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PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY UNDER ISOCRATIC CONDITIONS

II. THE ROLE OF COLUMN VARIABLES

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SUMMARY

In order to understand better the complex interaction of different separation variables in preparative high-performance liquid chromatography, general equations were derived that relate production rate and run time plus optimum column length and flow-rate to maximum operating pressure, particle size and sample molecular weight. Computer simulation was also used to illustrate optimum conditions for representative cases.

INTRODUCTION[a]

Previous theoretical work (see discussion in Part I[1]) makes it clear that the separation achieved in a preparative high-performance liquid chromatographic (HPLC) run is related to the column conditions (column length L and diameter d_c, column-packing particle size d_p and flow-rate F) in terms of two quantities: the plate number N_0 (for a small sample) and the column capacity w_s. That is, provided that N_0 and the column loading (w/w_s) are constant, the separation (recoveries and purities of various sample components) will not change. This in turn means, other conditions remaining equal[b], that the production rate is given by

$$P_R = \text{constant} \cdot [Ld_c^2/t_{R_0}]_{N=\text{constant}} \quad (1)$$

Because N_0 is a function of column length, particle size and flow-rate, the question then arises as to which column conditions are optimum for a particular preparative

[a] A list of all symbols used in Parts I–III is included in ref. 1.

[b] *i.e.*, same retention (values of k' and α for a small sample) and a constant ratio of sample mass to column capacity (w/w_s) in the preparative run.

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separation. This requires that we be able to describe production rate P_R quantitatively in terms of column conditions.

The relationship between production rate and column conditions has been addressed previously by Knox and Pyper[2], Snyder *et al.*[3] and (as this paper was being completed) Golshan-Shirazi and Guiochon[4]. The essential features of this question are largely covered in ref. 2, with some additonal details and quantitative refinements in refs. 3 and 4. In examining these various treatments, however, it appears to us that there is room for further discussion, especially as some confusion still exists in the minds of many chromatographers (*e.g.*, see ref. 5).

THEORY

Our treatment assumes that sample retention (k', α) is fixed by the choice of experimental conditions. For simplicity we shall consider a sample composed of two components X and Y, where the small-sample retention times are designated as t_x and t_y ($t_x < t_y$). However, this will not affect the generality of the following discussion. We shall also assume a fixed column diameter, as this parameter can be varied independently (any change in d_c will result in a change in production rate by the factor d_c^2, provided that the flow-rate is changed by the same factor). Under these conditions, eqn. 1 becomes

$$P_R = \text{constant} \cdot [L/t_y]_{N=\text{constant}} \tag{2}$$

i.e., the production rate is proportional to the column length divided by the (small-sample) retention time of the later-eluting band Y.

Knox equation

In the following treatment we are interested mainly in the so-called column conditions: column length L, particle size d_p and flow-rate F. Together with the choice of mobile phase and sample, these variables determine the column plate number N_0 (small sample). We can interrelate these different parameters (L, d_p, etc.) in terms of their effect on N_0, which can in turn be described quantitatively by means of the well known Knox equation[6]:

$$h = Av^{1/3} + B/v + Cv \tag{3}$$

where h is the reduced plate height, v is the reduced velocity and A, B and C are constants for a given HPLC system (defined sample and separation conditions). The reduced parameters h and v are further defined as

$$h = H/d_p = L/(N_0 d_p) \tag{4}$$

and

$$v = ud_p/D_m \tag{5}$$

where H is the plate height (L/N_0), u is the linear velocity of the mobile phase and D_m is the solute diffusion coefficient in the mobile phase.

Knox and Pyper[2] used eqn. 3 to describe how the production rate varies with the column conditions, including pressure, but they simplified their treatment by assuming that $h \approx$ constant $\cdot\ v^a$. This is a reasonable first approximation; however, it is possible to predict values of the constants A, B and C in eqn. 2 so as to obtain more accurate estimates of N_0 as a function of the experimental conditions for a wide range of reversed-phase HPLC systems: small molecules in isocratic elution[7,8] or gradient elution[9,10] and large molecules such as peptides and proteins in similar HPLC systems[10]. We shall make use of eqn. 3 (without assuming $h \approx Cv$) to obtain a more complete description of the effects of column conditions on production rate.

