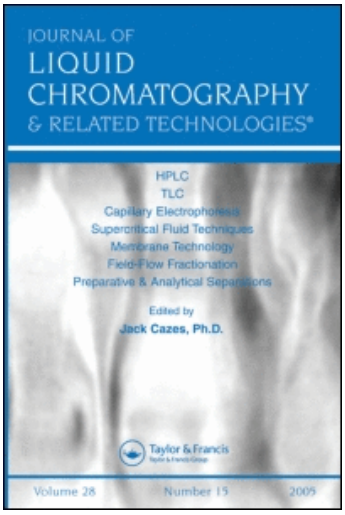

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Extra-Column Volume in CCC

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Abstract: Countercurrent chromatography (CCC) is used by natural products and medicinal chemists for fractionation of extracts of flora and fauna to screen for pharmacological activity, and in isolating and purifying gram quantities of individual natural and synthetic compounds. The large extra-column volume in CCC apparatus, particularly in the coil-planet centrifuge designs employing an anti-twisting flow line assembly, may lead to errors in the calculation of the distribution ratios of eluting fractions. The effect of extra-column volume is to delay the time required for a peak to reach the detector. This delay is equal to the transit time of the extra-column volume plus half of the mobile-phase portion of the sample volume. The effect is negligible for columns of large volume (300 mL) but becomes more significant for small columns (15 mL) and for large sample volumes. Manual correction for the effect is very tedious, but results obtained using a generally applicable spreadsheet are presented and discussed. A second spreadsheet is also indicated wherein peak retention times, along with sample and extra-column volume, are entered for calculation of distribution constants, which are summarized on a single page convenient for entry in a notebook.

Keywords: CCC, Countercurrent chromatography, Extra-column volume, Sample injection, Coil-planet centrifuge, K, K_c, Partition coefficient, Distribution constant

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INTRODUCTION

IUPAC Nomenclature

The distribution constant, K_c, in chromatography is defined by the IUPAC as the ratio of the analytical concentration of solute in the stationary phase, divided by its analytical concentration in the mobile phase.[1] Analytical concentration includes all forms (unionized, ionized, complexed, associated, etc.) in which the solute may exist. The term distribution constant is recommended by the IUPAC in preference to partition coefficient, which has been widely used in partition chromatography. They also recommend upper case subscripts, M, S, R, to refer to mobile and stationary phases and peak retention, and to place more than a second subscripted letter in parentheses. Lower case subscripts are recommended for physical parts of the system. These recommendations are largely followed in this paper. But, in view of the complexity of some of the subscript notation, the subscript c is dropped from K in some symbols. However, K in this paper is equivalent to K_c as defined above.

The IUPAC also recommends the use of the term extra-column volume (in preference to the term dead-volume) for the flow-line volume exterior to the column itself from the injection point to the detector. This term, with the symbol V_{fD}, will be used here. An additional term, to represent the total extra-column volume, V_{fT}, between the injection point and the effluent collection point is also introduced.

Solute Retention and Distribution Constant

If the extra-column volume and sample injection volume are negligibly small, the distribution constant, K_c, can be calculated directly from the general equation for chromatographic retention

$$V_R = V_M + K_c V_S \tag{1}$$

after recasting Eq. 1 as

$$K_c = [V_R - V_M]/V_S \tag{2}$$

All symbols are defined in a list following the text. Equation 2 allows calculation of K_c using measurements of volume, distance or time, measured from the sample injection point, constant flow rate being assumed.[2] Equations presented here will be expressed in volume, but units can be converted to time by dividing by the flow rate.

Extra-Column Volume

All countercurrent chromatographs contain extra-column volume, which can be appreciable, particularly in small units of the coil-planet centrifuge

configuration.[2,3] A dynamic method to determine the extra-column volume in CCC, based on measurement of the stationary phase volume as a function of flow rate, has been published.[4] CCC provides an absolute method for determining solute distribution constants, particularly octanol/water distribution constants.[5] The volume of the inlet and outlet flow lines of a typical coil-planet centrifuge CCC may range from 0.1 mL to almost 2 mL, depending on the internal diameter and length, as summarized in Table 1. The system may include several other extra-column volumes illustrated in Figure 1, in addition to the flow lines to and from the coil (a, b, and e, f), These include a link (i, a), of negligible volume, between the injection valve and a flow-switching valve; short lengths of tubing (b, c and d, e) from the coil inlet and outlet ports to the helical winding itself; accessories, such as an effluent heater; inlet and outlet leads for the detector, and tubing (k, p) ending at the collection vessel. These volumes are conveniently combined into those prior to the coil, $V_{f(i.c)}$, those between the coil and the detector cell, $V_{f(d.h)}$, and those from the detector cell to the collection vessel, $V_{f(h.p)}$. For calculations, the first two of these are added to give V_{fD},

$$V_{fD} = V_{f(i.c)} + V_{f(d.h)} \tag{3}$$

representing all of the pre-detector extra-column volume. Post-detector extra-column volume, V_{fE}, also exists between the detector and the collection vessel, $V_{f(h.p)}$, in Figure 1. Adding this post-detector volume to the pre-detector volume V_{fD}, gives the total extra-column volume, V_{fT}, of the CCC system (Eq. 4).

$$V_{fT} = V_{fD} + V_{fE} \tag{4}$$

The Generalized Countercurrent Chromatogram

All countercurrent chromatograms can be represented in terms of the distribution constants of the solutes, as shown in the generalized format illustrated in Figure 2, for a system with negligible extra-column volume. Figure 2 is a

Table 1. Typical volume of inlet and outlet flow tubes

Tubing	Internal diameter (mm)	Internal cross-section area, (mm^2)	Length (cm)	Volume, mL	
				1 tube	2 tubes
Zeus no. 20	0.86	0.58	150	0.87	1.74
Zeus no. 20	0.86	0.58	72	0.41	0.82
1/16 PTFE	0.50	0.20	72	0.14	0.29
1/16 PTFE	0.30	0.07	72	0.05	0.10

