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Instrument Parameters Controlling Retention Precision in Gradient Elution Reversed-Phase Liquid Chromatography

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Abstract

The precision of retention time in RPLC is important for compound identification, for setting peak integration time windows and in fundamental studies of retention. In this work, we studied the effect of temperature (T), initial (ϕ_0) and final mobile phase (ϕ_f) composition, gradient time (t_G), and flow rate (F) on the retention time precision under gradient elution conditions for various types of low MW solutes. We determined the retention factor in pure water (k'_w) and the solute-dependent solvent strength (S) parameters of Snyder's linear solvent strength theory (*LSST*) as a function of temperature for three different groups of solutes. The effect of small changes in the chromatographic variables (T , ϕ_0 , ϕ_f , t_G and F) by use of the *LSST* gradient retention equation were estimated. Peaks at different positions in the chromatogram have different sensitivities to changes in these instrument parameters. In general, absolute fluctuations in retention time are larger at longer gradient times. Drugs showed less sensitivity to changes in temperature compared to relatively less polar solutes, non-ionogenic solutes. Surprisingly we observed that fluctuations in temperature, mobile phase composition and flow rate had less effect on retention time under gradient conditions as compared to isocratic conditions. Overall temperature and the initial mobile phase composition are the more important variables affecting retention reproducibility in gradient elution chromatography.

1. Introduction

The precision of chromatographic retention time is an important analytical metric [1-7]. Irreproducible retention times have many possible sources including irreproducible flow rates, fluctuations in the system temperature and variations in eluent composition (when controlled by the instrument). The potential for fluctuations in retention time under gradient as compared to isocratic elution may be increased by the added complexity of the eluent program. Flow rate, column temperature and the eluent program are all chromatographic variables set by the user and the accuracy and precision of these parameters are dependent on instrument performance. The effect of these parameters was studied in this work. We first

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looked at the equations governing the relationship between fluctuations in the gradient retention time and fluctuations in various instrumental variables such as flow rate, initial and final eluent composition, gradient time and temperature. We next measured the actual fluctuations in a specific instrument and computed the contribution to the retention times for three distinct sets of solutes. Other instrumental factors impacting retention precision such as electronic noise in the peak signal [8-11], and long term drift [12] have been studied elsewhere.

There are number of critical issues in chromatography that mandate good retention time precision. In automated quantitation, the integration start and stop times are specified within defined limits to assure that the peak is properly identified. These limits vary broadly depending on how narrow the peak is and how close it may be to neighboring peaks [13]. Perhaps the most rigorous application requiring extremely precise retention time data are multivariate chemometric curve resolution methods where data from two or more chromatographic runs are combined. In the most severe cases (e.g. using chemometric methods that combine two or more chromatographic runs with univariate spectral information) retention precision better than 0.2 to 0.3 times the peak half-width is required [13]. When more advanced methods are employed the additional information (e.g. PARAFAC2 [14]) relaxes the retention precision to approximately 0.7 – 1.4 times the peak half-width. Assuming a plate count of 10,000 and a retention time of 2 minutes, chemometric techniques may require retention times to be precise to 0.001 to 0.01 minutes. Retention precision is also a figure of merit to prove that an instrument is qualified for use [5, 15]. Another use of retention time is to chemically verify the presence or absence of a compound [16-18]. In this regard the more precisely the retention time can be defined the fewer will be the number of compounds that can be confused with the analyte of interest. Additionally precise measurement of retention times allows the prediction of separations by using gradient elution training data through methods described by Snyder [19]. There have been a number of studies of the reproducibility of retention time in liquid chromatography that emphasize the importance of column equilibration [12, 20-32]. It is possible to achieve run to run gradient elution retention reproducibility to ± 0.002 min (standard deviation) in a bit more than two column volumes of re-equilibration (so-called “repeatable equilibrium”) [20]. Given a stable column and a good signal to noise ratio [8] the parameters that control retention time precision are: temperature (T), initial (ϕ_0) and final (ϕ_f) mobile phase composition, gradient time (t_G) and flow rate (F).

To maintain retention repeatability care has to be taken to ensure that the instrument is functioning as intended. For example, several studies reported difficulties in precise column temperature control in liquid chromatography especially when the instrument is operated at higher temperatures [33-39]. Zhu and coworkers pointed out that if the source of the problem is the column thermostat the oven set-point temperature may differ from the actual temperature by several degrees. There have been a few reports on the stability of the column temperature which is what controls retention reproducibility [33]. Peasen et al. explored thermal effects that can induce baseline fluctuations. They reported that temperature can cycle with time [40]. Another source of problems is the differences between the actual column temperature and oven temperature set point, which may not be the same when using

different equipment for the same method[41, 42]. In prior work we showed that at flow rates of 1 mL/min or less temperature control of the column was not as tight as at higher flow rates (2-3 mL/min). This was attributed to quite evident changes in the duty cycle of the oven column compartment temperature control system [20].

The consistency of the mobile phase composition is another important parameter that influences retention precision. When a fresh batch of eluent is prepared a small change in composition can have a significant effect on the absolute value of the retention time. In gradient elution, the retention reproducibility may suffer if the instrument is not consistently delivering the same eluent profile (e.g. gradual changes in solvent composition with time) [25]. Extremely good isocratic retention reproducibility can be achieved by gravimetric metering of the eluent [20]. When an improperly functioning pump is used disturbances in mobile phase composition or flow rate and changes in gradient time have a direct impact on the “gradient steepness” and will change the retention time [43-45].

Herein we attempt to better understand the effect of the various instrumental parameters on retention time precision using three different, hopefully representative, test groups of solutes: the first is a group of 14 of the more retained standard solutes of the 16 solutes used by Snyder and Dolan in their hydrophobic subtraction method [46, 47], the second is a set of 18 common pharmaceuticals, and the third is a set of 22 simple derivatives of benzene which have been used extensively to study retention mechanisms in RPLC [48-50]. Based on measured characteristics of these compounds we can compute their retention times under a wide variety of gradient conditions. We then investigated the effect of small changes in the chromatographic parameters (temperature, initial and final eluent strength, gradient time and flow rate) on the precision in retention time.

2. Theory and computational methods

2.1. Predicting gradient elution retention time

The logarithmic retention factors of analytes ($\ln k'$) in RPLC generally decrease in a *quasi*-linear fashion as the volume fraction of organic modifier (ϕ) in the eluent is increased under isocratic conditions. This behavior is approximated by the basic retention equation of linear solvent strength theory (*LSS*) [51].

$$\ln k' = \ln k'_w - S\phi \quad (1)$$

where S is the solute's sensitivity to changes in ϕ and $\ln k'_w$ is its extrapolated retention in pure water. Other relationships could be used but we feel they would simply complicate the mathematics below and not do much to enhance our understanding of the principles involved.

We assume that the above equation is accurate and thus used the linear solvent strength theory to predict gradient retention times (t_R). Schoenmakers and coworkers derived the following equation for predicting the retention time of a solute under linear gradient elution conditions [52]:

$$t_R = t_o + t_D + \frac{t_o}{b} \ln \left(b \left(k'_o - \frac{t_D}{t_o} \right) + 1 \right) \quad (2)$$

where t_o is the column dead time, t_D is the system dwell time, and k'_o is the retention factor in the initial eluent. The gradient steepness (b) is defined as:

$$b \equiv \frac{S \Delta \phi V_m}{F t_G} \quad (3)$$

where ϕ is the change in the initial (ϕ_o) and final (ϕ_f) eluent strength, V_m is the column dead volume (mL), F is the flow rate (mL/min) and t_G is the gradient time (min). It is important to note that Eq. (2) is only valid for those peaks eluting between $t_o + t_D$ and $t_o + t_D + t_G$.

To utilize Eq. (2) and predict the retention times of many solutes under gradient elution conditions, training runs are used to obtain values of S and k'_w as a function of column temperature. In principle one needs only two gradient runs to determine S and $\ln k'_w$ where only t_G is varied, but three runs provide more reliable results [53]. Thus, we collected solute retention time data using gradient times of 10, 30 and 60 minutes under otherwise fixed conditions. Further, we measured retention times in this fashion at three different column temperatures (35, 50 and 65 °C). In principle we could then obtain S and $\ln k'_w$ values at each temperature studied by using the Solver add-in function of Excel to minimize the sum of the squares of the residuals of t_R , which were predicted against the experimental values

(i.e. $\sum_{i=1}^n (t_{R,predicted} - t_{R,experimental})_i^2$) [53, 54]. This procedure would entail determining 6 parameters (3 values of S and $\ln k'_w$) from a total of 9 measurements. In plotting $\ln k'_w$ vs. temperature we always observed quite good straight lines as expected when using three values of T spanning a range of 35 °C-65 °C. Thus, we made the reasonable approximations that over this narrow range in temperature both S and $\ln k'_w$ would be reasonably linear functions of temperature:

$$S = a_s + b_s T \quad (4)$$

$$\ln k'_w = a_w + b_w T \quad (5)$$

We then used Solver with the 9 data points (3 values of t_G at 3 temperatures) to obtain the best values of the 4 parameters of Eqs. (4) and (5). This is statistically more satisfying and in our experience the error in using Eqs. (4) and (5) is almost certainly no greater than incurred in the use of Eq. (1) at each temperature.