It is also useful to generalize the role of column conditions by recourse to another approximation for eqn. 3[11]:

$$h = \text{constant} \cdot v^n \tag{6}$$

Eqn. 6 (used also to a limited extent by Knox and Pyper[2]) provides a good fit to eqn. 3 over a fairly wide range of flow-rate (or values of v[b]). For practical conditions ($v > 3$), the parameter n lies between 0 and 1. Eqn. 6 will allow additional insight into how production rate, run time and other separation characteristics are affected by changes in separation conditions. The treatment of Knox and Pyper[2] is equivalent to assuming $n = 1$.

"Primary" separation variables

We shall arbitrarily define certain separation variables as "primary" and others as "secondary" (*i.e.*, dependent on the primary variables). The column plate number N_0, column pressure P, particle size d_p and solute molecular weight M are designated as "primary" variables[c]. Because of the form of eqn. 6, it will be seen that we can express other ("secondary") variables [P_R, run time (t_R), L, F, etc.] in terms of a single general function of these primary variables:

$$\text{secondary variable} = \text{constant} \cdot N_0^w p^x d_p^y M^z \tag{7}$$

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[a] Knox and Pyper[2] examined the error in assuming $h \approx Cv$, but they did not pursue its consequences in detail. Golshan-Shirazi and Guiochon[4] use eqn. 3 (rather than assuming $h \approx Cv$) with "average" (fixed) values of A, B and C.

[b] Eqns. 57–59 of ref. 1 (based on our eqn. 6) provide optimum values of the particle size, production rate and reduced velocity v as a function of experimental conditions. However, the practical consequences of these relationships are not explored in ref. 1, and it is easy to draw misleading conclusions. In following derivations based on eqn. 6 (eqns. 13–17), the proportionality constant of eqn. 6 cancels. A reviewer has questioned what effect this assumption will have on our later conclusions derived from eqns. 13–17. This question is answered in Table IV, where predictions based on eqns. 13–17 are compared with model calculations from the more accurate eqn. 4. It is found that predictions based on eqn. 6 agree with those derived from eqn. 4.

[c] By "primary" we mean "effectively independent", and by "secondary" we mean "dependent". Thus our choice of conditions (α, k_0) other than L, d_p and F determines N_0 for the separation, *i.e.*, N_0 becomes an independent variable. Similarly, equipment considerations determine the pressure P, the sample fixes the molecular weight M, and we can vary particle size d_p independently of L and F. Our choice of these parameters (N_0, P, M and d_p) then determines the remaining variables discussed here.

TABLE I
APPROXIMATE DEPENDENCE OF EMPIRICAL FACTOR n ON REDUCED VELOCITY v
Assumes Knox equation with $A = 2$, $B = 1$ and $C = 0.05$[8].

v	n
3	0.0
10	0.4
30	0.5
100	0.7
300	0.8
1000	0.9

The exponents w–z will be shown to be functions of the parameter n, which is defined by the value of v (see Table I).

"Secondary" separation variables

Equations that describe various secondary variables can be derived from the following well known relationships[12]:

$$P = \text{constant} \cdot uL/d_p^2 \tag{8}$$

$$t_R = t_0(1+k_y) = \text{constant} \cdot t_0 \tag{9}$$

$$t_0 = L/u = V_m/F = \text{constant} \cdot (L/F) \tag{10}$$

$$D_m = \text{constant} \cdot M^v \tag{11}$$

where u is the mobile phase velocity, k_y is the capacity factor of the last band Y, t_0 is the column dead time, V_m is the column dead volume, D_m is the solute diffusion coefficient and M is the solute molecular weight; eqn. 11 is an empirical relationship, where the exponent v varies between 0.3 and 0.6 depending on molecular weight[10]; here we shall assume $v \approx 0.5$.