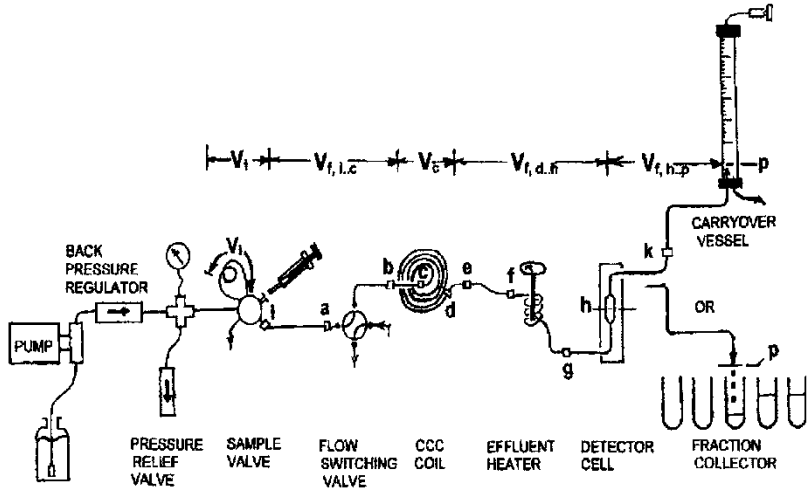

Figure 1 Extra-column volume in a CCC system.

visual representation of Eq. 1, where intervals on the horizontal axis are presented as multiples of V_S beyond the mobile phase front, V_M, where a solute with a K_c of zero elutes. The solute with K_c of 1 always elutes at a retention volume equal to one column volume, V_C. A non-retained solute, with a K_c of 0, elutes one stationary phase volume earlier, at the mobile phase front, with a retention volume of V_M. Solutes with integrally greater K_c values elute at integral multiples of the stationary phase volume, V_S, beyond V_M. Once the index points for V_0 or t_0 and V_1 or t_1 are indicated on the chromatogram, the K_c values of the peaks can be readily estimated by visual linear interpolation between and beyond these indices, or calculated as

$$K_c = [V_R - V_0]/[V_1 - V_0] = [t_R - t_0]/[t_1 - t_0] \tag{5}$$

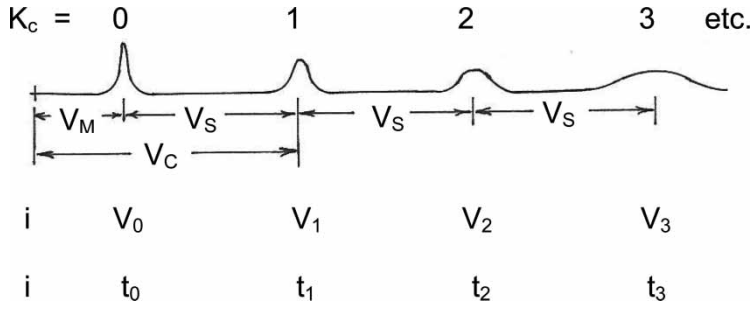

Figure 2. Generalized countercurrent chromatogram.

The exact location of the V_0 or t_0 and V_1 or t_1 points on a chromatogram will permit accurate calculation of solute distribution ratios or, conversely, will allow accurate prediction of the retention times of solutes for which K_c is known.

Effects of Sample Volume and Extra-Column Volume

When appreciable extra-column volume is present, or a sample of significant volume is injected, the effect will be to delay arrival of each peak at the detector by a volume V_{dD}, equal to the extra-column volume, V_{fD}, prior to the detector, plus a volume that depends on the phase used for sample injection, the injection volume, and in the case of stationary phase or mixed-phase injection, on the phase volume ratio, V_M/V_S, in the column. This delay will be evaluated for three situations: 1, injection of a sample in mobile phase; 2, injection in stationary phase; and 3, injection in a mixture of mobile phase and stationary phase. The exact solution in the third case will be limited to dissolution of a sample in a mixture of mobile and stationary phases in the phase volume ratio, β, existing in the column and will be referred to as a β-mixed-phase injection. The equation for the third case will reduce to that applicable to cases 1 or 2, where only one phase is injected.

Sample Injection in the Mobile Phase

The formation of the sample zone, and subsequent chromatography, for mobile phase injection is illustrated in Figure 3. A particular case is depicted, where the phase volume ratio, β, is $1/2$, corresponding to a stationary phase retention, S_F, of 0.67. The sample contains a nonretained marker plus a component which elutes later. The column (with the detector) is moved to the right in successive views so that the effluent stream and the associated chromatogram start from zero in each view. Figure 3A shows a pre-detector extra-column volume, V_{fD}, of 1 mL and a sample volume, V_i, of 2 mL, in mobile phase only, prior to sample injection. Figure 3B shows the extra-column volume of 1 mL having entered the column and sample half-injected, at which time 2 mL of mobile phase has exited the column, generating a baseline 2 mL long. In Figure 3C, the midpoint of a nonretained marker is eluting at a retention volume of 7 mL. Since the marker must elute at a V_M of 5 mL after entering the column, it has been delayed in reaching the detector by V_{dD}, which equals the pre-detector extra-column volume, V_{fD}, plus half the sample injection volume, V_i. But, to express the relationship more generally, it is written in Eq. 6 in terms of $V_{i(MZ)}$, the volume of the mobile phase sample zone after injection. $V_{i(MZ)}$ equals V_{iM} when mobile phase is part or all of the injected sample solution.