2.2. Theoretical uncertainty in retention time due to variations in the instrumental variables

When the contributions to the total retention imprecision ($\sigma_{t_{Rtotal}}^2$) from small fluctuations in temperature (σ_T^2), initial mobile phase ($\sigma_{\phi_o}^2$), final mobile phase ($\sigma_{\phi_f}^2$), gradient time

$(\sigma_{t_G}^2)$ and flow rate (σ_F^2) are uncorrelated, the equation of error propagation gives the following result:

$$\sigma_{t_{R_{total}}}^2 = \left(\frac{\partial t_R}{\partial T}\right)^2 \sigma_T^2 + \left(\frac{\partial t_R}{\partial \phi_o}\right)^2 \sigma_{\phi_o}^2 + \left(\frac{\partial t_R}{\partial \phi_f}\right)^2 \sigma_{\phi_f}^2 + \left(\frac{\partial t_R}{\partial t_G}\right)^2 \sigma_{t_G}^2 + \left(\frac{\partial t_R}{\partial F}\right)^2 \sigma_F^2 \quad (6)$$

Thus if one knows the various derivatives which represent the sensitivity of the retention time to a change in some instrument parameter as well as the actual change (fluctuation) then the variance in retention time can be predicted.

2.2.1. Theoretical effect of a change in temperature—A change in temperature will impact both k'_o and S . Any change in S will change the gradient steepness. To be able to

calculate $\sigma_{t_{R,T}}^2$ we need $\frac{dk'_o}{dT}$ and $\frac{db}{dT}$ (see Eq. 2):

$$k'_o = k'_w \exp(-\phi_o S) \quad (7)$$

$$b = \frac{S(\phi_f - \phi_o)t_o}{t_G} \quad (8)$$

$$\frac{dk'_o}{dT} = \frac{d(k'_w \exp(-S\phi_o))}{dT} \quad (9)$$

$$\frac{dk'_o}{dT} = k'_o \left(\frac{d \ln k'_w}{dT} - \phi_o \frac{dS}{dT} \right) \quad (10)$$

$$\frac{db}{dT} = \frac{d(S\Delta\phi t_o/t_G)}{dT} \quad (11)$$

$$\frac{db}{dT} = \frac{b}{S} \frac{dS}{dT} \quad (12)$$

$$\sigma_{t_{R,T}}^2 = \left[\left(\frac{\partial t_R}{\partial k'_o} \right)^2 \left(\frac{\partial k'_o}{\partial T} \right)^2 + \left(\frac{\partial t_R}{\partial b} \right)^2 \left(\frac{\partial b}{\partial T} \right)^2 \right] \sigma_T^2 \quad (13)$$

Substitution of Eqs. (7)-(12) in Eq. (13) and taking the limit as k'_o becomes very large yields Eq. (14):

$$\sigma_{t_{R,T}}^2 = \left(\frac{t_G}{S\Delta\phi} \right)^2 \left[\left(\frac{d \ln k'_w}{dT} - \phi_o \frac{dS}{dT} \right)^2 + \left(\left(\frac{1}{S} \right)^2 (1 - \ln b k'_o)^2 \left(\frac{dS}{dT} \right)^2 \right) \right] \sigma_T^2 \quad (14)$$

For peaks that elute well after the gradient arrives at the column inlet it is a common assumption in gradient theory that k'_o is quite large. From Eq. (14) it is obvious that retention changes due to temperature changes are bigger when the temperature coefficients of S and k'_o are larger. We also note that holding all else constant, larger values of b and S reduce the effects of temperature changes. Finally since b gets smaller as t_G increases we expect to see the retention precision worsen (become larger) as t_G increases.

2.2.2. Theoretical effect of changes in initial mobile phase composition—

Changes in the initial mobile phase change both k'_o and the gradient steepness (see Eq. 2).

Therefore we need both $\frac{dk'_o}{d\phi_o}$ and $\frac{db}{d\phi_o}$:

$$\frac{dk'_o}{d\phi_o} = -k'_o S \quad (15)$$

$$\frac{db}{d\phi_o} = \frac{St_o}{t_G} \quad (16)$$

$$\sigma_{t_{R,\phi_o}}^2 = \left[\left(\frac{\partial t_R}{\partial k'_o} \right)^2 \left(\frac{\partial k'_o}{\partial \phi_o} \right)^2 + \left(\frac{\partial t_R}{\partial b} \right)^2 \left(\frac{\partial b}{\partial \phi_o} \right)^2 \right] \sigma_{\phi_o}^2 \quad (17)$$

The limiting value at very large k'_o is

$$\sigma_{t_{R,\phi_o}}^2 = \left(\frac{t_G}{\Delta\phi} \right)^2 \left[1 + \left(\frac{1}{(S\Delta\phi)^2} (1 - \ln b k'_o)^2 \right) \right] \sigma_{\phi_o}^2 \quad (18)$$

Clearly the retention precision improves as ϕ and S increase and worsens as t_G increases.

2.2.3. Theoretical effect of a change in final mobile phase composition—

Holding all else constant changes in final mobile phase composition only change the

gradient steepness. Therefore we only need $\frac{db}{d\phi_f}$:

$$\frac{db}{d\phi_f} = \frac{St_o}{t_G} \quad (19)$$

$$\sigma_{t_R, \phi_f}^2 = \left[\left(\frac{\partial t_R}{\partial b} \right)^2 \left(\frac{\partial b}{\partial \phi_f} \right)^2 \right] \sigma_{\phi_f}^2 \quad (20)$$

The limiting value at very large k'_o is

$$\sigma_{t_R, \phi_f}^2 = \left[\left(\frac{t_G}{S \Delta \phi} \right) (1 - \ln b k'_o) \right]^2 \sigma_{\phi_f}^2 \quad (21)$$

Obviously this contribution gets worse as t_G increases and improves with larger S and ϕ .

2.2.4. Theoretical effect of a change in gradient time—Changes in the gradient time

will change only the gradient steepness which requires finding $\frac{db}{dt_G}$:

$$\frac{db}{dt_G} = - \frac{b}{t_G} \quad (22)$$

$$\sigma_{t_R, t_G}^2 = \left[\left(\frac{\partial t_R}{\partial b} \right)^2 \left(\frac{\partial b}{\partial t_G} \right)^2 \right] \sigma_{t_G}^2 \quad (23)$$

The limiting value at very large k'_o is

$$\sigma_{t_R, t_G}^2 = \left[\left(\frac{1}{S \Delta \phi} \right) (1 - \ln b k'_o) \right]^2 \sigma_{t_G}^2 \quad (24)$$

Interestingly this contribution unlike all the others is independent of t_G but like the others improves as S and ϕ increase.

2.2.5. Theoretical effect of a change in flow rate—Changes in the flow rate will change the dead time, dwell time and the gradient steepness. From this point on we will neglect the system dwell time. Changes in gradient steepness requires the determination of

$\frac{db}{dF}$ from Eq. (3)

$$\frac{db}{dF} = - \frac{b}{F} \quad (25)$$

$$\sigma_{t_R, F}^2 = \left[\left(\frac{\partial t_R}{\partial b} \right)^2 \left(\frac{\partial b}{\partial F} \right)^2 \right] \sigma_F^2 \quad (26)$$

The limiting value at very large k'_o is

$$\sigma_{t_{R^*F}}^2 = \left[\left(\frac{t_G}{FS\Delta\phi} \right) (1 - \ln bk'_o) \right]^2 \sigma_{t_G}^2 \quad (27)$$

This term increases with t_G but decreases with increasing F , S and ϕ .

3. Experimental

3.1. Instrumentation

All chromatographic experiments were conducted using an Agilent 1090 chromatograph controlled by version A.10.01 Chemstation software (Agilent Technologies; Palo Alto, CA). This instrument was equipped with a binary solvent delivery system, thermostated column compartment, autosampler, vacuum degasser, low pressure mixing chamber and photo diode array UV detector. The intrinsic dwell volume of the instrument was determined as 0.34 mL with a previously established method [55]. The extra-column volume (V_{ex}) was measured at 1mL/min flow rate by replacing the column with a zero dead volume connector.

