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A practical approach to transferring linear gradient elution methods

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Abstract

Attempts to theoretically address the problems involved in transferring linear gradient elution methods have been somewhat ad hoc due to the simplifying assumptions usually made in conventional gradient elution theory. Until now, all equations based on the k^* parameter of linear gradient elution theory used as the basis for predicting the separation selectivity have not explicitly included the effect of the dwell volume (V_D). Using an exact equation for predicting k^*, that is, one which fully accounts in an a priori fashion for V_D, we find a set of simple yet exact equations which unequivocally *must be satisfied* to transfer an optimized linear gradient elution method from one system (column or instrument or both) to another. These relationships absolutely mandate that a change in the instrument dwell volume requires a proportional change in the column volume; in turn, a change in the column volume requires a proportional change in the flow rate and/or gradient time to maintain a constant gradient steepness. Although we are not the first to suggest these guidelines, this work provides a complete theoretical foundation for these exact guidelines for the maintenance of gradient selectivity for the case of transferring a linear gradient elution method between different columns packed with the same particles and/or between different instruments.

Keywords: Gradient elution; Method transfer; Dwell volume; Selectivity

1. Introduction

Gradient elution RPLC is a powerful technique required to separate samples that otherwise exhibit the general elution problem under isocratic conditions [1]. Transferring an optimized gradient elution method between instruments, columns, laboratories, etc. is notoriously more difficult than transferring isocratic elution methods [2–4]. The main impediment to transferring a gradient elution method is the fact that different models of HPLC instruments, and perhaps local adaptations of different units of the same model, rarely have the same "dwell" volumes (V_D) [5], which can vary by as much as an order of magnitude between makes and models. Although other types of errors in gradient formation due to gradient rounding or other differences in the solvent delivery systems exist [2,6,7], this work focuses on the *importance of the dwell volume in relation to the column volume when transferring a gradient elution method between systems and columns*.

To obtain the same separation using exactly the same column on instruments that produce the same gradient profiles but have different dwell volumes, one must adjust the "*effective*" dwell volumes so that they are identical on each instrument. The "effective" dwell volume is the total volume of starting eluent delivered to the column inlet after injecting the solutes. Some instruments allow the sample to be injected after the gradient starts; this delayed injection decreases the "effective" dwell volume. Alternatively, an isocratic hold at the initial eluent composition can be introduced after sample injection to increase the "effective" dwell volume. In the absence of such deliberate machinations, the "effective" dwell volume and "intrinsic" dwell volume are equal. The *intrinsic* dwell volume ($V_{D,intr}$) is the volume of starting eluent delivered to the column inlet before the front of the gradient arrives at the column inlet; clearly $V_{D,intr}$ is an instrument constant whereas the "effective" dwell volume is readily adjustable [6]. Furthermore, the "effective" dwell volume controls the

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amount of solute pre-elution (i.e. elution under isocratic conditions) before the solute starts to move within the column under gradient conditions (see Appendix I). The pre-elution time ($t_{\text{pre-elution}}$) corresponds to the period of time after the sample is injected but before the gradient, which is delayed in the dwell volume, catches up to and overtakes the solute. The distance of solute pre-elution ($z_{\text{pre-elution}}$) corresponds to the distance the solute moves along the column at the initial eluent strength before being overtaken by the gradient. Thus, the "effective" dwell volume is what truly matters when transferring a gradient elution method; further references to V_D in this paper imply the "effective" dwell volume unless specifically noted.

Using an initial isocratic hold and delaying the injection are practical ways to adjust V_D. Unfortunately, many commercial instruments do not allow for delayed injection. Pre-column flow splitting will decrease V_D although the accuracy of the method degrades [8] and a significant amount of eluent is wasted. Another way to adjust V_D is by modifying $V_{D,intr}$ through changes in the eluent mixing chamber and/or the amount of tubing placed between the eluent mixing chamber and column inlet. This is clearly more difficult but decreasing V_D in this fashion is especially important when small volume columns (1 mm or 2.1 mm diameter) are being used [8–10]. We recommend the use of computer simulation programs such as Drylab 2000 Plus® to investigate the effect of V_D on selectivity before any impractical changes in V_D are considered [5].

2. Theory

Snyder and Dolan have shown that in the absence of a dwell volume effect (i.e. $V_D = 0$ and/or the same column and instrument are used), the selectivity of a gradient separation is controlled by the gradient steepness parameter (b) [9,11]. Eq. (1) shows that b relates to a property of the solute (S), the column (V_m) and the gradient profile (F, t_G and $\Delta\phi$); S is the slope of the $\ln k'$ (isocratic retention factor) versus ϕ (eluent strength; i.e. volume fraction of organic

$$b = \frac{S\Delta\phi V_m}{Ft_G} \tag{1}$$

$$\ln k' = \ln k'_w - S\phi \tag{2}$$

modifier) plot which assumes the linear solvent strength theory (LSST; see Eq. (2)) is accurate where k'_w is the retention of the solute in pure water, $\Delta\phi$ is the difference in the final (ϕ_f) and initial (ϕ_0) eluent strengths, F is the flow rate (in mL/min) and t_G is the total gradient time (in min). Obviously, b (see Eq. (1)) is independent of V_D.

Snyder and Dolan also addressed the issue of the effect of the dwell volume on the selectivity [2,11]. As these authors explained, when a dwell volume is introduced (i.e. $V_D \neq 0$; different columns and/or instruments are used) in principle all solutes, but pragmatically only the most weakly retained solutes, initially move under effectively isocratic conditions before the gradient, which is delayed by the dwell volume, catches up with the already displaced band of analyte. Clearly pre-elution only significantly effects the retention time of very weakly retained species. They argue that this composite elution process (i.e. solute pre-elution and subsequent gradient elution) established an "*effective gradient steepness*" (denoted b' in their work) that lowers the value of b for all solutes but most especially that of the weakly retained analytes [11].

They go on to correctly assert that the *selectivity can only be maintained constant by keeping both b and the ratio* V_D/V_m *constant*. This important conclusion was demonstrated experimentally for a mixture of pharmaceuticals using one instrument [11]. One of the major objectives of this work was to prove from first principles that their conclusions are correct and to clarify some related issues and practical consequences.