$$V_{dD(iM)} = V_{fD} + V_{i(MZ)}/2 \tag{6}$$

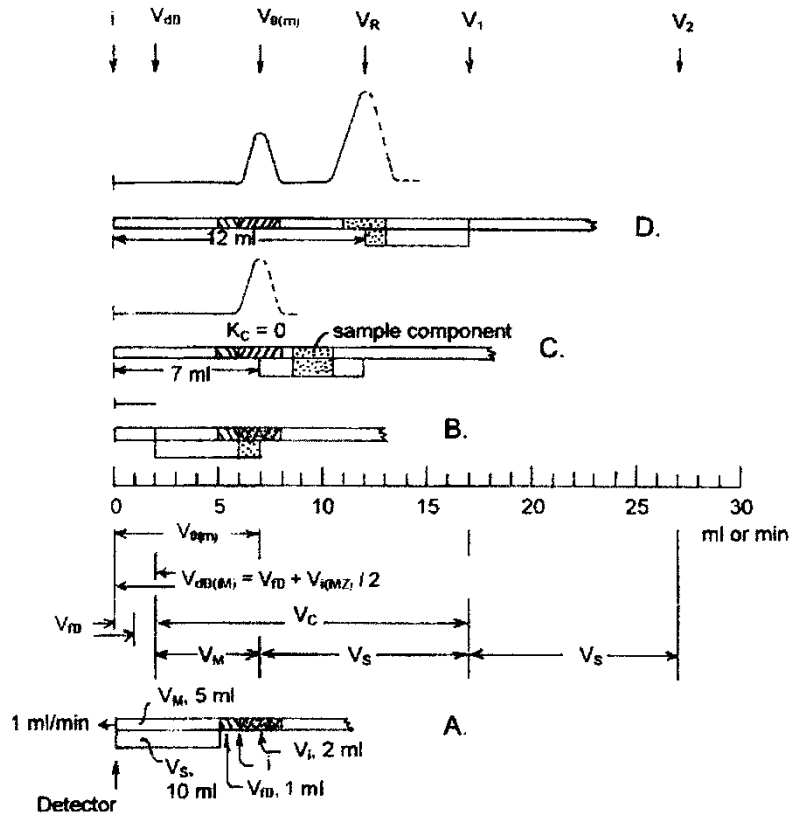

Figure 3. Peak delay in a CCC with extra-column volume. Column volume, V_c, 15 mL. Pre-detector extra-column volume, V_{fD}, 1 mL. Sample injection volume, V_i, 2 mL. Flow rate, F, 1 mL/min. Stationary phase retention, S_F, 0.67. A. Prior to sample injection. B. After 2 minutes flow. C. V_0 marker peak with K of 0 elutes at 7 minutes. D. Sample component elutes at 12 min. The column and the detector are moved to the right in each view.

The same relationship can also be expressed in a still more general way as

$$V_{dD(iM)} = V_{fD} + V_{iM} - V_{i(MZ)}/2 \tag{7}$$

This distinction between Eq. 6 and 7 is immaterial for samples injected in only mobile phase, but the relevance of Eq. 7 will be clarified in the discussion of cases 2 and 3.

In units of time, the delay will be

$$t_{dD(iM)} = V_{dD(iM)}/F \tag{8}$$

where F is the mobile phase flow rate. A second sample component is shown in Figure 3D, eluting at 12 mL. This peak has also been shifted by the delay volume V_{dD}.

Sample Injection in Stationary Phase

For comparison, the geometry of sample injection of a nonretained marker in 2 mL of mobile phase is again illustrated in Figure 4A and 4B. Conditions are the same as in Figure 3, except that extra-column volume is assumed to be negligibly small. The distance V_M signifies the mobile phase volume in

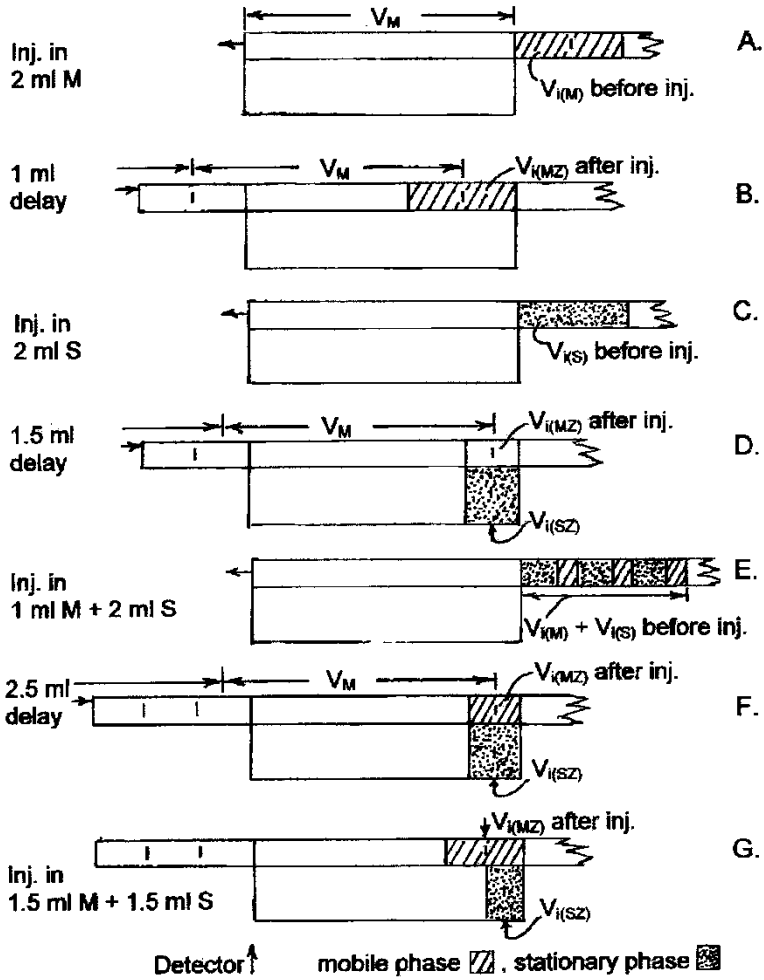

Figure 4. Geometry of on-column sample zones after sample injection in various modes. Extra-column volume is assumed to be zero. S_F is 0.67. A, before sample injection in mobile phase; B, after injection; C, before injection in stationary phase; D, after injection; E, before injection in β-mixed-phase; F, after injection; G, after injection in 1 : 1-mixed-phase. Injection volumes, V_i, and delay volumes, V_{dD}, are shown at left.

Figure 4A and is the same in Figures 4C and 4E. The shift after injection in V_M, in Figure 4B, shows a delay of 1 mL for the center of the V_0 zone to reach the detector.