Flow-rate, column length and run time. Eqns. 4–6 and 8–10 can be combined to yield

$$N_0 = \text{constant} \cdot Pd_p^{1-n}F^{-1-n}D_m^n \tag{12}$$

which can be rearranged to

$$F = \text{constant} \cdot P^{1/(1+n)}d_p^{(1-n)/(1+n)}D_m^{n/(1+n)}N_0^{-1/(1+n)} \tag{13}$$

Similarly, eqns. 8, 10 and 13 give

$$L = \text{constant} \cdot P^{n/(1+n)}d_p^{(1+3n)/(1+n)}D_m^{-n/(1+n)}N_0^{1/(1+n)} \tag{14}$$

and Eqns. 9, 10, 13 and 14 yield

$$t_R = \text{constant} \cdot P^{(n-1)/(1+n)} d_p^{4n/(1+n)} D_m^{-2n/(1+n)} N_0^{2/(1+n)} \quad (15)$$

Production rate. Eqns. 2, 11, 14 and 15 give an expression for the production rate:

$$P_R = \text{constant} \cdot N_0^{-1/(1+n)} P^{1/(1+n)} d_p^{(1-n)/(1+n)} M^{2n/(1+n)} \quad (16)$$

The effect on the production rate of changes in pressure, particle size, sample molecular weight or plate number is given by eqn. 16. Table II summarizes these relationships (eqns. 13–16) and gives numerical values of the coefficients w–z for $n = 0$ and 1.

Reduced velocity. A similar description of v can be obtained from eqns. 5, 10 and 13 (also derived in eqn. 2):

$$v = \text{constant} \cdot N_0^{-1/(1+n)} P^{1/(1+n)} d_p^{n/(1+n)} D_m^{-1/(1+n)} \quad (17)$$

RESULTS AND DISCUSSION

The preceding treatment allows an examination of production rate, run time and other separation characteristics for both the general and specific cases.

General relationships

Production rate. Eqn. 16 and Table II are of interest here. First, consider the role of operating pressure in preparative HPLC. According to Table II, P_R varies with pressure as $P^{0.5-1.0}$, depending on the reduced velocity v. This means, other factors remaining equal, that there is always a substantial advantage to operating at higher pressures. This in turn has motivated some manufacturers to stress a high-pressure capability for their preparative HPLC columns, *e.g.*, 2–4000 p.s.i. The use of higher pressures requires a corresponding increase in column length and flow-rate in order to maintain an optimum value of N_0 for the separation[a].

The question of which particle size is best for a given preparative separation has not yet been answered to everyone's satisfaction (the discussion in ref. 5 is revealing). According to Table II, production rate varies with particle size as $d_p^{0.0-1.0}$; *i.e.*, larger particles generally favor higher values of P_R, other factors remaining equal. At first glance, this is a surprising finding[b]. However, when we examine specific separations

[a] Golshan-Shirazi and Guiochon[4] pointed out that there is an optimum pressure for a given preparative separation, which is true but misleading. Their optimum pressure corresponds to the case when $v \ll 3$, which is never a good choice in practice (*i.e.*, this case corresponds to lower N_0 and longer run-time). In any case, pressures larger than their "optimum" can in principle always be used to achieve higher production rates by using larger particles (so as to increase v). Of course, practical considerations of various kinds impose very definite limits on maximum pressure.

[b] The reason for a smaller production rate with smaller particles can be seen as follows. Knox and Pyper[2] have shown, based on the assumption $h = Cv$, that production rate is independent of particle size. This approximation will be valid for larger particles, because then the reduced velocity v is large (*cf.*, eqns. 3 and 5). As the particle size is decreased, v decreases (see the examples in Table III) and eventually the term $Av^{1/3}$ in eqn. 3 becomes significant compared with Cv, *i.e.*, h is larger than predicted from the approximation $h = Cv$. This means a smaller value of N_0, other factors remaining equal, which translates into a longer time required to increase N_0 back to the value predicted assuming $h = Cv$.