3.2. Reagents

Acetonitrile was obtained from Burdick and Jackson (Muskegon, MI). 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 (± 0.01 g) based on the density (at 25 °C) of acetonitrile and passed through a 0.45 μ m nylon filtration apparatus (Lida Manufacturing Inc.; Kenosha, WI) immediately before use. Eluent compositions are reported as the v/v ratio. Potassium phosphate monobasic was from Chempure (Houston, TX). Phosphoric acid (85%) was obtained from Sigma-Aldrich (St. Louis, MO). Homologous series of alkylphenones (acetophenone, propiophenone, valerophenone and hexaphenone) and uracil were obtained from Aldrich (Milwaukee, WI) as a solid and was used without further purification. Uracil was used to measure the kinetic dead volume of the column.

3.3. Column

The columns used in this work were a 5 cm $\times$ 4.6 mm i.d. column with 5 μ m Zorbax SB-C18 particles with a pore size of 80 Å which was a gift from Agilent Technologies.

3.4. S-D Compounds, drug standards and low polarity solutes

All solutes were of reagent grade or better and were used as obtained from the manufacturer without further purification. Standards of all benzodiazepine drugs studied in this work were obtained from the Minnesota Bureau of Criminal Apprehension (BCA) as 1 mg/mL stock solutions in methanol (originally from Cerilliant (Round Rock, TX, USA)). Buclizine (antihistamine drug) was purchased from Sigma-Aldrich (St. Louis, MO) as a solid and was used without further purification. Snyder-Dolan (*S-D*) compounds (acetophenone, anisole, amitriptyline, benzonitrile, 4-n-butylbenzoic acid, cis chalcone, trans chalcone, 5-diphenylhydantoin, ethylbenzene, mefenamic acid, 4-nitrophenol, nortriptyline, 5-phenylpentanol and toluene) and low polarity solutes (benzyl formamide, benzylalcohol, phenol, 3-phenyl propanol, acetophenone, benzonitrile, p-chlorophenol, nitrobenzene,

methyl benzoate, anisole, benzene, p-nitrotoluene, p-nitrobenzyl chloride, toluene, bromobenzene, benzophenone, naphthalene, ethylbenzene, p-xylene, p-dichlorobenzene, propylbenzene, butylbenzene) were of reagent grade or better and were used as obtained from Aldrich (Aldrich Chemical Co., Milwaukee, WI) without further purification. We refer to the *S-D* solutes as solute set A, drug standards as solute set B and low polarity solutes as solute set C (Table 1A-1C).

3.5. Chromatographic conditions

The work for solute sets A and B was carried out with the following gradient program. Mobile phase A contained 30 mM potassium phosphate buffer at pH 2.8. Mobile phase B contained a total of 30 mM potassium phosphate buffer in acetonitrile (70/30 (v/v) acetonitrile/buffer). A concentrated stock potassium phosphate buffer was prepared by mixing phosphoric acid and potassium monobasic phosphate in a ratio that gave a solution of pH 2.8 in water. The appropriate amount of purely aqueous buffer was taken so that both the A and B solvents were 30 mM in phosphate. The gradient profile was from 28.6 to 100% B from 0 to the desired gradient time (10, 30 and 60 min) at a flow rate of 1.0 mL/min. The work for solute set C was carried out with (30/70, 40/60, 50/50, 60/40 (v/v) acetonitrile/water) under isocratic elution. Each isocratic elution was performed at 28.5, 37.5, 49.7, 61.1, 67.6 and 74.8 °C. The work described in section 4.3 was carried out under gradient and isocratic elution. The column was stored in (50/50, v/v) acetonitrile/water. Mobile phase A was pure water and mobile phase B was pure acetonitrile for gradient and machine mixed isocratic elution. Gradient profile was from 50-100 % B from 0-10 min at a flow rate of 1.0 mL/min. In one study premixed mobile phase for isocratic elution was prepared from (50/50 (v/v) acetonitrile/water. The *S* and $\ln k'_w$ values of solute set C were determined under isocratic conditions as described elsewhere [54].

3.6. Study of the equipment performance

Equipment operational qualifications were checked to verify key aspects of the equipment performance, including the tolerance of the solvent delivery system and the column oven in the absence of effects that could be introduced by a specific analytical method. Therefore the effects of pump, machine eluent mixer and temperature control of the high pressure pump on retention repeatability were tested.

The flow rate precision was measured using a 10 mL volumetric flask and a stop watch which was readable to 1/100 of a second (see below).

We used a digital thermometer (Model 15-077-59 Fisher Scientific, Waltham, MA) which has a resolution of 0.1 °C to monitor temperature fluctuations. The thermometer was placed in good thermal contact with the column at approximately the column center. The set point of the instrument was 35 °C but once temperature stabilized it was measured at 35.5 °C. Temperature was checked a few times throughout the day. The instrument was not turned off over night but the pumps were stopped. The column temperature did not ever deviate by more than the resolution of the thermometer. No retention or temperature measurements were made during the night so larger fluctuations than reported here may have taken place (see below). We believe that the with-in day temperature is constant to 0.1 °C.

Temperature changes were further studied by using a thermistor (Omega Model 44031) which had a much higher temperature resolution than the digital readout thermometer (see Fig. 1). The temperature changes at the exit of the 5 cm $\times$ 4.6 mm i.d. column were logged at 0.3 min. intervals over a 24 hour period. The flow rate was set to 1 ml/min and the instrument operated under isocratic conditions (50/50 (v/v) acetonitrile/water) with the column thermostat was set to 35 °C. The thermistor was placed in good thermal contact with the column and the column compartment was kept closed throughout the study. The resistance was logged and converted to temperature using the resistance-temperature conversion given by the manufacturer (see Fig. 1). It is evident that while the short term peak-to-peak fluctuations were certainly less than 0.1 °C there was long term drift during the day of about 0.1 °C and an overnight shift of nearly 0.25 °C.

Additionally the temperature of the high pressure pump was monitored by means of a mercury-in-glass thermometer (± 1 °C). The temperature of the laboratory was also measured throughout the day at the end of each chromatographic run with a mercury-in-glass thermometer. Over a working day room temperature typically rose by approximately 4 °C despite this the temperature at the column remained constant within about 0.1 °C (maximum fluctuation not standard deviation). All gradient and isocratic runs were repeated 3 times. After each set of measurements the instrument was shut down completely. After one hour the instrument was started again and the column was again equilibrated for another hour. At the end of the day it was shut down and left off overnight and the tests were repeated the next day.

3.6.1 Variations in mobile phase composition—In order to assess the small fluctuations in the mobile phase composition, as made by the instrument, we recorded the absorbance signal at the exit of the column generated by mixing methanol with methanol containing 3 % (v/v) acetone. The flow rate was 1 mL/min. Detection was performed at 265 nm; the column thermostat was set to 35 °C. The instrument was set to make four different mobile phase compositions from 0.05 to 0.20 volume fraction of the B solvent in 0.05 increments. The time between composition changes was approximately 20 min. The resulting trace is shown in Fig. 2A. A “blow-up” of the results for $\phi = 0.15$ is shown in Fig. 2B. A 1.0 milliabsorbance change corresponds to about 0.00029 volume fraction (VF) units. The pressure fluctuations were less than 2 bar at a total of 94 bar during these measurements. Fig. 2A and B show complex periodic fluctuations. The period of the fluctuation varied with the volume fraction of the B solvent; the frequency was exactly proportional to the volume fraction.

The standard deviation in the absorbance was computed making sure that *it was measured over an integral number of full cycles*. As long as the measurement is made over more than 4-5 cycles the standard deviation in the absorbance is quite reproducible. The absorbance is easily converted to a volume fraction and thus we can estimate the standard deviation of the eluent composition in volume fraction units. This was reasonably constant at all four volume fractions and was about 0.0002 (0.02% v/v). This corresponded to about one half of the up going peaks in Fig. 2B.

We also estimated the precision of the pumps ability to control the eluent composition by measuring the precision of retention of a series of alkylphenones under gradient elution and two different isocratic conditions (see Table 2). The two isocratic conditions involved a machine prepared mobile phase and a hand mixed mobile phase. Estimating *the average compositional sensitivity* of the solutes as about 0.031 min per 0.001 VF (see last line in Table 5) a retention time fluctuation of 0.003 min (approximately the average of the isocratic and gradient elution results (see Table 2)) corresponds to a fluctuation of about 0.0001 VF. We estimate the worst case within day value to be twice this (0.0002 VF).