In developing an *exact general theory of gradient elution* that allows for an initial delay before the gradient reaches the column inlet, three cases of solute elution must be considered: isocratic elution, gradient elution *before* the gradient leaves the column and gradient elution followed by isocratic elution at $\phi = \phi_f$ *after* the gradient has left the column. To determine the elution mode of a solute within the column in the case of a finite dwell volume, we first developed equations that describe the amount of solute pre-elution (i.e. $t_{\text{pre-elution}}$ and $z_{\text{pre-elution}}$) that occurs before the gradient catches the solute (see Appendix I). Using these equations, we find that a solute will elute under isocratic conditions when the retention factor of the solute at ϕ equal to ϕ_o (i.e. k'_0) is less than or equal to the ratio of the dwell time (i.e. $t_D = V_D/F$) divided by the kinetic dead time of the column (i.e. $t_0 = V_m/F$). Therefore, the retention time (t_R) of a solute eluting isocratically (i.e. when $k'_0 \leq t_D/t_0$) is determined by Eq. (3).

$$t_R = t_0(1 + k'_0) \text{ when } k'_0 \leq \frac{t_D}{t_m} \tag{3}$$

Schoenmakers derived an exact equation for the gradient retention time (see Eq. (4)) making the LSST assumption as did Snyder (that is, Eq. (2) is true) [12].

$$t_R = t_0 + t_D + \frac{t_0}{b}\ln\left(b\left(k'_0 - \frac{t_D}{t_0}\right) + 1\right)$$

$$\text{when } k'_0 > \frac{t_D}{t_m} \text{ and } t_R \leq t_G + t_D + t_0 \tag{4}$$

Snyder also presented two equations for predicting gradient retention time: one equation assumes that solute pre-elution is negligible while the other equation accounts for the amount of solute pre-elution [13]. After some algebraic rearrangements and some notational changes, we have shown that Snyder's equation which includes solute pre-elution and the Schoenmakers equation are identical (see Appendix II). Thus, Eq. (4) is the exact equation for predicting the gradient retention time provided that $k'_0 > t_D/t_0$ (i.e. the solute does not elute

completely isocratically) and that the solute elutes before the tail of the gradient leaves the column (i.e. $t_R \leq t_G + t_D + t_0$; see Appendices II and III). Schoenmakers also derived an equation for the retention time of a solute eluting after the gradient leaves the column assuming that LSST is accurate [12]; we have rewritten this equation in more conventional notation (see Appendix IV).

For solutes moving under gradient conditions, we have derived an equation that predicts the retention factor of the solute at any point (z) within the column (see Appendices I and III). Although any value of z can be used to calculate the retention factor and thus selectivity in gradient elution, Snyder and Dolan have suggested that one should calculate the selectivity when the solute has traveled halfway through the column (i.e. $z = L/2$); this value of the retention factor is usually denoted k^* [13]. It appears that Snyder and Dolan have only given an equation for k^* based on the assumption that V_D equals zero (see Appendix III) [13]. Here we present an exact equation for k^* (see Eq. (5)) without making any assumptions based on the exact theory of gradient elution. Since our equation includes the dwell volume and because we know that V_D has an effect on the selectivity (i.e. k^*) in gradient elution, we believe our derivation of k^* is exact and complete.

$$k^* = \frac{k'_0}{b((k'_0/2) - (V_D/V_m)) + 1} \tag{5}$$

Using Eq. (5), we can control for differences in V_D by setting k^* for two different systems (denoted with a subscript '1' and '2') to be equal (see Eq. (6)). As changes in the initial eluent strength have a complex effect on selectivity in gradient elution [11], we require the same value of ϕ_0 in both systems which makes k'_0 for a given solute the same in each system if the columns are packed with identical phases. Mathematical rearrangement of Eq. (6) leads to Eq. (7). From the work of Dolan and Snyder we know that one can maintain the selectivity constant using one

$$\frac{k'_0}{b_1((k'_0/2) - (V_{D,1}/V_{m,1})) + 1} = \frac{k'_0}{b_2((k'_0/2) - (V_{D,2}/V_{m,2})) + 1} \tag{6}$$

$$\frac{b_2}{b_1} = \frac{((k'_0/2) - (V_{D,2}/V_{m,2}))}{((k'_0/2) - (V_{D,1}/V_{m,1}))} \tag{7}$$

instrument (i.e. V_D is constant) and one column (i.e. V_m is constant) by keeping b constant [11]. Thus, we set the ratio of $b_2/b_1 = 1$ in Eq. (7); we refer to this as *condition I*. Next, to get the right hand side of Eq. (7) equal to 1, we must set $V_{D,2}/V_{m,2} = V_{D,1}/V_{m,1}$; we refer to this as *condition II*. Combining *conditions I and II* with Eq. (1) and keeping $\Delta\phi$ (i.e. ϕ_0 and ϕ_f) constant in each system, to avoid complex changes in the selectivity, results in Eq. (8). Thus, *when V_D is changed one absolutely must also change V_m proportionately* (*and vice versa*); *when V_m is changed one must keep b constant by adjusting F and/or t_G*. Although most chromatographers and instrument manufacturers have recognized that one must change the dwell volume in proportion to the

$$\frac{V_{D,2}}{V_{D,1}} = \frac{V_{m,2}}{V_{m,1}} = \frac{F_2 t_{G,2}}{F_1 t_{G,1}} \tag{8}$$

column volume to produce an acceptable separation and/or successfully transfer a gradient elution method, we believe the above treatment is the first exact theoretical verification of this concept. We were also pleased that these guidelines for transfer of a gradient elution method are identical to those proposed by Dolan and Snyder [11] based on their concept of *effective gradient slope*. Although we recommend exact adherence to the guidelines in Eq. (8), small errors in the assumed values of V_m, V_D and b will not significantly affect the selectivity in many situations. In other cases, the dwell volume has a small effect on the selectivity. For example, Zmak et al. maintained the resolution of protein and peroxidase enzyme mixtures using monolithic columns of different volume by keeping b constant and allowing the ratio of V_D/V_m to vary [14].

Successful transfer of an optimized gradient elution method requires that one must maintain the two constants shown in Eqs. (9) and (10). With this in mind, we have devised two experiments to verify that the guidelines in Eq. (8) qualitatively maintain the band spacing in

$$\text{constant} = \frac{V_m}{F t_G} \tag{9}$$

$$\text{constant} = \frac{V_D}{V_m} \tag{10}$$

gradient elution. In the first experiment, we deliberately change V_D on one instrument in proportion to V_m; we adjusted V_m by combining in series up to three 5 cm × 4.6 mm columns packed with the same type of particles. We show that the selectivity on each column is qualitatively identical which allows us to use the columns in any combination to vary V_m. Also, we vary F and t_G to satisfy Eq. (9) while holding V_D/V_m constant. In the second experiment, we use the same column and adjust the "effective" dwell volume on two different instruments to be equal to satisfy Eq. (10). The first experiment uses a methodology similar to that used by Dolan and Snyder [11] to verify the guidelines in Eq. (8). However, we believe the second experiment is of great importance to chromatographers as the transfer of a gradient elution method between instruments and/or labs is notoriously difficult. In both experiments, we qualitatively maintained the separation and confirmed the validity of the guidelines in Eq. (8) for maintaining band spacing in gradient elution.