TABLE II

DEPENDENT VARIABLES AS A FUNCTION OF THE "PRIMARY" VARIABLES N_0, P, d_p AND M (Eqn. 6)

Secondary variable = constant · $N_0^w P^x d_p^y M^z$.

Secondary variable	n	*Primary variable*			
		N_0 $w =$	P $x =$	d_p $y =$	M $z =$
Production rate P_R			$\frac{1}{(1+n)}$	$\frac{(1-n)}{(1+n)}$	$\frac{-n}{2(1+n)}$
	0	[a]	1	1	0
	1		1/2	0	−1/4
Run time t_R		$\frac{2}{(1+n)}$	$\frac{(n-1)}{(1+n)}$	$\frac{4n}{(1+n)}$	$\frac{n}{(1+n)}$
	0	2	−1	0	0
	1	1	0	2	1/2
Column length L		$\frac{1}{(1+n)}$	$\frac{n}{(1+n)}$	$\frac{(1+3n)}{(1+n)}$	$\frac{1}{2(1+n)}$
	0	1	0	1	0
	1	1/2	1/2	2	1/4
Flow-rate F		$\frac{-1}{(1+n)}$	$\frac{1}{(1+n)}$	$\frac{(1-n)}{(1+n)}$	$\frac{-n}{(1+n)}$
	0	−1	1	1	0
	1	−1/2	1/2	0	−1/4
Reduced flow-rate v		$\frac{-1}{(1+n)}$	$\frac{1}{(1+n)}$	$\frac{2}{(1+n)}$	1/2
	0	−1	1	2	1/2
	1	−1/2	1/2	1/4	1/2

[a] The dependence of production rate on N_0 is not of interest, as there is a optimum value of N_0 for maximum P_R.

having different values of α (see the following section), we shall see that this apparent advantage of larger particles is often minor. Further, larger particles require longer run times and longer columns (larger amounts of column packing), which can represent offsetting factors.

The effect of molecular weight on production rate (Table II) is predicted to be minor: P_R will vary as (mol. wt.)$^{0.0-0.25}$. Hence an increase in sample molecular weight from 500 to 5000 should lead to a decrease in P_R by a factor of about two. However, this assumes that the large-pore columns which are used for large-molecule separations will have the same capacity (w_s) as columns used for small-molecule chromatography, which is not the case. For this reason, and because of the so-called "non-ideal" behavior[10,13] of many such separations, production rates can be expected to be significantly lower for most large-molecule samples.

Run time. Although production rate is of major interest in preparative HPLC, total run time is also important. Some combinations of operating conditions (see

below) lead to differences in run time, while holding the production rate constant. In these cases, the production of a given amount (batch) of purified product will require more runs in separations in which the run time is shorter.

Long run times can lead to lower recoveries of purified products, owing to losses that occur as a result of the separation process. Longer run times can also be inconvenient to schedule and to support, particularly in R&D applications. Eqn. 15 and Table II provide insight into the effects of pressure, particle size and sample molecular weight on run time.

Run time varies with pressure as $P^{0.0-(-1.0)}$. Therefore, other factors remaining equal, higher pressures mean shorter run times. Hence higher pressures are generally advantageous, for both maximum production rate and short run times. Run time varies with particle size as $d_p^{0.0-(-1.0)}$, *i.e.*, smaller particles favor shorter run times. Faster run times represent a significant advantage of small particles in many applications. Run time varies with sample molecular weight as (mol. wt.)$^{0.0-0.5}$, meaning that large molecules usually require longer run times, other considerations remaining the same. As large biomolecules are especially prone to separation-related reaction and loss, small particles (and short run times) are particularly favored for this class of compounds.