Similar diagrams in Figure 4C and 4D show the geometry of injection for a sample in 2 mL of stationary phase. No mobile phase enters the column during stationary phase injection and the sample volume simply displaces 2 mL of stationary phase in the coil, which will result in 2 mL of effluent. Because of the phase volume ratio illustrated, $V_M/V_S = 1/2$, the stationary phase sample zone, $V_{i(SZ)}$, on the column is only half the length of the zone formed when 2 mL of mobile phase was injected into a system with the same phase volume ratio. There will be a mobile phase zone, $V_{i(MZ)}$, adjacent to the on-column stationary phase zone, and it will be the movement of this mobile phase zone which will carry the contents of the sample zone to the detector. A nonretained marker cannot be injected with stationary phase since it is, by definition, insoluble. However, one can imagine that some is present, for the purpose of calculating the position of V_0. The volume of stationary phase injected is represented in Figure 4D by the equivalent on-column volume, $V_{i(SZ)}$. Adjacent to $V_{i(SZ)}$ is a volume of mobile phase, $V_{i(MZ)}$. The volume of $V_{i(MZ)}$ depends on the phase volume ratio as expressed in Eq. 9.

$$V_{i(MZ)} = V_{i(SZ)}[V_M/V_S] = V_{i(SZ)}[(1 - S_F)/S_F] \tag{9}$$

When only stationary phase is injected, $V_{i(SZ)}$ equals the injection volume, V_{iS}, but, $V_{i(SZ)}$ is retained in Eq. 9 to preserve the generality of the equation.

In the example shown in Figure 4D, $V_{i(MZ)}$ is 1 mL, and the sample portion of the delay volume is $V_{i(MZ)}/2$ or 1.5 mL. In general, the delay volume for sample injection in stationary phase can be calculated from Eq. 10, which has the same format as Eq. 7.

$$V_{dD(iS)} = V_{fD} + V_{iS} - V_{i(MZ)}/2 \tag{10}$$

Sample Injection in Mixed Phases with V_M/V_S Ratio

A mixture of mobile and stationary phases is often used to dissolve crude samples containing solutes having a wide range of polarity. For reasons illustrated in Figures 4E and 4F, it is desirable to use a mixture of phases in the same proportion as the phase volume ratio present in the CCC. This phase volume ratio, V_M/V_S, has been defined as β by the IUPAC.[1] To simplify notation, sample injections using the V_M/V_S ratio will be referred to as β-mixed-phase injections. It should also be advantageous to inject the mixed-phase sample as a series of alternating segments of each phase, as shown in Figure 4E. This will ensure a uniform distribution of solutes along

each phase in the injected zone. As the segments enter the column, those composed of stationary phase will flow to the stationary phase layer, while those in mobile phase will push the mobile phase in the column forward. Though effluent flows continuously from the column, the mobile phase sample zone does not move forward while the stationary phase segments enter the column. The injected sample zone in Figure 4F illustrates a sample made up in 1 mL of mobile phase plus 2 mL of stationary phase, a β of 0.5. The sample zone geometry for the 3 mL injection is identical with that shown in Figure 4D for injection of 2 mL of stationary phase. But the mixed phase injection can contain nonretained solutes. During the injection, 3 mL will have exited the column, and the delay volume attributed to sample injection is seen in Figure 4F to be 2.5 mL, 1 mL greater than obtained with a 2 mL injection of stationary phase in Figure 4D. The general expression for V_{dD} when a sample is injected in β-mixed-phases is

$$V_{dD(iM,i\beta)} = V_{f(D)} + V_{iM} + V_{iS} - V_{i(MZ)}/2 \qquad (11)$$

Equation 11 is a very general expression for the solute delay volume. It reduces to Equations 6 and 10 when samples are injected in only mobile phase or only stationary phase, respectively. The subscript in $V_{dD(iM,\ i\beta)}$ indicates that V_{dD} calculated from Eq. 9 applies to mobile phase and β-mixed-phase injection. When mobile phase is part of the injection solution, either alone or as part of a β-mixed-phase solution, the $V_{i(MZ)}$ term is equal to the mobile phase portion of the injected solution, V_{iM}. But, $V_{i(MZ)}$ is never zero; when only stationary phase is injected, $V_{i(MZ)}$ is the on-column mobile phase zone in immediate contact with the injected stationary phase and its volume must be calculated from Eq. 9.

Equation 9 can be substituted for $V_{i(MZ)}$ in Eq. 11 to obtain an equivalent general expression (Eq. 12).

$$V_{dD(iS)} = V_{fD} + V_{iM} + V_{iS} - [V_{i(SZ)}/2][(1 - S_F)/S_F] \qquad (12)$$

Either V_{iM} or V_{iS} in Eq. 12 will be zero if injection is made in a single phase. However, neither $V_{i(MZ)}$ nor $V_{i(SZ)}$ is ever zero. If not included in the injection, the on-column zone of a phase is equal to the on-column volume of the counterphase in immediate contact with the injected phase. When a sample in β-mixed-phase is injected, V_{iM} will equal $V_{i(MZ)}$ and V_{iS} will equal $V_{i(SZ)}$. Although Eq. 12 is quite general, it is more direct to employ Eq. 11, for situations other than the injection of only stationary phase, as indicated by the subscript in $V_{dD(iS)}$ in Eq. 12. Note that the $(1\text{-}S_F)/S_F$ term in Eq. 12 is the phase volume ratio, V_M/V_S, or β.

Comparison of Methods 1, 2, and 3

The stationary phase fraction, S_F, is frequently greater than 0.5 in CCC. In such a case, injection of a sample in stationary phase will result in a

narrower sample zone than injection in an equal volume of mobile phase. This should produce greater resolution, especially with larger sample volumes. Inclusion of a portion of mobile phase, in proportion to the phase volume ratio, β, will increase the sample capacity without further increasing the size of the on-column sample zone. When equal sample volumes, V_i, are compared, the peak delay volume increases from mobile phase injection, to stationary phase injection, to injection of β-mixed-phase samples.

Sample Injection in Mixed Phases of Equal Volume

The geometry of the injected sample zone following injection of a mixture of equal volumes of each phase is illustrated in Figure 4G. With a column phase volume ratio, β, of $1/2$, as illustrated, (or, in general, any β less than 1), the injected mobile phase zone, $V_{i(MZ)}$, will be longer than the adjacent stationary phase zone, $V_{i(SZ)}$, resulting in a displacement to the left (if the mobile phase is less polar) for peaks with low K_c. The discrepancy will be greater as S_F increases. There does not appear to be a simple formula to characterize cases where the ratio of phases in the sample differs from that in the column. The resulting error in calculating K_c may be small if the injection volume is small, compared to the column volume and anticipated band spreading.