4. Results and Discussion

4.1. $\ln k'_w$ and S of the compounds

We measured the $\ln k'_w$ and S values at different temperatures in order to predict retention times under various conditions. We found that these solute parameters vary in a reasonably linear fashion with temperature as the S values range from as low as 3 to as high as 18. Only in the case of the simple benzene derivatives (solute set C) is an approximate linear relationship between S and $\ln k'_w$ observed [56-59]. For solute sets A and B there is a rather poor correlation between S and $\ln k'_w$ [19, 51, 60] due to the high chemical diversity of the analytes.

S and $\ln k'_w$ and all low polarity solutes decrease linearly with temperature. For the S - D solutes all $\ln k'_w$ values decrease with temperature whereas some S values increase (4-n-butylbenzoic acid, mefenamic acid and ethylbenzene) and others either decrease or do not vary much with temperature

4.2. Sensitivity of retention time to instrument parameters

In order to estimate the sensitivity of the retention time to the gradient elution variables we made very small changes in each variable (denoted T , t_G , ϕ_o , ϕ_f etc.) one at a time and computed by means of Eq. (2) the resulting changes in the retention times. For notational simplicity a change in any of these instrument variables is denoted as X . The difference in retention times resulting from X is referred to as t_R . In preliminary calculations we determined X values that were sufficiently small such plots of t_R versus X were extremely linear. This allowed us to compute the slope of t_R vs. each variable hereafter called the sensitivity. Under these conditions the changes in t_R are perfectly proportional to the small changes in temperature. The same linear relationship was observed for each solute against each instrument variable.

This calculation was done for all the conditions given in Table 3. These sensitivities let us compute the change in absolute retention time for a given presumptive change in the instrument parameter. Inspection of Tables 4-8 shows that with the exception of changes in t_G all sensitivities increase as t_G increases. *This is in agreement with the predictions one would make based on the theoretical equations of sections 2.2.1-2.2.5.*

The sensitivities did vary considerably with solute type and gradient time (see Table 4-8) *but did not vary much with flow rate or the initial and final mobile phase composition.* In order to summarize all the results we computed the average sensitivity for all solutes within a

given solute set at a given gradient time. Furthermore we averaged the sensitivities over all values of the four secondary variables (ϕ_o , ϕ_f , F and T , see Table 3). We also determined the maximum sensitivity (using the most sensitive solute) within a given solute set that showed the greatest sensitivity at a given gradient time.

It is important and interesting to note that at the short gradient times solute set B (the drug set) is less sensitive to temperature fluctuations than are the other two solute sets. The average over all three solute sets and all gradient times indicates that a 1 °C change in temperature will change the retention time by 0.052 min. Furthermore if we look at the worst case scenario (i.e. the most temperature sensitive solute at a gradient time of 60 minutes) the retention time changes by 0.24 min for a 1 °C change in temperature. Since changes in retention time are proportional to the magnitude of the perturbation it is correct to say that if one requires a 0.1% precision in retention time the requisite control would need to be 10-fold tighter than for 1% precision in retention. For example, at a t_G of 60 minutes and with temperature control to 0.1 °C the change in retention time becomes 0.024 min for the most temperature sensitive solute and 0.0052 min for an average solute.

4.2.1. Sensitivity to changes in temperature (T)—The effect of a 1 °C temperature change on retention times at fixed ϕ_o , ϕ_f , t_G and F are shown in Fig. 3 (A-C). In Fig. 3 C the intermediately retained solutes appear to be somewhat more sensitive to temperature than either the weakly or strongly retained solutes. On the other hand, in Fig. 3 A and B there is a weak upward, scattered correlation of t_R with t_R .

Retention variations due to fluctuations in temperature propagate through changes in both k'_o and S (see Eqs. (7)-(14) and Table 9). Although the sensitivity of S to temperature is larger than $\ln k'_w$ for solute sets B and C, the data in Table 9 indicate that changes in $\ln k'_w$ with temperature dominate the effect of temperature control. This is because the contribution to the effect of temperature fluctuations resulting from thermal changes in S must be multiplied by ϕ_o (see Eq. 14). Because temperature fluctuations change S , this means that temperature effectively changes the gradient steepness. From the comparisons in Fig. 3, it is interesting to note that 1) the maximum sensitivity of the compounds in sets A and C are more than twice as great as in set B and 2) the compounds in set C have a maximum sensitivity at intermediate t_R values whereas the sensitivity for compounds in set A and intermediately retained solutes in set B are generally scattered versus retention.

It is evident that the drugs are generally less sensitive to temperature than the other solutes. The question arises: is this due to a smaller sensitivity of k'_w and S to temperature for this set of solutes or is it due to some cancellation of changes (see Eq. (14), note the presence of the difference terms)? To settle this issue, we need to examine the magnitude of each term in Eq. (14). The numerical results are summarized in Table 9. The symbols T_1 and T_2 will

denote the terms $\left[\left(\frac{t_G}{S\Delta\phi} \right)^2 \left(\frac{d\ln k'_w}{dT} - \phi_o \frac{dS}{dT} \right)^2 \right]$ and $\left[\left(\frac{t_G}{S^2\Delta\phi} \right)^2 (1 - \ln bk'_o)^2 \left(\frac{dS}{dT} \right)^2 \right]$ of Eq. (14), respectively. Inspection of Table 9 shows that although the term T_1 is larger than T_2 for solute sets A and B, the opposite is true for solute set C where T_2 exceeds T_1 . T_2 represents the variation in gradient steepness with temperature (see Eq. 2). T_1 arises from

the variation of k'_o with temperature. Term T_I is therefore impacted by the effect of temperature on both the k'_w and S parameters. From Table 9 it is clear that T_I is smallest for solute set B at all gradient times. The small value of T_I for the drugs, when ϕ_o is equal to 0.23 (based on the average of all conditions used in Table 3), is not due to any cancellation

between $\left(\frac{d \ln k'_w}{dT}\right)$ and $\left(\frac{dS}{dT}\right)$. Rather the overall size of T_I for solute set B results from the larger average value of S (14.1) compared to solute sets A (11.1) and C (6.7). The variance in retention time due to a temperature change (see Eq. (14)) can have as much as a 4th power dependence on S thus even a marginally larger value of S has a big effect and consequently the drug data set is less sensitive to temperature due to their overall larger S values. This bodes well for peptides solutes as they generally have larger S values than low MW solutes.

4.2.2. Sensitivity to changes in the initial mobile phase composition (ϕ_o)—

Referring to Table 5 we see that the average sensitivities to a 0.001 change in the initial volume fraction are 0.033, 0.036 and 0.023 min for solute sets A, B and C, respectively. Fig. 4A shows the effect of a 0.0002 change in the initial volume fraction on the absolute change in retention time, when ϕ_o is 0.2. It is clear that variations in the initial eluent composition have a greater effect on the intermediately eluting compounds for all three sets of solutes.

We tested the effect of a 1% change in the initial mobile phase composition at several ϕ_o values (0.2, 0.3 and 0.4) under a variety of experimental conditions (different temperatures, gradient times and final mobile phase compositions). The results (not shown here) confirm that variations in the initial mobile phase composition have a direct effect on both k'_o and the gradient steepness as clearly shown by comparing Eqs. (17) and (20). *Thus, changes in ϕ_o will change the retention time more than do changes in ϕ_f , t_G and F* (see Eqs. (15) - (18)) because these three variables do not influence k'_o . Also note that retention time changes are larger for higher values of ϕ_o for a given absolute change in ϕ_o . We also observe that changes in k'_o and b (resulting from changes in ϕ_o) influence the retention time differently depending on the degree of solute retention. Not unexpectedly for early eluting peaks k'_o has more impact on retention whereas b becomes more controlling for the intermediate and later eluting peaks. Similar to what was observed for the effect of temperature, we see that the sensitivities of the three solute sets to changes in initial mobile phase are lower for the 10 min gradients than for the longer gradients.

4.2.3. Sensitivity to changes in the final mobile phase composition (ϕ_f)—

The effect of a 0.0002 v/v fluctuation in ϕ_f on the retention time for a 30 min gradient when ϕ_f is 0.7 is shown in Fig. 4B. All three solute sets show the same trends with retention. Obviously, weakly retained compounds should be and are less sensitive to fluctuations in the final mobile phase composition and the effect of variations in ϕ_f is more pronounced for the late eluting peaks. Fig. 4B shows that at very high retention the curves for the three solute classes exhibit similar trends as expected.

For all three solute sets a 0.001 volume fraction change in ϕ_f shifts retention time on average by 0.007 min (see Table 6). For the most sensitive solutes this value increases to 0.026 min.

Note that on average retention is almost five times as sensitive to a change in ϕ_o as to a change in ϕ_f .

