the minimum possible value of σ_t (d_t, n, F_t optimized) will vary between about 0.5 and 1.5 sec. Let us assume a typical value of $\sigma_t \approx 1$ sec, for which $t = 100$ sec. Since σ_t is proportional to $t^{1/2}$, for an optimized reaction colorimeter

$$\sigma_t \approx 0.1\, t^{1/2} \tag{14}$$

Substitution of eqn. 14 into eqn. 13 then gives t as a function of variables of direct interest:

$$t = 200\, x\, t_0^2 (1 + k')^2/N \tag{14a}$$

In isocratic separations, k' for the last eluted band is typically 3–15. Separation time t_s (equal t_R in eqn. 12a) is then usually 4–16 times greater than t_0. Since separation times in modern LC are typically of the order of 5–30 min, t_0 will usually vary from about 20–500 sec. Column plate numbers N usually vary from 200–10,000 plates.

Let us first assume the most stringent possible condition on extra-column band broadening in the reaction colorimeter, which is usually taken as no more than a 5% increase in $\sigma_{s,t}$ for the first-eluted band; *i.e.*, $x = 0.05$ and $k' = 0$. For this case, the following allowable reaction times t for different values of t_0 and N can be calculated from eqn. 14:

t_0 (sec)	t (sec)		
	$N = 200$	$N = 1000$	$N = 10{,}000$
25	31	6	0.6
100	500	100	10
500	12,500	2500	250

For other than very fast reactions, t will usually vary from 10–1000 sec. This means that very fast separations ($t_0 = 25$ sec, $t_s = 1.5$–7 min) are apparently precluded, except for rather low column plate numbers. Similarly, separations with very efficient columns appear to require either very fast reactions, or long separation times. In practice, such conclusions are overly restrictive, since reaction colorimetry confers great selectivity for many separations, and this selectivity can be traded for more than a 5% increase in bandwidth as a result of dispersion in the reaction coil. In many cases,

values of x equal to 0.1–0.2 are acceptable. In many other applications, loss of resolution at the beginning of the chromatogram (*i.e.*, for $k' = 0$) is of no great concern; rather, maximum resolution and minimum bandwidths in the region of k' equal 2–3 are required. An increase in k' from 0 to 2, coupled with an increase in x from 0.05 to 0.1, leads to an increase in all the above values of t by a factor of about 20. This practical adjustment greatly increases the range of reaction colorimetry, and precludes only reactions that are slow ($t > 200$ sec) in combination with separations that are either very fast and/or require a very large value of N. However, the preceding analysis does suggest that the use of reaction colorimetry should be properly coordinated with the design of a particular LC separation; *i.e.*, the separation/detection scheme should be optimized as a whole.

CONCLUSIONS

The preceding analysis allows us to calculate the dispersion of a reaction colorimeter as a function of experimental variables, and to minimize overall dispersion in the colorimeter by optimizing key variables. While general rules can be derived from calculations based on model cases, the final conditions for particular applications should be checked against eqn. 9. If dispersion within the reaction colorimeter is marginal at this point, further optimization should be pursued, using eqn. 9 in conjunction with possible changes in the LC separation system.

Generally the flow-rate of effluent from the column will have been chosen in order to optimize the separation within the column. Where a smaller flow-rate F_t is preferable from the standpoint of the reaction colorimeter, stream-splitting of the column effluent can be used. If larger values of F_t are advantageous, column diameter can usually be increased (maintaining linear velocity through the column constant) to give larger values of F_t without otherwise affecting the column separation. Alternatively, greater flow-rates of added reagents can be used to achieve larger final values of F_t, if the resulting increased sample dilution is acceptable.

Reaction colorimetry with segmented flow is compatible with high-performance LC separations for most applications. However, in some cases the overall LC system —including column separation and detector system— must be optimized *together* to limit dispersion and achieve the necessary resolution of all compounds of interest. The pressure drop across the reaction colorimeter has not been considered in this paper. In most cases it will be small, *e.g.*, less than a few p.s.i.

In the present paper it has been assumed that a photometer will be used in conjunction with chemical reaction. However, it should be obvious that other detection principles (*e.g.*, electrochemical, fluorescence, etc.) can also be used in conjunction with on-line post-column chemical reaction. The present theoretical analysis of dispersion in segmented-flow reaction detectors is equally applicable to any final detection principle.

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LIST OF SYMBOLS

d_t tubing internal diameter (cm)
D_m diffusion coefficient of sample in flowing liquid (cm²/sec)
D'_m effective diffusion coefficient in segmented-flow; see Table I and ref. 9
$D_{w,25}$ value of D'_m for water as liquid and 25°
F flow-rate of liquid plus air (ml/sec)
F_a flow-rate of air (ml/sec); $F = F_a + F_l$
F_c liquid flow-rate (ml/sec) through the flowcell, after debubbling; it is assumed that $F_c \approx 0.7\ F_l$
F_l flow-rate of liquid (ml/sec)
k' sample capacity factor in LC separation; equal to total quantity of sample in stationary phase divided by total quantity of sample in moving phase (see ref. 1)
L tubing length (cm)
n segmentation frequency, equal to number of air-bubbles injected per unit time (sec^{-1})
N column theoretical plate number
t holdup time of reaction coil; time spent by a liquid segment in the coil (sec)
t_0 time required for mobile-phase molecules to pass through an LC column (sec); see ref. 1
t_R retention time for an eluted sample band (sec); see ref. 1
t_s time for completion of an LC separation (sec); equal to retention time for last eluted band in a chromatogram (see eqn. 12a)
u linear velocity of moving segments (cm/sec)
V_a volume of an air-bubble (ml)
V_c volume of the colorimeter flowcell (ml)
V_s volume of a liquid segment (ml)
x allowable increase in bandwidth (or $\sigma_{s,t}$) of an eluted band after passage through the reaction coil, expressed as a fraction; see eqn. 11 and preceding discussion
γ liquid surface tension (dynes/cm)
η liquid viscosity (P)
σ measure of spreading or dispersion of sample after flow through a given tube, equal to square root of variance (measured as number of actual segments); dimensionless; see ref. 9
$\sigma_{s,t}$ width of eluted band as it leaves the column, in time units (sec), and expressed as the square root of the band variance
σ_t value of σ in time units (sec); eqn. 1a
σ'_t dispersion resulting from flow through reaction coil plus flowcell; equal to $(\sigma_t^2 + \sigma_{t,c}^2)^{1/2}$ (sec)
$\sigma_{t,c}$ dispersion arising from flow through flowcell alone (unsegmented flow) (sec)
σ_V value of σ in volume units (ml); see eqn. 1
$\sigma_{V,c}$ value of $\sigma_{t,c}$ in volume units (ml)