3. Experimental

3.1. Instrumentation

All experiments were conducted using two instruments: an HP 1090 Series I and an HP 1100. Each instrument was controlled by version A.10.01 Chemstation software (Hewlett-Packard S.A., Wilmington, DE). The HP 1090 was equipped with an autosampler, binary pump and photodiode array UV detector; the HP 1100 was equipped with a vacuum degasser, an autosampler, quaternary pump, block heater and variable wavelength UV detector. The $V_{D,intr}$ of the HP 1090 and the HP 1100, including all tubing required to connect the column, were determined to be 0.31 mL and 0.75 mL, respectively, using the technique described in [2,7]. A prototype eluent pre-heater and column heating jacket obtained from Systec Inc. (New Brighton, MN) were used to pre-heat the mobile phase and maintain the column at $40.0 \pm 0.1\,^{\circ}C$ using the HP 1090 instrument; a thermocouple and Omega CN9000 display (Omega Engineering Inc., Stamford, CT) were used to monitor the eluent temperature at the column exit. The eluent temperature was not monitored using the HP 1100 but the oven was set to $40.0 \pm 0.1\,^{\circ}C$. The flow rate of each instrument was checked using a 10 mL volumetric flask and a stopwatch, and was determined to be consistently accurate to within 1% of the set point. The extra-column volume (V_{ex}) for each instrument was measured at various flow rates by replacing the column with a zero dead volume connector and injecting 10 μL of a 0.1 mg/mL solution of acetone in acetonitrile.

3.2. Reagents

All solutes were reagent grade or better and were used as obtained from the manufacturer without further purification. Uracil, acetone, *N*-benzylformamide, benzylalcohol, phenol, 2-phenylethanol, bromobenzene, 4-bromotoluene, acetophenone, ethylbenzene, nitrobenzene, benzene, 4-chlorophenol, 3-nitrotoluene, biphenyl and butylbenzene were obtained from Aldrich (Milwaukee, WI). Benzo(*k*)fluoranthene and benzo(*g*,*h*,*i*)perylene were obtained from Accustandard (New Haven, CT). These solutes were diluted into one sample using the initial eluent (see below); the concentration of nitrobenzene, 3-nitrotoluene, acetophenone and biphenyl was 20 μg/mL; all other solutes were approximately 1 mg/mL.

The eluent reservoirs and filtration apparatus glassware were scrupulously cleaned, rinsed with water then acetone, and dried using nitrogen before use. The organic co-solvents in this study were used as obtained from the manufacturer; acetonitrile was obtained from Burdick and Jackson (Muskegon, MI) and *n*-propanol, *n*-butanol and tetrahydrofuran were obtained from Fisher (FairLawn, NJ). HPLC grade water was obtained in-house from a Barnstead Nanopure Deionizing system (Dubuque, IA). This water was boiled to remove carbon dioxide and cooled to room temperature before use.

All eluents were prepared gravimetrically (±0.001%) based on the density (17) at room temperature (25 °C) of acetonitrile, *n*-propanol and water were eluent composition is reported as the (v/v) ratio. The ternary solvents were made by first adding the *n*-propanol to acetonitrile followed by dilution with water. The eluents were stirred magnetically until they reached room temperature. All eluents were passed through a 0.45 μm nylon filtration apparatus (Lida Manufacturing Inc., Kenosha, WI) immediately before use. These eluents were not degassed to any extent beyond the degassing that occurred during filtration. The initial eluent (i.e. channel A) consisted of 3/10/87 *n*-propanol/acetonitrile/water and the final eluent (i.e. channel B) consisted of 3/90/7 *n*-propanol/acetonitrile/water.

3.3. Columns

Three 5 cm × 4.6 mm columns (designated A–C) were packed with 5 μm Prontosil 200-C_{18} particles (pore sizes of 200 Å) obtained from Bischoff Chromatography (Leonberg, Germany). The stainless steel column hardware was obtained from Isolation Technologies (Hopedale, MA). The Prontosil 200-C_{18} particles were slurried in 10/90 *n*-butanol/tetrahydrofuran and sonicated (model PC3, L&R Manufacturing, Kearny, NJ) for 20 min before packing. The column was packed using the downward slurry method technique at a packing pressure of 34.5 MPa using pure tetrahydrofuran as the driving solvent and a Haskel 16501 high-pressure pump (Haskel International Inc., Costa Mesa, CA).

The kinetic dead volume of each column was measured with uracil using the initial eluent and a flow rate of 1 mL/min and was found to be, on average, 0.599 ± 0.008 mL; this volume, considered to be 0.60 mL for simplicity, will be referred to as the column volume (V_m). V_m was "adjusted" by combining columns A–C in series to obtain a V_m = 1.20 mL (i.e. columns A and B) or a V_m = 1.80 mL (i.e. columns A–C).

3.4. Chromatographic conditions

All gradient elution conditions were as follows unless stated otherwise. Detection was performed at 254 nm and 10 μL injections of sample were made. The instrument was programmed to form a linear gradient from 100% channel A to 100% channel B in 8.50 min at a flow rate of 1.00 mL/min followed by an isocratic hold at 100% B for 1.20 min and then a step change back to 100% channel A. The instrument was flushed with 100% channel A for 5 min before ending the run (i.e. stopping data collection and beginning data analysis). We optimized the gradient time using Drylab 2000 Plus© (LC Resources, Walnut Creek, CA) and three gradient training runs performed on the HP 1100 with t_G = 5 min, 10 min and 20 min; all combinations of two training runs indicated the optimum t_G was 8.5 min based on the resolution of the critical pair and the analysis time.

When transferring a method between instruments or columns, one must properly adjust V_D. As the HP 1090 and

HP 1100 do not allow us to delay the injection of the sample mixture, which would allow V_D to be less than $V_{D,intr}$, we used an isocratic hold to make V_D = 1.11 mL when V_m was 0.60 mL. An isocratic hold is achieved by programming the pump to deliver the initial eluent to the column for a desired time (t_{ϕ_0}) which is calculated using Eq. (11). We chose V_D = 1.11 mL to be higher than $V_{D,intr}$ on each instrument to allow us to implement an

$$t_{\phi_0} = \frac{V_D - V_{D,intr}}{F} \quad (11)$$

isocratic hold and to provide a reasonable separation of the sample mixture.