4.2.4. Sensitivity to changes in the gradient time (t_G)—Fig. 4C shows the effect of a 0.03 min change in gradient time (with $t_G = 30$ min) on absolute retention time. In this plot, the three solute sets show similar patterns which resemble the pattern of changes due to variations in ϕ_f (see Fig. 4B). Inspection of Eqs. (2) and (3) shows that a change in b due to a fluctuation in ϕ_f or t_G will result in the same change in t_R . All solute sets show that the sensitivity to changes in t_G increase as retention increases. On average, retention time fluctuates by 0.12 min for all the solutes using a 30 min gradient when the gradient time is varied by 1 min. Mefenamic acid, buclizine and butylbenzene are the most sensitive to fluctuations in both gradient time and final mobile phase composition (see Table 7) in their respective solute sets. This is as expected because these compounds are the most retained in their respective solute sets.

4.2.5. Sensitivity to changes in flow rate (F)—Changes in flow rate modify the column dead time, dwell time and the gradient steepness. Fig. 4D shows the effect of a 0.002 mL/min change in flow rate on the absolute value of the retention time when F is 0.5 mL/min. For all solute sets the less retained compounds are less affected by changes in flow rate than the more retained solutes although there is a lot of scatter in the plots. There is no great difference in sensitivity to flow rate among the three solute sets (see Table 8). Furthermore, the retention time sensitivities due to flow rate changes for all three solute sets are less at shorter gradient times (see Table 8). This is once again in good agreement with the equations in section 2.2.5.

4.3 Estimation of the typical gradient retention time imprecision

We felt it important to obtain estimates of the reproducibility of the flow rate, the precision of the system to prepare eluent and the column temperature. These results are reported in the next three sub-sections.

4.3.1. Flow rate reproducibility—We first examined the reproducibility of the pump. We hypothesized that stopping the instrument might induce changes in various instrument components, such as the pump, piston and valve seals and therefore impact the precision of the flow rate. However, we saw the same flow rate repeatability as measured by use of the volumetric flask approach when we turned the pump on and off during a single day (± 0.002 mL/min at 1 mL/min) as we obtained from repeated flow rate measurements during a single day without turning the instrument on and off. Starting the instrument fresh at the start of each day appears to perturb the instrument components differently than turning it off repeatedly within one day. This is shown by the fact that the inter-day (over 3 days) repeatability of the flow rate was about ± 0.008 mL/min. Thus gradient flow rate reproducibility within one day regardless of whether it is turned on and off is about 0.002 mL/min at a flow of 1 mL/min but can be much poorer (0.008 mL/min) between days.

4.3.2 Column Temperature Reproducibility—The temperature of the column changed within ± 0.1 °C during the within day flow rate measurements reported above; however, the

inter-day repeatability of the column temperature was only about ± 0.7 °C. We also monitored the temperature fluctuations at the column exit over the course of a day. We found that the average column temperature fluctuation for a *continuously* working system was approximately 0.25 °C (see Fig.1)

4.3.3 Temperature of the high pressure pump—We wanted to see if retention precision was related to the thermal environment of the high pressure pump. The temperature of the pump was 32 °C at room temperature. When we deliberately induced a 40 °C increase of the high pressure pump by heating it with a hot air gun to 72 °C we saw no change in isocratic retention times of the alkylphenones. It was surprising to see that retention did not change despite the large temperature excursion. This allowed us to eliminate the temperature of the high pressure pump as a cause of imprecision in retention time. *Clearly we have been unable to pin point why the inter-day precision in retention is worse than the intra-day precision.*

4.4 Estimated contributions from each variable and the total gradient retention precision

Given Eq. (6), the slopes of t_R vs. X (see Tables 4-8) and estimates of the reproducibility in each of the instrument parameters we can compute the individual contribution of each term and the expected retention reproducibility. We will use the average and the worst case sensitivity in each solute set. The results and the estimated degree of control of each instrumental variable are given in Table 10 and Fig. 5. We estimate the degree of control of each variable as: 0.1 or 0.25 °C, 0.0002 volume fraction, 0.01 min and 0.002 mL/min for temperature, mobile phase composition, and gradient time and flow rate respectively.

If we focus first on the average and worst case of the total reproducibility (see columns 5 and 6 in Tables 10A and B) we do not see much difference between any of the solute sets. Clearly the reproducibility in time units gets poorer as the gradient time increases although it is evident that the relative reproducibility improves considerably as gradient time increases. Furthermore when we compare the average total fluctuation in retention time to that due only to temperature (see Fig. 5A and B) it becomes clear that temperature control is overall the most significant variable when the assumed changes in temperature are 0.25 °C (see below).

Table 10A was computed based on the assumption that to the absolute retention the change in temperature is 0.1 °C and that the total imprecision in retention from all sources can be computed based on Eq. (6). The other columns in Table 10A/B give the ratio of the estimated fluctuation due to the indicated variable relative to the fluctuation caused by temperature. Assuming that temperature changes are only 0.1 °C then temperature, the initial mobile phase composition and flow rate are the major variables that control the precision in retention. The drug solutes are an exception in that gradient time is also important because drugs are so insensitive to temperature thus temperature becomes less important. However, when temperature control worsens to about 0.25 °C (as observed in over night and between day runs) then column temperature becomes the major variable in controlling retention precision (see Table 10B). In only 7 of the 18 cases do the initial mobile phase, gradient time and flow rate exceed temperature as the major variable.

We see in Fig. 5 A-B that the total fluctuation is generally worse for the intermediate and late eluters as compared to the early eluters. The very late eluters appear to be slightly more precise than the intermediate eluters. This is frequently observed in practice. In order to further explore the existence of a maximum in the precision vs. retention plot the gradient conditions (flow rate, initial and final ϕ) used for each solute set were adjusted so that the retention times for each of the three solutes sets ranged all the way to the end of the 60 min gradient. The results are shown in Fig 11 A-D. We see that there is a clear maximum in the low polarity solute set (Fig. 6D), a strong suggestion of a maximum in the drug set (Fig. 6C) but only a monotonic upward trend in the S-D set (Fig. 6B). Note that under the conditions used in all plots temperature fluctuations (assumed to be 0.25 °C) were dominant. The uncertainty in retention time is clearly highest for solute set C (see Fig. 6A).

4.5 Comparison of precision in isocratic and gradient elution chromatography

Based on the fundamental equation of isocratic chromatography:

$$t_R = \frac{V_m(1+k')}{F} \quad (28)$$

and the equation of error propagation, Eq. (6), one can easily estimate the precision in retention time due to fluctuations in temperature, mobile phase composition and flow rate.

4.5.1 Effect of flow rate changes in isocratic chromatography—The relative precision in retention time is equal to the relative fluctuations in the flow rate:

$$\frac{\sigma_{t_R}}{t_R} = \frac{\sigma_F}{F} \quad (29)$$

Thus, a 1% change in F will give a 1% change in t_R under isocratic conditions. It is interesting to note that gradient elution retention times are, by and large, less sensitive to fluctuations in flow rate than are isocratic retention times. For solute sets A, B and C (based on the flow sensitivities reported in Table 8) the retention times under gradient conditions vary by 0.61%, 0.60% and 0.71% respectively given a 1% change in F . Thus gradient elution is about a factor of two times less sensitive to flow changes than is isocratic elution. This results from cancellation of the flow rate effect between the t_o and b terms in the third term of Eq. (2). Although the flow rate changes have an effect on the argument of the logarithmic term in Eq. (2) the dependence of t_R on such changes is weaker than the first order dependence of t_R under isocratic conditions shown in Eq. (29).

4.5.2 Effect of changes in eluent composition in isocratic chromatography—

We can use Eq. (1) to estimate the effect of fluctuations in composition on changes in t_R . For simplicity we will assume that the solutes are well retained such that k' is the dominant term in Eq. (28). In this situation it is easy to show that:

$$\frac{\sigma_{t_R}}{t_R} = S\sigma_\phi \quad (30)$$

The average values of S for solute sets A, B and C at 35 °C are 11.1, 14.1 and 6.7 (see Table 1A-1C) respectively. As explained in section 3.6 the volume fraction fluctuation in isocratic composition is about 0.0002 (see Fig.2 and the discussion in section 3.6). Based on this we obtain a precision of 0.22%, 0.28% and 0.13% for solute sets A, B and C respectively under *isocratic* conditions at a mobile phase volume fraction of 0.2. The estimated relative precisions in the *gradient* retention times at an initial mobile phase composition of 0.2 for solute sets A, B and C, are 0.09%, 0.11% and 0.06% respectively. Since the final mobile phase composition has a much smaller effect on precision than the initial mobile phase composition we conclude that the retention precision in isocratic elution is more sensitive to mobile phase composition than in gradient elution.