Location of V_0 Using a Marker or the Carryover Volume

The foregoing discussion of Figs. 3 and 4 indicates that, when sample injection is done using small sample volumes of either mobile or mixed phases (but not stationary phase only), the location of V_0 on the chromatogram can be determined by addition of a nonretained marker to the sample. Indigenous sample constituents of zero or very low K_c may also suffice, allowing the first-eluting peak to serve as a V_0 marker. A marker automatically corrects for both the sample volume and the extra-column volume in locating V_0 and provides a means to calculate V_M. The relationships between the retention times for various general index points, the extra-column volume, sample volume, column volume, and phase volumes are summarized in Figure 3. When a marker is used to locate V_0, one can write

$$V_{0(m)} = V_{dD(iM,i\beta)} + V_{M(m)} \tag{13}$$

Transcribing Eq. 13 allows $V_{M(m)}$ to be estimated as

$$V_{M(m)} = V_{0(m)} - V_{dD(iM,i\beta)} \tag{14}$$

The subscript m indicates that the location of V_0 and the subsequent estimation of V_M are based on use of a nonretained marker. $V_{dD(iM,i\beta)}$ is calculated using Eq. 11.

The position of V_0 can also be calculated from the measured carryover volume, V_{CO}, of stationary phase. If a CCC system at startup is filled with stationary phase from point i or a in Figure 1, (the volume of the i.a connection being negligible); then, after starting rotation and beginning mobile phase flow, V_{CO} represents the stationary phase carried out of the system. V_{CO} includes the total extra-column volume, V_{fT}, as well as a volume of stationary phase equivalent to the on-column mobile phase volume, $V_{M(CO)}$, displaced from the coil, and any stationary phase, V_{iS}, contained in the sample (Eq. 15).

$$V_{CO} = V_{fT} + V_{M(CO)} + V_{iS} \tag{15}$$

Recasting Eq. 15 as

$$V_{M(CO)} = V_{CO} - V_{fT} - V_{iS} \tag{16}$$

allows $V_{0(CO)}$ to be calculated (Eq. 17) from the carryover volume by adding the pre-detector delay volume, V_{dD}, as

$$V_{0(CO)} = V_{M(CO)} + V_{dD} \tag{17}$$

The V_{dD} appropriate to the injection mode (Eq. 11 or 12) must be employed in Eq. 17, leading to two Equations (18) and (19).

$$V_{0(CO,iM,i\beta)} = V_{M(CO)} + V_{dD(iM,i\beta)} \tag{18}$$

$$V_{0(CO,iS)} = V_{M(CO)} + V_{dD(iS)} \tag{19}$$

Calculation of Distribution Constants, K_c

The numerical values of distribution constants calculated from either Eq. 2 or the generalized Eq. 5 will differ, depending on the corrections applied and the means used to estimate the parameters employed. Four basic approaches, distinguished by descriptive subscripts, will first be described and then summarized in Table 2. To simplify matters, the subscript c is dropped from K_c.

Table 2. Equations for calculating K_c

Symbol	Equation No.	V_0 Marker	V_{dD} Correction[a]	V_i Correction[a]	V_{CO} Required
$K_{CO(nds)}$	20	No	No	No	Yes
$K_{m(nds)}$	21	Yes	No	Yes[a]	No
$K_{m(dD,iM,i\beta)}$	24	Yes	Yes	Yes	No
$K_{CO(dD,iM,i\beta)}$	27	No	Yes	Yes	Yes

[a]Corrects for V_0 position only

Calculation of $K_{CO(nds)}$

If V_S in Eq. 2 is replaced by $V_c - V_M$, and the total, uncorrected, V_{CO} used as an estimate of V_M, the parameter $K_{CO(nds)}$ is obtained as shown in Eq. 20.

$$K_{CO(nds)} = [V_R - V_{CO}]/[V_c - V_{CO}] \quad (20)$$

The subscript, $_{CO(nds)}$, implies that K is based on the uncorrected carryover volume and also provides no correction for extra-column volume, sample injection volume, or stationary phase contained in the sample. The uncorrected V_{CO} is too high as an estimate of V_M, leading to a denominator that is too small. In the numerator, that part of the shift in V_R resulting from extra-column volume is compensated by the increased volume of carryover. However, additional increase in V_R resulting from the sample volume leads to a higher numerator value as well. The value of $K_{CO(nds)}$ will, therefore, usually be high.

Calculation of $K_{m(nds)}$

The $K_{m(nds)}$ calculation is based on addition of a nonretained marker to the sample. This corrects for pre-detector extra-column volume and sample volume to accurately locate V_0 when the sample is injected in only mobile phase. The marker retention volume, $V_{0(m)}$, is used in Eq. 21 to designate the V_0 position on the chromatogram.

$$K_{m(nds)} = \frac{[V_R - V_{0(m)}]}{[V_c - V_{0(m)}]} \quad (21)$$

Crude samples often contain nonretained substances, which permit the retention volume of the first peak to be used for $V_{0(m)}$ without the addition of an extrinsic marker. But, the use of V_c to estimate the V_1 position (without correcting for V_{dD}) underestimates V_1 and leads to high estimates of K_c. The term $_{nds}$ in the subscript of these K_c values implies that no extra-column volume or sample volume corrections are applied to peaks beyond the marker peak.

Calculation of $K_{m(dD)}$

The $K_{m(dD)}$ calculation employs a nonretained marker and also incorporates corrections for pre-detector extra-column volume, V_{fD}, sample volume, V_i, and any stationary phase component, V_{iS}, in the sample injection to accurately locate V_1. As seen in Figure 3, the correct elution volume, designated $V_{1(dD)}$ in Eq. 22, of the peak with a K_c of 1, is found by adding V_{dD} to V_c.

$$V_{1(dD)} = V_{dD} + V_c \quad (22)$$

Substituting Eq. 11 for V_{dD} gives Eq. 23, appropriate for either mobile phase or β-mixed-phase injection, and Eq. 24 for calculation of $K_{m(dD)}$.

$$V_{1(dD,iM,i\beta)} = V_{dD(iM,i\beta)} + V_c \tag{23}$$

$$K_{m(dD,iM,i\beta)} = \frac{[V_R - V_{0(m)}]}{[V_{1(dD,iM,i\beta)} - V_{0(m)}]} \tag{24}$$

No equation for stationary phase injection is presented because the marker would be insoluble in stationary phase. Calculation of $K_{m(dD)}$, Eq. 24, is believed to be the most accurate of the four approaches, but it is limited when using a large injection volume, which yields a $V_{0(m)}$ peak too broad to accurately locate the marker peak center (see later $K_{m^*(dD)}$ Eq. 33 and 34.