Column length. In general, we desire a maximum production rate with the shortest possible columns, especially in the research laboratory. The required column length is seen (Table II) to vary with pressure as $P^{0.0-0.5}$, so that higher pressures require longer columns. Column length varies with particle size as d_p^{1-2}. Therefore, smaller particles allow us to work with shorter columns, and this is often desirable[a]. The required column length varies with sample molecular weight as (mol. wt.)$^{0.0-0.25}$, that is, there is little need to change the column length for samples of different molecular weight.

Other variables. The major contributions to separation have now been examined in general terms. The effects of other, less important parameters can be derived in a similar fashion. For example, a higher temperature lowers pressure (lower viscosity) and increases D_m. This can be shown by similar derivations as above to increase P_R, decrease run time, and increase column length. The use of a less viscous mobile phase will have similar effects on production rate, run time and column length. However, the choice of temperature and mobile phase is usually dictated by other concerns.

Specific examples. Further insights into the best combination of conditions for a given separation can be obtained from illustrative examples. It is possible (for maximum production rate) to accurately predict column plate number as a function of conditions[7–10,b]; this in turn permits the selection of optimum conditions for any required value of the column plate number N_0. Given a particular separation with specified values of k_y and α and some required recovery and purity values for the final product, it is possible to specify an optimum plate number and sample size for the

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[a] An exception occurs when the optimum value of N_0 is fairly low, in which event small-particle columns may require inconveniently short lengths.

[b] The calculations of plate number required in Table III were carried out using DryLab I software (LC Resources, Lafayette, CA, U.S.A.). This software uses accurate predictions of the Knox parameters A, B and C (eqn. 3) as a function of all experimental conditions[7,8], in contrast to the usual assumption of "average" values of A, B and C.

TABLE III

COLUMN LENGTH, FLOW-RATE, SEPARATION TIME (t_y) AND PRODUCTION RATE AS A FUNCTION OF COLUMN-PACKING PARTICLE SIZE, COLUMN PRESSURE AND α; CALCULATIONS BASED ON USE OF DRYLAB I COMPUTER SIMULATION SOFTWARE

Conditions and separation parameters: mobile phase; methanol–water (1:1); column temperature, 25°C; column diameter, 0.46 cm; column parameters: $A = 0.8$, $X = 0.75$, $Y = 0.70$; mol. wt. of solute, 500. Optimum plate numbers N_0 and sample sizes (w_y/w_s) from Part I[1]; 95% recovery of 99% pure product.

Conditions	d_p *(μm)*	L *(cm)*	F *(ml/min)*	t_R *(min)*	n	w_y/w_s	P_R *(mg/h)*
$\alpha = 1.2$, $N_0 = 2100$, $P = 500$ p.s.i.	5	7	0.8	1.5	0.5	0.0048	25
	10	24	0.9	5	0.6		27
	20	90	1.0	17	0.7		29
	40	340	1.1	60	0.8		31
$\alpha = 1.2$, $N_0 = 2100$, $P = 2000$ p.s.i.	5	12	1.8	1.2	0.6		55
	10	45	2.0	4	0.7		59
	20	170	2.1	15	0.8		62
	40	67	2.2	58	0.9		65
$\alpha = 1.5$, $N_0 = 500$, $P = 500$ p.s.i.	5	3	1.9	0.3	0.6		250
	10	11	2.0	1.0	0.7		270
	20	40	2.1	4	0.8		280
	40	160	2.2	14	0.9		290
$\alpha = 1.5$, $N_0 = 500$, $P = 2000$ p.s.i.	5	5	4	0.2	0.7		540
	10	20	4	0.9	0.8		560
	20	80	4	3	0.9		580
	40	320	5	14	1.0		590

separation[1] (for a maximum value of P_R). Here we shall assume that 95% recovery of 99% pure product is required, from a mixture of two compounds present in equal concentrations. As detailed in Part I[1], for $k_x = 1$ we have the following plate numbers and sample sizes as a function of α: $\alpha = 1.2$, $w_x/w_s = 0.0048$, $N_0 = 2100$; $\alpha = 1.5$, $w_x/w_s = 0.021$, $N_0 = 500$. Other conditions are given in Table III.