4.5.3 Effect of changes in temperature in isocratic chromatography—Using 0.1 °C for the temperature fluctuation in Eqs. (4) and (5) and again assuming well retained solutes we derive the following expressions for the relative fluctuation in t_R due to temperature changes:

$$\frac{\sigma_{t_R}}{t_R} = (b_w - \phi^* b_s) \sigma_T \quad (31)$$

The question arises as to what value of ϕ should be taken to allow a proper comparison of isocratic to gradient chromatography. We decided to take ϕ as ϕ^* which is the average eluent composition during gradient elution as defined by Snyder [19]. This particular ϕ corresponds to that which gives k^* , the average retention factor during gradient elution. Thus we compute the value from the equation:

$$\ln k^* = \ln k_w - S\phi^* \quad (32)$$

$$k^* \cong 2/b \quad (33)$$

$$\phi^* = (\ln k_w - \ln k^*)/S \quad (34)$$

We then computed the effect of a 1 °C temperature uncertainty using calculated value of b_w and b_s . For solute sets A, B and C we obtain a precision of 1.38, 1.07 and 1.83 % respectively (see Table 11) for *isocratic* chromatography. These can be compared to 0.71, 0.42 and 0.90 % computed for *gradient* elution assuming the sensitivities in Table 4 and a 1 °C change in temperature. As expected, the least and most temperature sensitive solute sets are the same for both isocratic and gradient chromatography. Thus when we use the average composition during the gradient as the basis on which we compare isocratic and gradient elution we see that gradient elution is less sensitive to temperature fluctuations than is isocratic elution.

The effect of temperature on retention over the whole range in isocratic composition is shown in Fig. 7A. Here we see quite different behaviors vs. composition. In the case of solute set A the effect of temperature is dominated by the temperature dependence of S , in the case of solute set C the $\ln k'_w$ is quite temperature dependent and finally we see that there is a cancelation of effects on the drugs so that in the range of 0.3-0.6 volume fraction the drugs are not very sensitive to temperature although in both water rich and water poor regions the drugs resemble one or the other two sets of solutes. It so happens that the overall average eluent composition (see Table 11 penultimate column) is about 0.3.

5. Conclusions

We have investigated the effect of fluctuations in various instrumental parameters in gradient elution liquid chromatography on retention precision for three groups of compounds. The main conclusions are as follows:

1. All instrument variables impact the retention precision when temperature fluctuations are 0.1 °C or less. However, temperature becomes the dominant variable when it changes by 0.25 °C or more as it can overnight or between days.
2. The second most influential parameter is the initial mobile phase composition which presumably could be significantly improved by making it up by hand using gravimetric metering and not machine proportioning. *The initial mobile phase composition is always more important than the final mobile phase composition.*
3. The flow rate is next in importance. Gradient time and final mobile phase are rarely more important than temperature or the other factors.
4. Within the three groups of solutes examined, the retention times of the drugs are, on average, significantly less sensitive to temperature changes than are the less polar solutes, especially at smaller gradient times. The retention times of the drugs will fluctuate by 0.03 min/°C compared to 0.05 min/°C for all solutes.
5. Low polarity solutes tolerate changes in initial mobile phase composition better than the S-D solutes or drugs. The retention time shifts by 0.02 min - 0.04 min when initial mobile phase composition is controlled to within 0.001 VF. Short term standard deviations in composition in volume fraction units were found to be 0.0002 for the instrument used in this work.
6. Changes in the initial mobile phase composition are nearly 5 fold more important to absolute retention time than is the final mobile phase composition. This suggests that the most reproducible results will be obtained by starting the gradient at 100% A solvent and preparing the A solvent gravimetrically. It is less important to prepare solvent B gravimetrically.
7. Changes in final mobile phase concentration and gradient time have similar effects on retention precision for the three groups of compounds.
8. The effect of changes in final mobile phase composition, gradient time and flow rate appear in the gradient steepness parameter. The retention time for all solutes changed by 0.007 min when the final mobile phase composition was controlled to

0.001 VF. A 0.01 minute change in gradient time and 0.001 mL/min in flow rate changes retention time by 0.0014 min and 0.0045 min respectively (when $F = 1$ mL/min)

9. The retention time changes due to changes in the instrumental parameters T , ϕ_o , ϕ_f and F are smaller at shorter gradient times (i.e. 10 min) times and increase at longer gradient times. The opposite trend is observed for t_G . The change in the relative retention time at the three gradient times tested is not at all proportional to the gradient time.
10. The retention times of the intermediately eluted compounds are more influenced by T , ϕ_o and F whereas ϕ_f and t_G have more effect on the retention times of the later eluting compounds.
11. Isocratic retention times are more sensitive to flow rate disturbances, temperature and mobile phase composition fluctuations than are gradient elution retention times.
12. We anticipate these results to be true for typical RP type phases but there have been few comparisons of the dependence of S , $\ln k_w$ and their temperature derivatives on the different RP materials [61].

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- We determined the retention factor in pure water (k'_w) and the solute-dependent solvent strength (S) parameters of Snyder's linear solvent strength theory (LSST) as a function of temperature for different groups of solutes (acidic, basic and neutral compounds).
- We estimated the effect of small changes in the chromatographic variables (T , ϕ_o , ϕ_f , t_G and F) by use of the LSST gradient retention equation.
- We found that absolute changes in retention time are larger at longer gradient times.
- Pharmaceuticals showed less sensitivity to changes in temperature compared to relatively less polar solutes, non-ionogenic solutes. Surprisingly we observed that fluctuations in temperature, mobile phase composition and flow rate had less effect on retention time under gradient conditions as compared to isocratic conditions.
- Overall temperature and the initial mobile phase composition are the more important variables affecting retention reproducibility in gradient elution chromatography.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Table 1A

Solute sets used in this study

Compounds Name	Formula	$\ln k'_w$ at 35°C	S at 35°C	Slope of $\ln k'_w$ vs $T(^{\circ}\text{C}^{-1})$	Slope of S vs $T(^{\circ}\text{C}^{-1})$
Standard Snyder-Dolan solutes ^d (Solute set A)					
4-Nitrophenol	C ₆ H ₅ NO ₃	3.56	7.85	-0.025	-0.030
Acetophenone	C ₈ H ₈ O	3.89	7.44	-0.010	0.0069
Benzonitrile	C ₇ H ₅ N	3.79	6.81	-0.013	0.0018
5,5-Diphenylhydantoin	C ₁₅ H ₁₂ N ₂ O ₂	5.86	13.57	-0.015	0.0076
Anisole	C ₆ H ₅ OCH ₃	4.73	7.65	-0.014	0.00076
Nortriptyline	C ₁₉ H ₂₁ N	7.51	17.95	-0.015	-0.0022
Amitriptyline	C ₂₀ H ₂₃ N	7.18	16.43	-0.014	-0.0014
Toluene	C ₇ H ₈	5.77	8.45	-0.016	-0.0065
5-Phenylpentanol	C ₁₁ H ₁₆ O	6.41	11.63	-0.011	0.0023
Ethylbenzene	C ₈ H ₁₀	6.81	9.50	-0.0076	0.017
4-n-Butylbenzoic acid	C ₁₁ H ₁₄ O ₂	7.54	12.56	-0.0079	0.021
Cis-chalcone	C ₁₅ H ₁₂ O	7.53	11.09	-0.017	-0.0048
Trans-chalcone	C ₁₅ H ₁₂ O	7.83	11.24	-0.016	-0.00081
Mefenamic acid	C ₁₅ H ₁₅ NO ₂	8.49	12.57	-0.0037	0.032
Average		6.21	11.05	-0.013	0.0031
Table 1B					
Drugs ^a (Solute set B)					
Aminoflunitrazepam	C ₁₆ H ₁₄ FN ₃ O	4.54	14.75	-0.021	-0.073
Chlordiazepoxide	C ₁₆ H ₁₄ ClN ₃ O	4.71	14.13	-0.015	-0.094
Bromazepam	C ₁₄ H ₁₀ BrN ₃ O	5.12	13.22	-0.017	-0.054
Nitrazepam	C ₁₅ H ₁₁ N ₃ O ₃	6.09	13.62	-0.027	-0.057
Oxazepam	C ₁₅ H ₁₁ ClN ₂ O ₂	6.57	15.48	-0.029	-0.072
Clonazepam	C ₁₅ H ₁₀ ClN ₃ O ₃	6.53	14.25	-0.030	-0.059