Calculation of $K_{CO(dD)}$

The $K_{CO(dD)}$ calculation, Eq. 25, does not employ a marker to locate V_0, but, instead, uses the equation appropriate for the injection mode (18 or 19) to calculate $V_{0(CO)}$ and the corresponding equations (23 or 26) to calculate $V_{1(dD)}$ in Eq. 25. This leads to two equations (27 and 28) for the three injection modes.

$$K_{CO(dD)} = \frac{[V_R - V_{0(CO)}]}{[V_{1(dD)} - V_{0(CO)}]} \tag{25}$$

$$V_{1(dD,iS)} = V_{dD(iS)} + V_c \tag{26}$$

$$K_{CO(dD,iM,i\beta)} = \frac{[V_R - V_{0(CO,iM,i\beta)}]}{[V_{1(dD,iM,i\beta)} - V_{0(CO,iM,i\beta)}]} \tag{27}$$

$$K_{CO(dD,iS)} = \frac{[V_R - V_{0(CO,iS)}]}{[V_{1(dD,iS)} - V_{0(CO,iS)}]} \tag{28}$$

If the various extra-column volumes are carefully measured, $K_{CO(dD)}$ should also be quite accurate and, in our experience, the $K_{m(dD)}$ and $K_{CO(dD)}$ values are very close when compared using mobile phase injection.

Characteristics of the estimates of K_c calculated from equations 20, 21, 24, and 27, using mobile phase injection, are summarized in Table 2. Consideration of the method of sample injection will be further discussed below.

Calculation of the Stationary Phase Fraction, S_F

When a marker is used in either mobile phase or a β-mixed-phase injection, S_F can be calculated as

$$S_{F(m,iM,i\beta)} = \frac{[V_c - V_{M(m)}]}{V_c} \tag{29}$$

where $V_{M(m)}$ is calculated using Eq. 14. The subscript, $_m$, indicates use of the marker, and the remaining symbols indicate that the equation applies to both mobile phase, $_{iM}$, and β-mixed-phase injection, $_{i\beta}$.

S_F based on carryover volume, V_{CO}, is applicable to all injection modes and is calculated as

$$S_{F(CO,iM,iS,i\beta)} = \frac{[V_c - V_{M(CO)}]}{V_c} \tag{30}$$

Where the $V_{M(CO)}$ term is calculated using Eq. 16.

Marker Injection Prior to Sample Injection

Sample injection in a small volume of mobile phase presents no problems in either locating $V_{0(m)}$ with a marker, or $V_{0(CO)}$ from the carryover volume. Mobile phase injection also permits continuous accumulation of carryover to correct sequentially-injected samples for bleed of stationary phase. A marker is not useful for stationary phase sample injection and the marker becomes less satisfactory in other injection modes as sample volume increases and the $V_{0(m)}$ peak becomes too broad, and the marker peak center may be obscured by other poorly-retained sample components.

A marker can be used to locate V_0 in these cases by injecting it in a negligibly small volume, V_{i^*}, of mobile phase, several minutes prior to injecting the sample in a larger volume, V_i, of any phase. This assumes that S_F remains constant for both the pre-sample and sample runs. The marker retention volume, $V_{0(m^*)}$, can then be used for calculation of S_F and sample $K_{0(m^*)}$ values. The asterisk designates that a pre-sample-injection marker is used in the determination. Limiting its use to mobile phase injection, $_{iM}$, Eq. 13 can be expressed as

$$V_{0(m^*,iM)} = V_{dD(iM)} + V_{M(m^*)} \tag{31}$$

Substituting Eq. 6 for $V_{dD(iM)}$ in Eq. 31 and replacing $V_{i^*(MZ)}$ by the equivalent $V_{i^*(M)}$ gives

$$V_{0(m^*,iM)} = V_{fD} + \frac{V_{i^*(M)}}{2} + V_{M(m^*)} \tag{32}$$

From which $V_{M(m^*)}$ can be calculated as

$$V_{M(m^*)} = V_{0(m^*,iM)} - V_{fD} - \frac{V_{i^*(M)}}{2} \tag{33}$$

Further simplification is not explored here, but in many instances, $V_{i^*(M)}$ in Eq. 32 and 33 will be negligibly small. A caveat for preparative samples of crude mixtures is that a significant volume of stationary phase will usually be carried over after sample injection.

The value of $V_{0(m^*,\,iM)}$ from Eq. 32 can be substituted for $V_{0(m)}$ in Eq. 24 to obtain

$$K_{m^*(dD,iM,i\beta)} = \frac{[V_R - V_{0(m^*,iM)}]}{[V_{1(dD,iM,i\beta)} - V_{0(m^*,iM)}]} \tag{34}$$

which is applicable to sample injection in either mobile or β-mixed-phase. The $V_{1(dD,iM,i\beta)}$ term in Eq. 34 is obtained using Eq. 23, which, in turn, is calculated using Eq. 11, wherein $V_{i(MZ)}$ is replaced by V_{iM}. The values of V_{iM} and V_{iS} in Eq. 11 are those employed for the sample injection.

There appear to be few, if any, situations where sample injection in only stationary phase is advantageous, since it is always possible to add mobile phase to the sample in the ratio of β. However, Eq. 35 applies to determining K_c based on a marker injection prior to sample injection in only stationary phase.

$$K_{m^*(dD,iS)} = \frac{[V_R - V_{0(m^*)}]}{[V_{1(dD,iS)} - V_{0(m^*)}]} \tag{35}$$

The required $V_{1(dD,iS)}$ term is calculated from Eq. 26, in which $V_{dD(iS)}$ is calculated from Eq. 12. $S_{F(m^*)}$ in Eq. 12 is calculated as

$$S_{F(m^*)} = \frac{[V_c - V_{M(m^*)}]}{V_c} \tag{36}$$

using $V_{M(m^*,iM)}$ from Eq. 32. S_F can also be calculated from the carryover volume using Eq. 30. The drift in S_F over the CCC run can be judged by comparing $S_{F(m^*,iM)}$ with $S_{F(CO)}$ for the sample run.