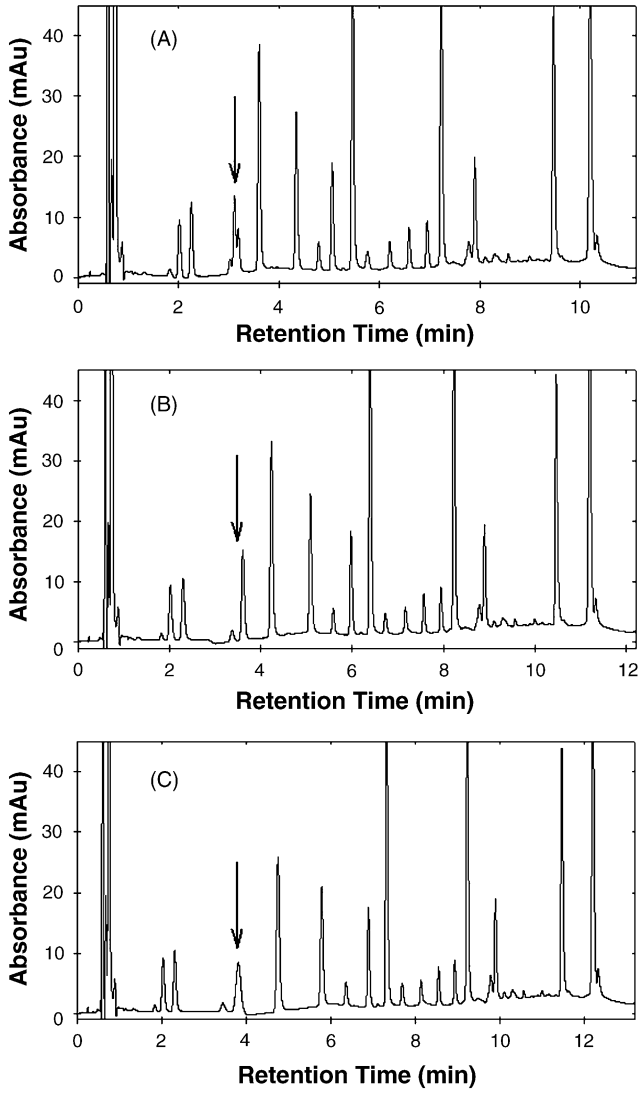

Fig. 1. The effect of an isocratic hold providing a change in the "effective" dwell volume. The three columns described in the experimental section were combined into a single column with V_m = 1.80 mL. Conditions: 3/10/87 to 3/90/7 *n*-propanol/acetonitrile/water in 8.5 min; hold at final eluent strength for 1.2 min then back to initial eluent strength; flow rate = 3 mL/min; detection at 254 nm; 10 μL injection. The "effective" dwell volume was varied between (A) 3.33 mL, (B) 6.33 mL and (C) 9.33 mL. The arrow denotes the region where the dwell volume has the greatest effect on selectivity.

To verify that a higher "effective" dwell volume was not required to obtain adequate separation, we combined the three columns in series (i.e. V_m = 1.80 mL) and varied V_D beyond 3.33 mL as shown in Fig. 1. Although the separation of some peaks improved, there is an obvious loss of separation for other peaks. Thus, we decided to use the constants of 0.60 mL/(1 mL/min × 8.5 min) = 0.071 and 1.11 mL/0.60 mL = 1.85 resulting from Eqs. (9) and (10), respectively, to maintain the "optimized" separation in Fig. 1A using other systems.

4. Discussion

4.1. Test of different column dead volumes

Before performing any experiments to determine if the guidelines in Eq. (8) satisfactorily maintain an optimized gradient elution separation when one changes the instrument (i.e. V_D), the column (i.e. V_m), F and/or t_G, we first had to confirm that the three columns used in this study were qualitatively identical. Therefore, we performed separations on each column using the HP 1090 and identical chromatographic conditions as shown in Fig. 2. Obviously, all three columns are qualitatively identical in terms of band spacing, which one would expect as the columns contain particles from the same batch of packing material. We performed a similar comparison of the columns using the HP 1100 and obtained similar results (data not shown). As the columns are qualitatively identical, we are able to use any column or combination of the three columns to adjust the column volume to values of 0.60 mL, 1.20 mL or 1.80 mL.

According to Eq. (9), band spacing is maintained in gradient elution using the same column and instrument by keeping Ft_G constant. Therefore, we maintained b by varying F and t_G on the HP 1090 as shown in Fig. 3. Obviously, proper adjustment of F and t_G while maintaining V_D/V_m constant on one instrument qualitatively maintains the gradient elution selectivity while allowing for simultaneous adjustment of the analysis time.

Maintaining the selectivity in gradient elution by satisfying Eq. (9) is trivial; our main objective was to obtain further evidence that the guidelines in Eq. (8) for maintaining the gradient separation are accurate. Therefore, we deliberately altered V_m, F and the "effective" dwell volume on the HP 1090 to maintain the separation according to Eqs. (9) and (10) as shown in Fig. 4. Although the column efficiency scales with V_m and F, it is clear that *the guidelines in* Eq. (8) *qualitatively maintain the selectivity of an optimized gradient separation*. Although we altered V_m by combining columns of the same dimension in series, other work in this lab showed that the separation is qualitatively maintained using columns of different dimensions (and thus different V_m) packed with particles from the same lot (data not shown). We believe the serial combination of columns provides a more practical approach for adjusting V_m compared to packing columns of various di-

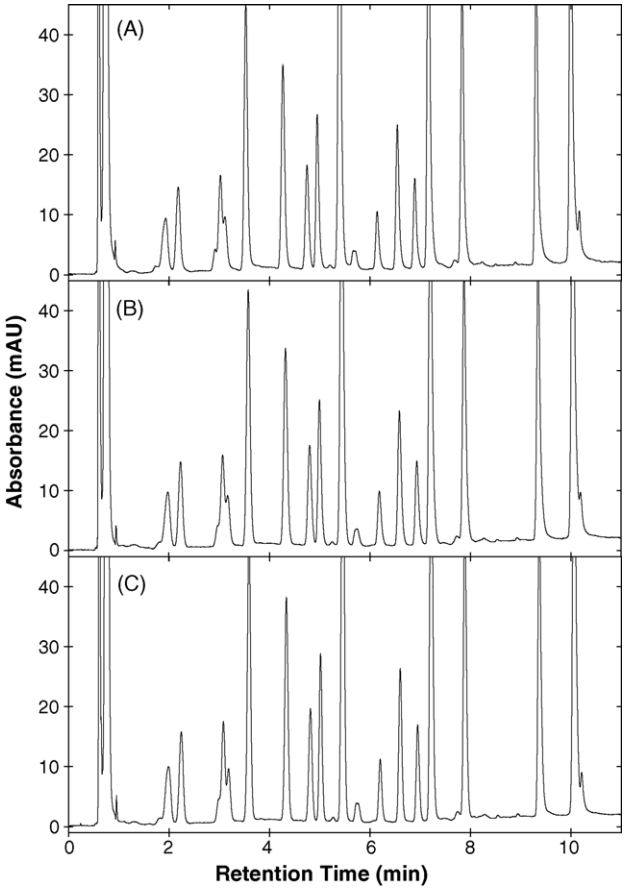

Fig. 2. Separation of the sample mixture on the three 50 mm × 4.6 mm columns A–C with V_m = 0.60 mL. Conditions: flow rate = 1 mL/min; HP 1090; "effective" V_D = 1.11 mL; other conditions are described in Fig. 1.