APPENDIX I

Change in air bubble size after injection

Normally the injected air-bubbles will not change size significantly after the

initial equilibration of pressure at the point of injection. However, several effects can lead to significant changes in air-bubble size following injection, and these lead to changes in F_a and a (normally) small change in σ_t (σ_t increasing with F_a, as discussed in the main text).

Since we normally operate with low-viscosity liquids, boiling perhaps 15–30° above the temperature of the reaction colorimeter system, these liquids will have an appreciable vapor pressure: P_1. If the injected air-bubbles are not pre-equilibrated with liquid, the final air-bubble volume V_a will be related to the initial value V_a^0 as:

$$V_a = (1 + P_1)\, V_a^0 \tag{A-1}$$

If the temperature of the system changes after injection of the air-bubble from T_1 to T_2 (°K), and the final liquid vapor pressure is P_1', the final value of V_a is then given as:

$$V_a = (T_2/T_1)\,(1 + P_1')\, V_a^0 \tag{A-2}$$

Where there is a pressure difference between the point of bubble injection and the flowcell, the air-bubbles will also increase in size as they move through the reaction coil. There is in principle some reduction in size of the bubbles as a result of dissolution of gas into the flowing liquid (assuming the liquid has not been pre-equilibrated with the gas). Where air is used for stream segmentation, this effect is usually quite small. One could conceivably use gases other than air, whose solubility is appreciable, but normally this would not be desirable.

APPENDIX II

Debubbling the reaction mixture before the flowcell

Normal segmentation rates in continuous-flow analysis do not exceed 1.5 sec^{-1}, and possible problems with debubbling (prior to the flowcell) at much higher rates may exist. However, the optimum n values suggested by Fig. 6 are in the range of 10–30 sec^{-1}, with little increase in σ_t for n equal 5–10. We tentatively conclude that segmentation rates of 5–10 are possible, which should result in little impact on the minimization of σ_t.

With debubbling of the reaction stream prior to the flowcell, we must also consider added extra-column dispersion as a result of flow through the debubbler and flowcell. If a tee-debubbler is used with minimal connector length between the air take-off leg and the flowcell, it appears that the debubbler should add little to overall dispersion relative to debubbler plus connector. It has been stated[18] that the dispersion due to the flowcell should be given as:

$$2\sqrt{3}\, V_c > \sigma_{V,c} > V_c$$

Here $\sigma_{V,c}$ is the volume dispersion due to the flowcell (same units as σ_V). The flowcell dispersion in time units $\sigma_{t,c}$ is then:

$$2\sqrt{3}\, V_c/F_c > \sigma_{t,c} > V_c/F_c$$

Here F_c is the volume flow-rate (ml/sec) of liquid through the flowcell. Typically about 50–70% of the liquid entering the debubbler is recoverable (free of bubbles),

and we will assume a recovery here of 70%. Calculating (eqn. 10a) $\sigma_{t,c}$ for various values of F_t, and $V_c = 0.002$ or 0.008 ml:

F_t	$\sigma_{t,c}$ (sec)	
	$V_c = 0.002$	$V_c = 0.008$
0.01	0.3–1.0	1.1–4.0
0.025	0.1–0.4	0.4–1.6
0.05	0.0–0.1	0.3–0.9

We can next calculate the total dispersion in reaction coil plus flowcell $\sigma_t' = (\sigma_t^2 + \sigma_{t,c}^2)^{1/2}$ from the above values plus data of Fig. 6:

F_t	σ_t' (sec)	
	$V_c = 0.002$	$V_c = 0.008$
0.01	0.6–1.1	1.2–4.0
0.025	0.6–0.7	0.7–1.7
0.05	0.8–0.9	0.9–1.2

These values can be compared with the previously optimized σ_t values for passage of segmented liquid through the flowcell: 0.85 sec for $V_c = 0.002$ and 1.45 sec for $V_c = 0.008$. It thus appears that debubbling before the flowcell can be marginally advantageous, particularly for flowcells larger than 0.002 ml in volume. This conclusion is somewhat surprising, since it might appear that segmentation within the flowcell should always decrease total dispersion. However, the latter conclusion overlooks the constraints on n imposed by the flowcell volume (*i.e.*, $V_s = 2\ V_c$). It should be emphasized, however, that this conclusion is based on particular experimental conditions and certain assumptions. Other experimental work has shown[19] that retention of segmented flow through the flowcell can be decidedly advantageous. Finally, it must be noted that debubblers are less reliable and more cumbrous to use than electronic debubbling.

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