EXPERIMENTAL

A PC Inc. (Potomac, MD) countercurrent chromatograph, orbital radius 10.6 cm, fitted with a spool (approx. 5 cm width, i.d.) containing a single-layer helical coil of Zeus no. 18 PTFE tubing at a radius ratio, β, of 0.85, having a volume of 13 mL, was used. Orbital speed was typically 1200 rpm and mobile phase flow rate was typically 0.5 mL/min. Samples illustrated were injected in 0.2 mL of mobile phase at concentrations ranging from 1 to 6 mg/mL along with the dye Poly R-478 (Sigma P1900, CAS 68550-77-6), at a concentration of approximately 30 μg/mL. An Isco V-4 monitor set at 254 nm with a 2 mm prep cell (2-mm path, 48 μL vol.), along with a Milton Roy model 196-31 pump and a Houston Omniscribe chart recorder were used. The prep cell, with a straight-through, vertical flow path, is desirable to reduce droplet noise and back pressure. Although not used for the samples reported here, the setup included an adjustable column effluent warmer (to suppress droplet noise in the monitor cell), Figure 1. A carryover vessel, constructed from a 10-mL volumetric pipette and readable

to 0.01 mL continuously collected stationary phase expelled from the coil. Effluent from the carryover vessel can be recycled to the solvent reservoir as described elsewhere.[6] System extra-column volumes were calculated by using a 1-mL tuberculin syringe to fill a measured length of tubing to obtain the i.d., or by filling the entire component with water containing a dye. The pre-detector dead volume, $V_{f(D)}$ was 0.59 mL; total system dead volume, $V_{f(T)}$, was 0.80 mL.

K values were determined for benzamide and a series of N-alkylbenzamides in the system ethyl acetate/ethylene glycol with ethylene glycol as the mobile phase, pumped in the head to tail direction. Synthesis of the standards is described elsewhere.[6] An Excel spreadsheet, configured for summarizing a series of related chromatograms, was used for calculations. The spreadsheet also calculates the stationary phase ratio, S_F, and the column efficiency, N, if the peak 4-sigma base width is entered. An IF function was used to select the equation appropriate for the injection mode used, depending on whether the volume of mobile phase in the injection solution is entered as zero or has a positive value. A second spreadsheet, more adapted to larger volume preparative separations, and suitable for transcribing data collected with a data logger, was also developed. A Cole-Parmer modular paperless recorder was used for analog/digital conversion and data logging. The spreadsheet is compatible with any data in digital format, however, and various methods could be employed to produce real-time K determination. The second worksheet summarizes the calculations and the chromatogram on a single page, convenient for insertion in a notebook. Copies of the spreadsheets are available from wdconway@buffalo.edu or LChadwick@kalsec.com.

RESULTS AND DISCUSSION

K_c values for a series of N-alkylbenzamides are summarized in Table 3. The series of six injections were made sequentially, over a period of about 4 hours, without refilling the CCC with stationary phase. Carryover of stationary phase was continuously monitored and column bleed is reflected in the gradual fall of S_F from 0.61 initially to 0.39 in run 6.

$K_{m(dD)}$ is expected to provide the most accurate value, since the V_0 point is directly indicated and correction is made for extra-column volume and sample volume. The $K_{CO(dD)}$ values are in close agreement with the $K_{m(dD)}$ values, indicating that dead volume corrections for pre- and post-detector flow lines are correct, and also confirming that the Poly-R dye, used to indicate $V_{0(M)}$ for the $K_{m(dD)}$ values is, indeed, not retained.

$K_{CO(nds)}$ values employ no corrections for sample volume or dead volume and no marker for V_0. $K_{m(nds)}$ values utilize a marker for V_0, but no dead volume or sample volume corrections for peaks beyond V_0. Both of these are higher than the $K_{m(dD)}$ values by about 8 to 16%, which is probably acceptable for routine solvent system optimization studies. However, the sample

Table 3. Comparison of K_c values of alkyl-benzamides in EtOAc/ethylene glycol solvent system

R =	0	-H (benzamide)	-Me	-Et	-Pr	-Bu	-Hex	-Oct
Inj no:	1	2, 5	3	4	5	6	6	6
$K_{CO(nds)}$	−.002	0.40, 0.46	0.66	1.00	1.34	1.82	3.15	5.27
$K_{m(nds)}$	0	0.40, 0.43	0.66	1.00	1.36	1.90	3.36	5.69
$K_{m(dD,iM,i\beta)}$	0	0.37, 0.38	0.59	0.89	1.20	1.64	2.90	4.91
$K_{CO(dD,iM,i\beta)}$	.01	0.38, 0.42	0.61	0.90	1.19	1.58	2.72	4.54
$S_{F(m,iM,i\beta)}$	.61	0.59, 0.46	0.55	0.51	0.46	0.39	0.39	0.39

Mobile phase: Lower phase EG in (H) → T direction; Markers: Alkyl-benzamides: C_6H_5-CO-NH-R.

Flow rate: 0.49 mL/min; Volumes: $V_i = 0.2$ mL; $V_c = 13$ mL; $V_{fD} = 0.59$ mL; $V_{fT} = 0.80$ mL; $V_{dD} = 0.69$ mL.

injection volume is small in this example and larger deviations can be expected as the injection volume is increased in preparative studies.

CONCLUSIONS

The countercurrent chromatogram is portrayed in a general way (Figure 2, Eq. 5), followed by a description of the differences in the geometry of the on-column sample zones that form when samples are injected in either mobile phase, stationary phase, or a mixture of both phases in the ratio, β, of the phase volumes in the column. The extra-column volume, in combination with the sample injection volume, causes an increase in the retention volume required to elute the chromatographic peaks, in effect delaying the time required for the peaks to reach the detector. Using the same injection volume, this peak delay is least with mobile phase injection and greatest with β-mixed-phase injection. However, the on-column sample zone length is smallest for injection in stationary phase and β-mixed-phase injection, which may provide greater sample resolution.