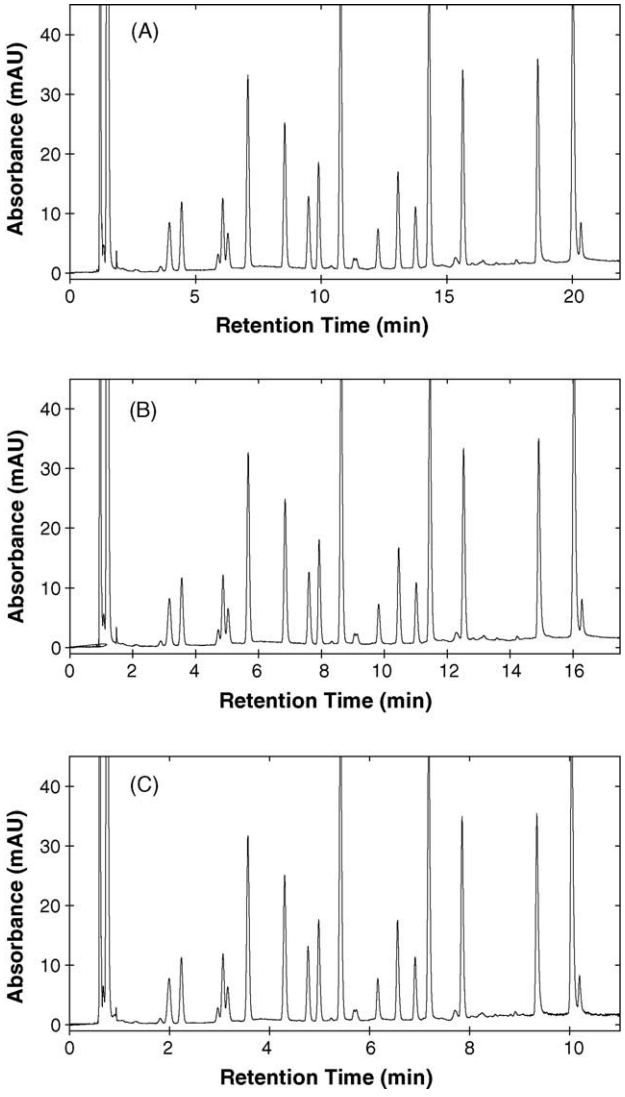

Fig. 3. Satisfying Eq. (9) by adjusting the F and t_G with V_m = 1.20 (columns B and C) and an "effective" V_D = 2.22 mL using an HP 1090. The values of F and t_G were (A) 1 mL/min and 20 min, (B) 1.25 mL/min and 16 min, and (C) 2 mL/min and 10 min; respectively. Other chromatographic conditions are described in Fig. 1.

mensions. Although a number of studies on column selectivity allow one to choose similar columns [15–22], we feel that one should use particles from the same lot to ensure column selectivity for different V_m values is qualitatively identical. When absolutely identical particles are not available, minor changes in the gradient steepness and/or initial eluent strength can often compensate for the small differences in the LSST parameters between the original and available particles.

4.2. Test of different instruments

Knowing that the guidelines in Eq. (8) are valid using one instrument, we decided to transfer the optimized gradient elution method from an HP 1090 to an HP 1100. The HP 1090 and HP 1100 have significantly different values of $V_{D,intr}$ (0.31 mL and 0.75 mL, respectively). Also, the solvent delivery, injection and detection systems are different which leads to differences in the extra-column volume on each instrument. Thus, any effort to minimize, match and/or account for the extra-column volume (V_{ex}) on each instrument as a function of the flow rate is highly recommended as V_{ex} is required to calculate an accurate value of V_m. Also, the dwell volume of both instruments varies with the back-pressure due to the design of the pulse dampener; this variation in V_D with back-pressure adds another degree of complexity when transferring a gradient elution method. Despite the potential difficulties in obtaining accurate values of V_D and V_m, Fig. 5 shows that we successfully transferred the gradient method from the HP 1090 to the HP 1100. We realize that errors in gradient formation (e.g. curvature in ϕ versus t) or very large differences in extra-column volume (V_{ex}) between other pairs of instruments may not allow one to obtain the excellent qualitative agreement between the two separations shown in Fig. 5. Errors in gradient formation or differences in extra-column volume relate to instrument design and/or maintenance issues which are not the focus of this paper.

In Figs. 2–5, we have effectively transferred the same linear gradient elution method between columns of different vol-

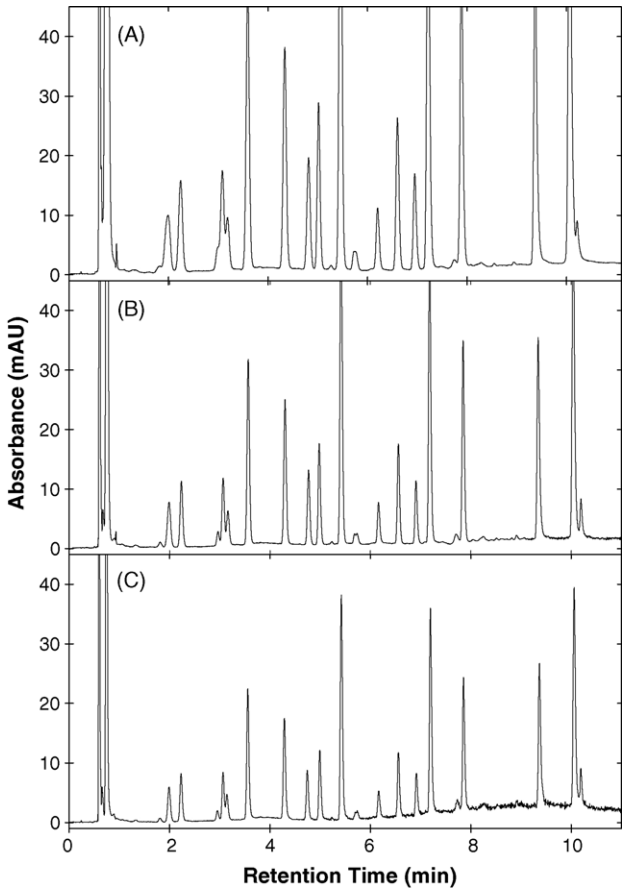

Fig. 4. Compensating for changes in V_m by properly adjusting the "effective" V_D to satisfy Eq. (10). The column volumes used were 0.60 mL (column C), 1.20 mL (columns B and C) and 1.80 mL (columns A–C). We adjusted only the flow rate ((A) 1 mL/min; (B) 2 mL/min; (C) 3 mL/min) to satisfy Eq. (9). Other chromatographic conditions used are described in Fig. 1.