Table III summarizes the required values of column length, flow-rate and resulting production rates P_R for $\alpha = 1.2$ or 1.5 and various particle sizes. These examples can be used both to illustrate and to confirm the relationships in Table II. Table III also provides additional insight into the question of the "best" particle size in preparative HPLC.

The n values in Table III vary between 0.5 and 1.0 for this range of α and N_0, and we shall assume an average value of $n = 0.8$ in the following discussion[a]. In the examples of Table III the pressure has been varied by 4-fold (from 500 to 2000 p.s.i.) and the particle diameters vary in steps of 2-fold. Table IV compares the change in production rate P_R, run time t_R, column length L and flow-rate F as predicted from Table II (with $n = 0.8$) and as calculated in Table III. Close agreement between these two sets of values is observed. The computer-simulation (DryLab) values in Table IV come from the original Knox equation (eqn. 3), so the resulting comparisons are meaningful.

[a] For smaller values of α the optimum value approaches $n \approx 0.5$, which was assumed in Part I[1].

TABLE IV

COMPARISON OF DATA IN TABLE III (COMPUTER SIMULATION) WITH PREDICTIONS FROM EQUATIONS IN TABLE II

Separation characteristic i	*Table*	*Change in i for 4-fold change in P*[a]	*Change in i for 2-fold change in* d_p[b]
Production rate	II	2.2-fold	1.1-fold
	III	2.1–2.6	1.0–1.1
Run time	II	0.8-fold	3.4-fold
	III	0.8–0.9	3.2–3.8
Column length	II	1.8-fold	3.7-fold
	III	1.8–2.0	3.5–3.9
Flow-rate	II	2.2-fold	1.1-fold
	III	2.0–2.2	1.0–1.1

[a] For change in pressure from 500 to 200 p.s.i.
[b] For a 2-fold increase in particle diameter.

Table III also illustrates the trade-offs between column length and run time as a function of particle size. For operation at 2000 p.s.i. and $\alpha = 1.2$, the column length varies from 12 to 670 cm as the particle size is changed from 5 to 40 μm, while the run time varies from 1.5 to 60 min. Depending on our preferences with respect to column length and run time, we have a variety of choices. We are also free to choose a plate number that is larger or smaller than the value of N_0 which is predicted to yield the optimum production rate; a change in N_0 of $\pm 50\%$ relative to the optimum value will usually reduce the production rate by no more than 10–20% (see Part I[1]).

The predictions in Table III are for a specific set of conditions. Before generalizing these results for other separations, it is necessary that any differences in experimental conditions be taken into account. The relationships in Table II can be useful in this regard.

CONCLUSIONS

For every preparative HPLC separation, there will be an optimum plate number N_0 and sample size that yields a maximum production rate of purified product. This value of N_0 is dictated by the retention characteristics of a small sample (k', α) and the desired recovery of product. Column-packing particles of any diameter can be used to achieve the required plate number N_0, but this in turn determines the column length, flow-rate and run time. The effect of particle size on production rate is generally minor, and the choice of the best particle size will usually be chosen for other reasons, *e.g.*, run time, column length or total amount of column packing required.

General relationships have been derived that show the effects of different conditions (column pressure, particle size, sample molecular weight) on production rate, run time, column length and flow-rate. These equations have been expressed in terms of the parameter $n = (\mathrm{d}[\log h]/\mathrm{d}[\log v])$, which allows additional insight into the effects of column conditions on the latter parameters. These relationships (plus the specific examples shown here) and the conclusions in Part I[1] allow a good understanding of how to choose the best conditions for a given preparative HPLC separation.

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