Four approaches for calculating partition coefficients are presented (Eq. 20, 21, 24, 27). The first two of these, for $K_{CO(nds)}$ and $K_{m(nds)}$, make no corrections for extra-column volume or sample injection volume. Estimates of K_c values from both Eq. 20 and Eq. 21 are usually high but, when compared using injection in mobile phase, both are still within about 16% of the more accurate values, $K_{m(dD,iM,i\beta)}$ and $K_{CO(dD,iM,i\beta)}$, in Table 3, calculated by the third and fourth methods. $K_{m(dD,\ iM,\ i\beta)}$ uses a nonretained marker (added to the sample in Table 3, but an indigenous marker, when present, can also be used) along with corrections for sample volume and extra-column volumes. The last approach, $K_{CO(dD,iM,i\beta)}$, uses stationary

phase carryover volume, sample volume, and extra-column volume for calculation. Both provide similar results. After sample injection in only stationary phase, the size of the on-column mobile phase zone depends on the phase volume ratio, β, and Eq. 35 must be used to calculate $K_{CO(dD,iS)}$. Three equations (Eq. 29, 30, 36) are presented for calculating the stationary phase fraction, S_F, which is required in Eq. 35. For large injection volumes, where the V_0 peak is very broad, or in cases where contamination by addition of a marker must be avoided, recourse may be had to injecting a marker in a small volume of mobile phase, prior to sample injection. K_{m^*} can then be calculated using Eq. 34 or 35, and $S_{F(m^*)}$ can be obtained using Eq. 36. For preparative separations of crude mixtures, only two injection modes are practical, namely mobile phase injection and β-mixed phase injection. In most cases, crude extracts will contain indigenous markers, and $K_{m(dD,iM,i\beta)}$ will be the preferred calculation.

Attention is called to the fact that, rather than injecting mixed phases sequentially, they should be loaded into the injection loop as alternating segments, so that both phases will, in effect, enter the column together. Using the spreadsheets that have been developed, there is no difficulty calculating K_c by all four methods, and it will be interesting to note their relative agreement under various experimental conditions.

SYMBOLS

β	phase volume ratio, V_M/V_S, in the column (official IUPAC denomination) commonly used as r (spool radius) over R (rotor radius) in the CCC world.
d	distance on a chromatogram.
dD	in subscript, refers to peak delay volume or delay time prior to the detector.
fd	in subscript, refers to flow line extra-column volume.
F	flow rate.
i	start of sample injection, in units of volume or time.
iM, iS, iβ	in subscript, to clarify whether equation or term is applicable to injection in mobile phase, stationary phase or β-mixed-phase sample injection.
S_F	stationary phase fraction (or ratio) $= V_s/V_c = V_S/(V_M + V_S) = 1/(\beta + 1)$.
t_i	time sample injection is begun.
K_c	solute partition ratio or distribution ratio, expressed as solute analytical concentration in stationary phase divided by solute analytical concentration in mobile phase.
$K_{CO(nds)}$	partition ratio based on uncorrected carryover volume, with no correction for extra-column volume or sample volume (Eq. 20).

$K_{m(nds)}$	partition ratio based on non-retained marker with no extra-column volume or sample volume correction (Eq. 21).
$K_{m(dD,iM,\ i\beta)}$	partition ratio based on nonretained marker, corrected for extra-column volume and sample volume (Eq. 24), for mobile phase and β-mixed-phase injection.
$K_{CO(dD)}$	partition ratio based on carryover volume, with corrections for extra-column volume, sample injection volume and stationary phase contained in injection (Eq. 25, 27, 28).
$K_{m^*(dD)}$	is K_c determined using a nonretained marker injected prior to sample injection (Eq. 34, 35).
M	in subscript, refers to mobile phase, as in V_M.
m	in subscript, refers to use of a nonretained marker to determine V_0.
m*	marker injected in a small volume of mobile phase prior to sample injections.
V_c	column volume.
V_M	mobile phase volume.
$V_{M(CO)}$	mobile phase volume estimated from corrected carryover volume (Eq. 16).
V_S	stationary phase volume.
V_R, t_R	retention volume or time measured from start of injection.
V_{CO}	total carryover volume of stationary phase, from extra-column volume, stationary phase displaced from the coil, and stationary phase content of injection (Eq. 15).
V_0, t_0; V_1, t_1	elution volume or time for solutes with K of 0 or 1.
$V_{0(m)}$	retention volume of solute with K of 0 determined with a nonretained marker (Eq. 13).
$V_{0(m^*,iM)}$	retention volume of solute with K of 0 determined with a nonretained marker injected in a small volume of mobile phase prior to sample injection (Eq. 32).
$V_{0(CO)}$	retention volume of solute with K of 0 calculated from V_{CO} by correcting for extra-column volume, sample injection volume and stationary phase contained in the injection (Eq. 18, 19).
V_{dD}	peak delay volume from start of injection to the detector midpoint (Eq. 6,7,10, 11, 12). Both extra-column volume, V_{fD}, and the sample volume, V_i, contribute to V_{dD}.
$V_{f(a.b)}$	extra-column volume in flow line segments a to b etc. (Figure 1).
V_{fD}	extra-column volume in extra-column flow line segments from point of sample injection to midpoint of detector cell (Eq. 3).
V_{fE}	post-detector volume ($V_{f(h.p)}$ in Figure 1).
V_{fT}	total extra-column volume, from injection point to collection vessel (Eq. 4).

$V_{1(dD)}$	retention volume of a solute with K of 1, corrected for extra-column volume and sample injection volume (Eq. 23, 26).
V_i	sample injection volume, including both phases for mixed phase injections.
V_{i^*}	negligibly small volume of mobile phase, containing a nonretained marker, injected prior to the sample injection.
V_{iM}	volume of mobile phase contained in the sample injection.
$V_{i(MZ)}$	The on-column zone of mobile phase at the column inlet following sample injection. This zone exists as the mobile phase in immediate contact with an injected zone of stationary phase, whether or not mobile phase is included in the sample injection,
V_{iS}	volume of stationary phase contained in the sample injection.
$V_{i(SZ)}$	The on-column zone of stationary phase at the column inlet following sample injection. This zone exists as the stationary phase in immediate contact with an injected zone of mobile phase, whether or not stationary phase is included in the sample injection.

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