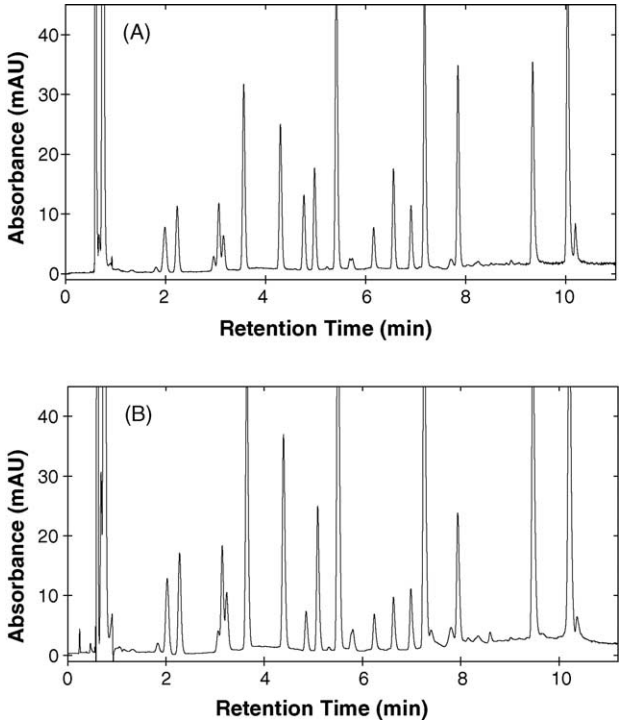

Fig. 5. Compensating for changes in V_D (i.e. the instrument) by properly adjusting the "effective" V_D to be 2.22 mL on the HP 1090 (A) and HP 1100 (B) using V_m = 1.20 mL (columns B and C). Other chromatographic conditions are described in Fig. 1.

ume packed with identical particles and/or between systems with significantly different values of $V_{D,instrument}$. To quantify our success of transferring the linear gradient elution method based on the guidelines in Eq. (8), we compared the apparent gradient selectivity (α_{app}; see Table 1) of each qualitatively identical separation performed. We chose not to compare values of k^* as this parameter is intrinsically identical for each solute in every separation that satisfies the guidelines in Eq. (8). Also, we did not compare values of the apparent retention factors (k'_{app}) as this parameter is sensitive to the extra-column column (V_{ex}), which is difficult to measure, whereas the value of α_{app} is not. Obviously, the selectivity of weakly retained solutes is more sensitive to the assumed values of V_m, V_D and F used to satisfy the method transfer guidelines (see Table 1). Regardless, we believe the percent relative variance in α_{app} is acceptable (average and median values of 0.63% and 0.31%, respectively) for the transference of a gradient method between different instruments and/or different columns. Further improvements in the quantitative accuracy of method transference are possible with minor re-optimization to account

Table 1
Apparent selectivity[a] and percent relative variance[b] for all separations performed[c]

Apparent selectivity[a]	Solute pair																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Average	9.75	1.18	1.46	1.04	1.04	1.16	1.25	1.13	1.05	1.10	1.06	1.09	1.07	1.06	1.04	1.10	1.21	1.08	1.02
Median	9.47	1.18	1.45	1.04	1.04	1.16	1.25	1.13	1.05	1.10	1.06	1.08	1.07	1.06	1.04	1.10	1.21	1.08	1.02
Maximum	11.14	1.19	1.50	1.05	1.04	1.16	1.25	1.13	1.06	1.10	1.08	1.10	1.07	1.06	1.04	1.10	1.21	1.09	1.02
Minimum	9.31	1.17	1.44	1.03	1.03	1.16	1.24	1.12	1.05	1.09	1.06	1.07	1.07	1.06	1.04	1.10	1.20	1.08	1.01
Percent relative variance[b]	5.49	0.66	1.06	0.51	0.35	0.25	0.22	0.31	0.46	0.36	0.69	0.66	0.11	0.08	0.08	0.09	0.24	0.19	0.18

[a] We use the apparent retention factor ($k'_{app} = (t_{R,g} - t_0)/(t_0 - t_{ex})$) to calculate the apparent selectivity ($\alpha_{app} = k'_{app,j}/k'_{app,I}$ and $k'_{app,j} > k'_{app,i}$) where $t_{R,g}$ is the gradient retention time and t_{ex} represents the extra-column time ($t_{ex} = V_{ex}/F$).

[b] The percent relative variance = $\sqrt{\text{variance in } \alpha_{app}/(\text{average } \alpha_{app})^2} \times 100$.

[c] We performed 14 separations, including those described in Figs. 2–5, using two different instruments and various combinations of columns following the guidelines in Eq. (8) (see Section 2).

for small errors in the assumed values of V_m, V_D and F in each system.

5. Conclusions

In this work we have derived an equation for k^* (see Eq. (5)) based on the exact theory of gradient elution which includes the effect of V_D and thus incorporates the effect of solute pre-elution. Using the k^* equation we were able to verify that the guidelines previously presented by Snyder and Dolan [11] based on the assumption of the equality of k^* are correct. This means that the selectivity is maintained during the transfer of a gradient elution method only when the gradient steepness (see Eq. (1)) and the ratio of V_D/V_m are held constant (see Eq. (8)).

Using these exact guidelines, we have successfully transferred an optimized linear gradient elution method between different columns packed with identical particles and between different instruments. Transferring a gradient elution method between two instruments that generate similar gradient profiles is not problematic as long as one can easily adjust the "effective" dwell volume on each instrument. If delayed injections are not possible, we recommend artificially increasing the dwell volume for larger volume columns to allow for a simple transfer of the optimized method to a smaller volume column. However, increasing V_D may adversely affect the selectivity; in this case, one should change the eluent mixing chamber and/or amount of tubing contributing to $V_{D,intr}$ to adjust V_D. Although *quantitatively* maintaining the resolution is difficult when changing the flow rate and column volume, we feel the above guidelines provide a practical means to *qualitatively* maintain a gradient separation transferred between two systems. Furthermore, we believe the quantitative transfer of a gradient elution method between various systems in this work was successful despite different values of V_{ex} on each instrument and small errors in the assumed values of V_m, V_D, and F. In the situation when a separation is not adequately transferred, we recommend minor re-optimization of the method to account for any errors in the parameters required to satisfy Eq. (8).