Compounds Name	Formula	$\ln k'_w$ at 35°C	S at 35°C	Slope of $\ln k'_w$ vs $T(^{\circ}\text{C}^{-1})$	Slope of S vs $T(^{\circ}\text{C}^{-1})$
Estazolam	$\text{C}_{16}\text{H}_{11}\text{ClN}_4$	6.68	14.99	-0.026	-0.060
Flunitrazepam	$\text{C}_{16}\text{H}_{12}\text{FN}_3\text{O}_3$	6.65	13.43	-0.027	-0.051
Desalkylflurazepam	$\text{C}_{15}\text{H}_{10}\text{ClFN}_2\text{O}$	6.79	14.17	-0.022	-0.044
Nordazepam	$\text{C}_{15}\text{H}_{11}\text{ClN}_2\text{O}$	6.49	13.29	-0.019	-0.047
Temazepam	$\text{C}_{16}\text{H}_{13}\text{ClN}_2\text{O}_2$	6.87	14.09	-0.025	-0.057
Triazolam	$\text{C}_{17}\text{H}_{12}\text{Cl}_2\text{N}_4$	7.24	15.17	-0.024	-0.045
Clobazam	$\text{C}_{16}\text{H}_{13}\text{ClN}_2\text{O}_2$	6.98	13.81	-0.027	-0.050
Lormetazepam	$\text{C}_{16}\text{H}_{12}\text{Cl}_2\text{N}_2\text{O}_2$	7.25	14.49	-0.025	-0.053
Diazepam	$\text{C}_{16}\text{H}_{13}\text{ClN}_2\text{O}$	6.98	12.41	-0.018	-0.032
Halazepam	$\text{C}_{17}\text{H}_{12}\text{ClF}_3\text{N}_2\text{O}$	8.301	12.93	-0.024	-0.033
Prazepam	$\text{C}_{19}\text{H}_{17}\text{ClN}_2\text{O}$	8.09	12.34	-0.018	-0.024
Bucizine	$\text{C}_{28}\text{H}_{33}\text{ClN}_2$	10.80	17.10	-0.036	-0.061
Average		6.82	14.09	-0.024	-0.05
Table 1C					
Low polarity compounds ^b (Solute set C)					
N-benzyl formamide	$\text{C}_8\text{H}_9\text{NO}$	1.15	2.83	-0.018	-0.048
Benzylalcohol	$\text{C}_7\text{H}_8\text{O}$	1.57	3.42	-0.012	-0.021
Phenol	$\text{C}_6\text{H}_5\text{OH}$	2.20	4.48	-0.025	-0.051
3-Phenyl propanol	$\text{C}_9\text{H}_{12}\text{O}$	3.27	5.82	-0.014	-0.016
Acetophenone	$\text{C}_8\text{H}_8\text{O}$	3.19	5.16	-0.016	-0.016
Benzonitrile	$\text{C}_7\text{H}_5\text{N}$	3.35	5.36	-0.022	-0.023
p-Chlorophenol	$\text{C}_6\text{H}_5\text{ClO}$	3.77	6.34	-0.026	-0.029
Nitrobenzene	$\text{C}_6\text{H}_5\text{NO}_2$	3.81	5.95	-0.019	-0.024
Methyl benzoate	$\text{C}_8\text{H}_8\text{O}_2$	4.21	6.16	-0.020	-0.017
Anisole	$\text{C}_7\text{H}_8\text{O}$	4.32	6.10	-0.022	-0.021
Benzene	C_6H_6	4.35	5.88	-0.022	-0.019
p-Nitrotoluene	$\text{C}_7\text{H}_7\text{NO}_2$	4.99	7.04	-0.027	-0.026

Compounds Name	Formula	$\ln k'_w$ at 35°C	S at 35°C	Slope of $\ln k'_w$ vs $T(^{\circ}\text{C}^{-1})$	Slope of S vs $T(^{\circ}\text{C}^{-1})$
p-Nitrobenzyl chloride	$\text{C}_7\text{H}_6\text{ClNO}_2$	5.34	7.77	-0.032	-0.033
Toluene	C_7H_8	5.44	7.03	-0.025	-0.023
Bromobenzene	$\text{C}_6\text{H}_5\text{Br}$	5.81	7.48	-0.028	-0.027
Benzophenone	$\text{C}_{13}\text{H}_{10}\text{O}$	6.16	8.45	-0.027	-0.026
Naphthalene	C_{10}H_8	6.49	8.38	-0.032	-0.031
Ethylbenzene	C_8H_{10}	6.53	8.22	-0.029	-0.029
p-Xylene	C_8H_{10}	6.53	8.12	-0.028	-0.025
p-Dichlorobenzene	$\text{C}_6\text{H}_4\text{Cl}_2$	6.64	8.33	-0.031	-0.030
Propylbenzene	C_9H_{12}	7.66	9.36	-0.032	-0.030
n-Butylbenzene	$\text{C}_{10}\text{H}_{14}$	8.67	10.48	-0.032	-0.032
Average		4.79	6.73	-0.025	-0.027

^a S and $\ln k'_w$ were determined in gradient experiments under the conditions explained in the experimental section

^b S and $\ln k'_w$ were determined by isocratic condition with SB-C18 as explained in the experimental section

Table 2
Precision of retention in gradient and isocratic elution

	Average t_R , min	Range, min	SD, min
Premixed Isocratic ^d			
Uracil	0.442	0.002	0.0012
Acetophenone	1.096	0.002	0.0010
Propiophenone	1.625	0.002	0.0010
Valerophenone	3.764	0.005	0.0025
Hexanophenone	6.113	0.006	0.0035
Average	2.608	0.003	0.0018
Median		0.002	0.0012
Instrument Mixed Isocratic Elution ^b			
Uracil	0.449	0.001	0.00058
Acetophenone	1.094	0.001	0.00058
Propiophenone	1.616	0.001	0.00058
Valerophenone	3.725	0.002	0.0010
Hexanophenone	6.041	0.004	0.0020
Average	2.585	0.002	0.00095
Median		0.001	0.00058
Gradient Elution ^c			
Uracil	0.448	0.002	0.0010
Acetophenone	1.083	0.000	0.0000
Propiophenone	1.541	0.002	0.0010
Valerophenone	2.831	0.007	0.0038
Hexanophenone	3.693	0.009	0.0047
Average	1.919	0.004	0.0021
Median		0.002	0.0010

Conditions at $F=1$ mL/min, $T=35$ °C, $\lambda=254$ nm on Agilent Zorbax SB-C18 column 50×4.6 mm, d_p 5 μ m.

^a Eluent A is 50/50 (v/v) acetonitrile/water metered gravimetrically and mixed by hand

^{b,c} Eluent A is 100% water, eluent B is 100% acetonitrile, a 50:50 mixture was metered by the instrument.

^c Gradient from 50 to 100 % B from 0-10 min.

Table 3
Summary of all gradient elution conditions used in the calculation of the sensitivities reported in Tables 4-8

t_G , min	10	30	60
T , °C	35, 65	35, 65	35, 65
ϕ_o , volume fraction (VF)	0.2, 0.3	0.2, 0.3	0.2, 0.3
ϕ_f volume fraction (VF)	0.7, 0.8	0.7, 0.8	0.7, 0.8
F , ml/min	1, 3	1, 3	1, 3

Table 4

Summary of sensitivity to a temperature change

Solute Set	t_G (min)	Average Relative Sensitivity ($\times 10^{-3}/^{\circ}\text{C}$)	t_R/t_R	Average Sensitivity ($\times 10^{-2}\text{min}/^{\circ}\text{C}/\mu$)	Highest Sensitivity ($\times 10^{-2}\text{min}/^{\circ}\text{C}$)	Most Sensitive Compound
A	10	5.7		2.3	3.1	Ethylbenzene
	30	7.6		5.3	7.9	Trans-chalcone
	60	8.9		8.3	14.6	Trans-chalcone
	Ave.	7.4		5.3	8.5	N/A
B	10	2.5		0.88	1.4	Clonazepam
	30	4.4		2.8	4.9	Clonazepam, Halazepam
	60	5.8		5.2	10.1	Halazepam
	Ave.	4.2		3.0	5.5	N/A
C	10	6.7		2.9	4.3	Nitrobenzyl chloride
	30	9.6		7.2	12.3	Propylbenzene
	60	11.3		11.7	23.7	Propylbenzene, Dichlorobenzene
	Ave.	9.2		7.3	13.4	N/A
Average of all solutes and gradient times		6.9		5.2	9.1	N/A

^aThe average sensitivity value at each gradient time in Tables 4-8 is the average value for all solutes in the indicated solute set over all of the temperatures, compositions and flow rates listed in Table 3.