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Appendix I. The isocratic pre-elution problem

To determine when the gradient "catches" the analyte zone within the column, one must derive equations to describe the time and distance of solute pre-elution for the case when a finite "effective" dwell volume (V_D) exists. The velocity of a solute moving under isocratic conditions (μ_i,) is defined in Eq. (A1) where μ_0 is the velocity of the mobile phase and k'_0 is the retention factor of the solute in the initial eluent strength (ϕ_0). The mobile phase velocity depends on the column length (L) and the kinetic dead time of the column (t_0) as shown in Eq. (A2). The distance the solute moves isocratically along the column (z_i) is defined in Eq. (A3) where t is the time after the sample is injected. The distance that the front of the gradient has traveled (z_g) is defined in Eq. (A4) where t_D is the dwell time as defined in Eq. (4). Obviously, the gradient first enters the column when $t = t_D$. Furthermore, the solute will move under isocratic conditions when $z_i \geq z_g$; thus, the solute is undergoing isocratic pre-elution before moving under gradient conditions. The time of solute pre-elution (i.e. $t = t_{pre\text{-}elution}$) is equal to the time when the gradient overtakes the solute (i.e. $z_i = z_g$). To ensure that the gradient catches the solute, the solute must be retained (i.e. $k'_0 > 0$) and the gradient must enter the column (i.e. $t > t_D$). Under these conditions, we can use Eqs. (A3) and (A4) to obtain an expression for $t_{pre\text{-}elution}$ (see Eq. (A5)). Furthermore, we can use the expression for $t_{pre\text{-}elution}$ and Eq. (A3) to obtain an expression for the distance of solute pre-elution ($z_{pre\text{-}elution}$, see Eq. (A6)) which also requires $k'_0 > 0$ and $t > t_D$.

Using the equations for $t_{pre\text{-}elution}$ and $z_{pre\text{-}elution}$ allows us to determine how the solute elutes from the column. Obviously, solutes with $k'_0 = 0$ always elute isocratically with a retention time (t_R) equal to t_0. Solutes with $k'_0 > 0$ will elute isocratically from the column when $z_{pre\text{-}elution} \geq L$; this condition is expressed in more conventional notation in Eq. (A7). Therefore, solutes with $k'_0 > 0$ will move under gradient conditions (after undergoing isocratic pre-elution) only when $k'_0 > t_D/t_0$. We stress that values of $z_{pre\text{-}elution} > L$ and $t_{pre\text{-}elution} > t_0(1 + k'_0)$ have no physical meaning; these values only indicate that the solute elutes under isocratic conditions (see Eq. (3)).

$$\mu_i = \frac{\mu_0}{1 + k'_0} \tag{A1}$$

$$\mu_0 = \frac{L}{t_0} \tag{A2}$$

$$z_i = \mu_i t = \frac{\mu_0 t}{1 + k'_0} \tag{A3}$$

$$z_g = \mu_0(t - t_D) \text{ for } t > t_D \text{ and } z_g = 0 \text{ when } t = 0 \tag{A4}$$

Equating Eq. (A3) and Eq. (A4) gives:

$$t_{pre\text{-}elution} = \frac{t_D(1 + k'_0)}{k'_0} \text{ for } k'_0 > 0 \text{ and } t > t_D \tag{A5}$$

$$z_{pre\text{-}elution} = \mu_0 \frac{t_D}{k'_0} \text{ for } k'_0 > 0 \text{ and } t > t_D \tag{A6}$$

Thus

$$k'_0 \leq \frac{t_D}{t_0} \tag{A7}$$

for 100% isocratic elution.

Appendix II. The exact equation for predicting gradient elution retention time

Schoenmakers derived an equation for predicting the retention time in gradient elution that fully and properly accounts for the extent of isocratic pre-elution before the gradient catches the solute assuming LSST ($\log k' = \log k'_w - S\phi$) is accurate [12]. We have re-written his equation in more conventional notation (see Eq. (4)). Snyder also derived an equation for predicting the retention time in gradient elution assuming LSST is accurate (see Eq. (A8)) [23]. However, Snyder's equation assumed that solute pre-elution was negligible for all solutes. To account for solute pre-elution, Snyder introduced an ad hoc correction factor χ (see Eq. (A9)) into Eq. (A8) to obtain Eq. (A10) [13]. Based on Eq. (A7), we know that $\chi > 1$ if the solute elutes under gradient conditions; Snyder made a similar claim.

To compare the Schoenmakers and Snyder equations, we converted Eq. (A10) from logarithmic (i.e. log) into natural logarithmic (i.e. ln) notation; the resulting equation is shown in Eq. (A11). Substituting χ into Eq. (A11) results in Eq. (A12). Further rearrangement of Eq. (A12) leads to the same equation derived by Schoenmakers (see Eq. (4)). Thus, Schoenmakers and Snyder arrived at the same conclusion although they both used different methods to solve the problem. Interestingly, an approach used by Jandera and Kucerova [24] to derive an equation for the gradient elution retention time by splitting the column into two serially coupled columns (the solute elutes isocratically on the first column and under gradient conditions on the second column) also leads to Eq. (4).

$$t_R = t_0 + t_D + \frac{t_0}{b}\log(2.3bk'_0 + 1) \tag{A8}$$

$$\chi = \frac{t_D}{t_0 k'_0} \tag{A9}$$

$$t_R = t_0 + t_D + \frac{t_0}{b}\log(2.3bk'_0(1-\chi) + 1) \tag{A10}$$

$$t_R = t_0 + t_D + \frac{t_0}{b}\ln(bk'_0(1-\chi) + 1) \tag{A11}$$

$$t_R = t_0 + t_D + \frac{t_0}{b}\ln\left(bk'_0\left(1 - \frac{t_D}{t_0 k'_0}\right) + 1\right) \tag{A12}$$

Appendix III. The exact equation for gradient elution selectivity

Although the Snyder and Schoenmakers theories provide the same gradient elution retention equations, Schoenmakers introduced an important parameter (τ; see Eq. (A13)) which determines whether the solute is moving under isocratic ($\tau \leq 0$) or gradient ($0 < \tau \leq t_G$)

$$\tau = t - \frac{zV_m}{LF} - \frac{V_D}{F} \tag{A13}$$

conditions [12]; we will refer to τ as the *transition time*, that is, the time at which elution changes from isocratic to gradient elution (see Fig. A1). When $\tau = t_G$, the tail of the gradient leaves the column and the solute will continue eluting from the column isocratically at $\phi = \phi_f$ if one programs a hold at the final eluent strength when the gradient ends.

Unfortunately, calculating the transition time is difficult as one can only know the distance the solute has moved within the column (z) at a specific time t (or vice versa). To determine the value of z or t, one must use the fundamental equation of gradient elution derived by Schoenmakers [12] (see Eq. (A14)) where $k'(\tau)$ is calculated using Eq. (A15) and $\phi(\tau)$ is calculated using Eq. (A16). Integration of Eq. (A13) leads to Eq. (A17) which allows one to determine a value of t at a distance z (or vice versa) for a solute moving under gradient conditions (i.e. $z > z_{pre\text{-}elution}$).

With the ability to predict the transition time, we are now able to predict the retention factor of the solute at a specific value of the transition time using Eqs. (A15) and (A16). However, predicting the transition time is still tedious; thus, we desired an equation for predicting the retention factor of a solute moving under gradient conditions (i.e. $z_g > z_i$) at a specific distance within the column ($z > z_{pre\text{-}elution}$). Snyder and Dolan had previously derived such an equation assuming that the amount of solute pre-elution is negligible for all so-

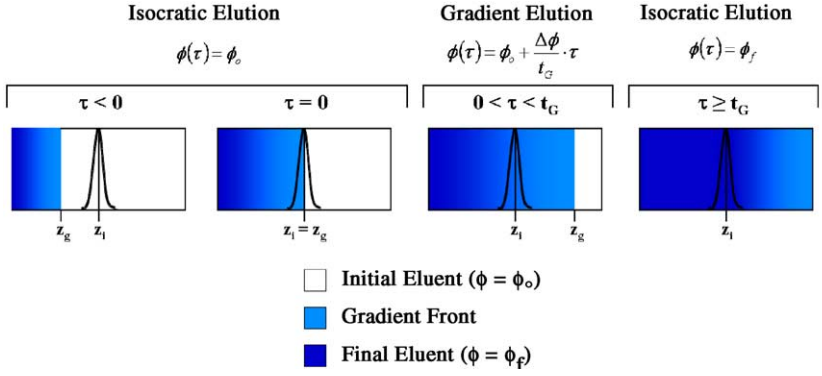

Fig. A1. Cartoon representation of a solute and the gradient moving along the column as a function of the transition time (τ; see Eq. (A13)). The initial eluent strength, gradient front and final eluent strength are distinguished using the colors white, light blue and dark blue, respectively.

lutes (i.e. $z_{\text{pre-elution}} \cong 0$); Eq. (A18) represents an equivalent form of their original Eq. [13]. Unfortunately, the assumptions made to derive Eq. (A18) resulting in an equation for the retention factor (and thus selectivity) in gradient elution *omits any effect of V_D on the selectivity*. This is why Snyder had to introduce the ad hoc "effective" gradient slope [11]. Obviously, Eq. (A18) is not exact as V_D is known to have a significant effect on the selectivity in gradient elution [2]. Therefore, we have derived an equation for predicting the gradient elution retention factor as a function of z (see Eq. (A19)) using the exact theory of gradient elution (i.e. Eqs. (A13)–(A17)).

$$\int_{-t_D}^{\tau} \frac{\partial \tau}{k'(\tau)} = \int_0^z \frac{\partial z}{u_0} \tag{A14}$$

$$\ln k'(\tau) = \ln k'_0 - S\phi(\tau) \tag{A15}$$

$$\phi(\tau) = \phi_0 + \frac{\Delta\phi}{t_G}\tau \text{ for } \tau > 0; \qquad \phi(\tau) = \phi_0 \text{ for } \tau \le 0 \tag{A16}$$

$$= \frac{zt_0}{L} + t_D + \frac{t_0}{b}\ln\left(b\left(\frac{z}{L}k'_0 - \frac{t_D}{t_0}\right) + 1\right)$$

$$\text{for } z > z_{\text{pre-elution}} \tag{A17}$$

$$k'(z) = \frac{k'_0}{bk'_0(z/L) + 1} \tag{A18}$$

$$k'(z) = \frac{k'_0}{b(k'_0(z/L) - (t_D t_0)) + 1} \text{ for } z > z_{\text{pre-elution}};$$

$$k'(z) = k'_0 \text{ for } z \le z_{\text{pre-elution}} \tag{A19}$$

Appendix IV. Predicting the retention time of a solute eluting after the gradient leaves the column

This section is included for the sake of completeness only as it is of no real value in the current work. In the case that the solute elutes isocratically with $\phi = \phi_f$ after the gradient exits the column (i.e. $\tau > t_G$; see Eq. (A13)), the integral equation for predicting gradient retention time (see Eq. (A14)) can be written as shown in Eq. (A20) where k'_f is the retention factor of the solute when $\phi = \phi_f$. Solving for t_R leads to Eq. (A21).

$$\int_{-t_D}^{0} \frac{\partial \tau}{k'_0} + \int_0^{t_G} \frac{\partial \tau}{k'(\tau)} + \int_{t_G}^{t_R - t_0 - t_D} \frac{\partial \tau}{k'_f} = \int_0^L \frac{\partial z}{u_0} \tag{A20}$$

$$t_R = t_0 + t_G + t_D + \frac{k'_f t_0}{k'_0}\left(k'_0 - \frac{t_D}{t_0} - \frac{[\exp((bt_G/t_0) - 1]}{b}\right) \tag{A21}$$

References

[1] L.R. Snyder, Proceedings of the International Symposium on Gas Chromatography, vol. 8, Europe, 1971, p. 81.
[2] L.R. Snyder, J.W. Dolan, LC–GC 8 (1990) 524.
[3] J.W. Dolan, LC–GC 20 (2002) 940.
[4] R. Lewis, J.W. Dolan, LC–GC 18 (2000) 1136.
[5] I. Molnar, L.R. Snyder, J.W. Dolan, LC–GC Int. 11 (1998) 374.
[6] L.R. Snyder, J.L. Glajch, J.J. Kirkland, Practical HPLC Method Development, Wiley-Interscience, New York, 1996.
[7] B.F.D. Ghrist, B.S. Cooperman, L.R. Snyder, J. Chromatogr. 459 (1988) 1.
[8] P. Jandera, L.G. Blomberg, E. Lundanes, J. Sep. Sci. 27 (2004) 1402.
[9] J.W. Dolan, LC–GC 18 (2000) 1228.
[10] H.E. Schwartz, B.L. Karger, P. Kucera, Anal. Chem. 55 (1983) 1752.
[11] J.W. Dolan, L.R. Snyder, J. Chromatogr. A 799 (1998) 21.
[12] P.J. Schoenmakers, H.A.H. Billiet, R. Tijssen, L. de Galan, J. Chromatogr. 149 (1978) 519.
[13] L.R. Snyder, J.W. Dolan, Adv. Chromatogr. 38 (1998) 115.
[14] P.M. Zmak, H. Podgornik, J. Jancar, A. Podgornik, A. Strancar, J. Chromatogr. A 1006 (2003) 195.
[15] J.W. Dolan, LC–GC 2 (1984) 668.
[16] J.W. Dolan, L.R. Snyder, T. Blanc, J. Chromatogr. A 897 (2000) 51.
[17] J.W. Dolan, L.R. Snyder, T.H. Jupille, N.S. Wilson, J. Chromatogr. A 960 (2002) 51.
[18] C.F. Poole, S.K. Poole, J. Chromatogr. A 965 (2002) 263.
[19] L.R. Snyder, J.W. Dolan, LC–GC 20 (2002) 1016.
[20] N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002) 171.
[21] N.S. Wilson, J. Gilroy, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 1026 (2004) 91.
[22] L.R. Snyder, J.W. Dolan, P.W. Carr, J. Chromatogr. A 1060 (2004) 77.
[23] L.R. Snyder, J.W. Dolan, J.R. Gant, J. Chromatogr. 165 (1979) 3.
[24] P. Jandera, M. Kucerova, J. Chromatogr. A 759 (1997) 13.