Table 5
Summary of sensitivity to a change in initial mobile phase composition

Solute Set	t_G (min)	Average Relative Sensitivity t_R/t_{RI} ($\times 10^{-3}/0.001VF$)	Average Sensitivity ($\times 10^{-2}\text{-min}/0.001VF$)	Highest Sensitivity ($\times 10^{-2}\text{-min}/0.001VF$)	Most Sensitive Compound
A	10	2.8	1.1	1.4	Nortriptyline
	30	4.5	3.2	4.2	Nortriptyline
	60	5.7	5.7	8.1	Nortriptyline
	Ave.	4.3	3.3	4.6	N/A
B	10	3.5	1.2	1.4	Oxazepam
	30	5.6	3.5	4.1	Triazolam
	60	7.1	6.2	8.0	Lormetazepam, Halazepam
	Ave.	5.4	3.6	4.5	N/A
C	10	2.1	0.84	1.1	Nitrobenzyl chloride
	30	3.2	2.2	3.5	Benzophenon
	60	3.8	3.8	7.2	Naphthalene
	Ave.	3.0	2.3	4.0	N/A
Average of all solutes and gradient times		4.3	3.1	4.4	N/A

Table 6
Summary of sensitivity to change in final mobile phase composition

Solute Set	t_G (min)	Average Relative Sensitivity ($\times 10^{-4}/0.001VF$)	Average Sensitivity ($\times 10^{-3}$ min/ 0.001VF)	Highest Sensitivity ($\times 10^{-2}$ min/ 0.001VF)	Most Sensitive Compound
A	10	9.4	5.0	1.0	Mefenamic acid
	30	7.5	7.8	2.1	Mefenamic acid
	60	6.0	9.3	2.9	Mefenamic acid
	Ave.	7.6	7.4	2.0	N/A
B	10	9.5	4.5	1.2	Bucizine
	30	7.7	7.1	2.7	Bucizine
	60	6.2	8.8	4.3	Bucizine
	Ave.	7.8	6.8	2.7	N/A
C	10	7.4	4.7	1.5	Butylbenzene
	30	5.4	6.7	3.0	Butylbenzene
	60	4.1	7.5	4.4	Butylbenzene
	Ave.	5.6	6.3	2.9	N/A
Average of all solutes and gradient times		7.0	6.8	2.6	N/A

Table 7

Summary of sensitivity to changes in gradient time

Solute Set	t_G (min)	Average Relative Sensitivity (min^{-1} in t_G) t_R/t_{RI}	Average Sensitivity (min/min in t_G)	Highest Sensitivity (min/min in t_G)	Most Sensitive Compound
A	10	0.046	0.25	0.50	Mefenamic acid
B	10	0.047	0.22	0.56	Bucizine
C	10	0.036	0.23	0.69	Butylbenzene
Average $t_G = 10$ min		0.043	0.23	0.58	N/A
A	30	0.013	0.13	0.34	Mefenamic acid
B	30	0.013	0.12	0.43	Bucizine
C	30	0.0091	0.11	0.48	Butylbenzene
Average $t_G = 30$ min		0.012	0.12	0.42	N/A
A	60	0.0052	0.082	0.25	Mefenamic acid
B	60	0.0054	0.077	0.35	Bucizine
C	60	0.0035	0.066	0.36	Butylbenzene
Average $t_G = 60$ min		0.0047	0.075	0.32	N/A
Average of all solutes and gradient times		0.0059	0.14	0.44	N/A

Table 8

Summary of sensitivity and changes in flow rate

Solute Set	t_G (min)	Average Relative Sensitivity (mL/min) ⁻¹	t_R/t_{RI}	Average Sensitivity (min/(mL/min))	Highest Sensitivity (min/ (mL/min))	Most Sensitive Compound
A	10	0.53		2.4	2.9	Toluene
	30	0.61		4.5	6.3	Ethylbenzene
	60	0.68		6.8	10.5	Ethylbenzene
	Ave.	0.61		4.6	6.6	N/A
B	10	0.53		2.1	2.5	Prazepam
	30	0.60		3.9	5.5	Prazepam
	60	0.66		6.0	9.5	Prazepam
	Ave.	0.60		4.0	5.8	N/A
C	10	0.63		2.7	3.4	Benzene
	30	0.71		4.9	7.2	Xylene
	60	0.78		7.1	12.4	Xylene
	Ave.	0.71		4.9	7.7	N/A
Average of all solutes and gradient times		0.64		4.5	6.7	N/A

Table 9
Individual terms contributing to variations in retention time due to 1 °C change in temperature^a

Solute Set	t _G , min	(t _G /S ϕ) ²	d(lnk' _w)/dT	(dS/dT)	ϕ ₀ (dS/dT)	T ₁ ^b	(t _G /S ² ϕ) ²	(1-ln bk' _o) ²	(dS/dT) ²	T ₂ ^c
A	10	4.5	0.013	0.010	0.0023	7.2 × 10 ⁻⁴	0.060	3.22	0.0002	3.4 × 10 ⁻⁵
	30	40	0.013	0.010	0.0023	6.5 × 10 ⁻³	0.54	2.23	0.0002	5.0 × 10 ⁻⁴
	60	160	0.013	0.010	0.0023	2.6 × 10 ⁻²	2.17	2.86	0.0002	3.0 × 10 ⁻³
Average		68	0.013	0.010	0.0023	1.1 × 10 ⁻²	0.88	2.8	0.0002	1.2 × 10 ⁻³
B	10	2.2	0.024	0.054	0.012	3.5 × 10 ⁻⁴	0.01	3.8	0.0031	8.1 × 10 ⁻⁵
	30	20	0.024	0.054	0.012	3.2 × 10 ⁻³	0.10	2.3	0.0031	7.7 × 10 ⁻⁴
	60	79	0.024	0.054	0.012	1.3 × 10 ⁻²	0.41	2.7	0.0031	4.6 × 10 ⁻³
Average		34	0.024	0.054	0.012	5.4 × 10 ⁻³	0.18	2.9	0.0031	1.8 × 10 ⁻³
C	10	13	0.025	0.027	0.0061	3.1 × 10 ⁻³	0.68	3.5	8.1 × 10 ⁻⁴	0.011
	30	116	0.025	0.027	0.0061	2.8 × 10 ⁻²	6	4.6	8.1 × 10 ⁻⁴	0.16
	60	463	0.025	0.027	0.0061	0.11	25	6.5	8.1 × 10 ⁻⁴	0.87
Average		197	0.025	0.027	0.0061	0.048	11	4.9	8.1 × 10 ⁻⁴	0.35
Average of all solutes and gradient times		99.71	0.021	0.030	0.0068	0.021	3.85	3.52	0.0014	0.12

^a All terms and symbols are defined in Eq. (14)

$$a_{T1} \text{ is the term } \left[\left(\frac{t_G}{S \Delta \phi} \right)^2 \left(\frac{d \ln k'_{w}}{dT} - \phi_o \frac{dS}{dT} \right)^2 \right] \text{ from Eq. (14)}$$

$$c_{T2} \text{ is the term } \left[\left(\frac{t_G}{S^2 \Delta \phi} \right)^2 (1 - \ln b k'_{o})^2 \left(\frac{dS}{dT} \right)^2 \right] \text{ from Eq. (14)}$$

Table 10A
Contribution to the computed retention precision from each variable and the resulting cumulative precision (min), assuming a 0.1 °C change in T

Solute set	t_G , min	Precision due to $T(\text{min})^{1a}$		Total t_R , precision (min) ^f		Precision due to ϕ_0^b relative to T		Precision due to ϕ_f^c relative to T		Precision due to t_G^d relative to T		Precision due to F^e relative to T	
		Ave	Worst	Ave	Worst	Ave	Worst	Ave	Worst	Ave	Worst	Ave	Worst
A	10	0.0023	0.0031	0.0064	0.0089	1.0	0.9	0.4	0.7	1.1	1.6	2.1	1.9
B	10	0.00088	0.0014	0.0055	0.0085	2.7	2.0	1.0	1.7	2.5	4.0	4.8	3.6
C	10	0.0029	0.0043	0.0068	0.011	0.6	0.5	0.3	0.7	0.8	1.6	1.9	1.6
A	30	0.0053	0.0079	0.012	0.018	1.2	1.1	0.3	0.5	0.3	0.4	1.7	1.6
B	30	0.0028	0.0049	0.011	0.015	2.5	1.7	0.5	1.1	0.4	0.9	2.8	2.0
C	30	0.0072	0.012	0.013	0.022	0.6	0.6	0.2	0.5	0.2	0.39	1.4	1.2
A	60	0.0083	0.015	0.020	0.031	1.4	1.1	0.2	0.4	0.1	0.2	1.6	1.4
B	60	0.0052	0.010	0.018	0.028	2.4	1.6	0.3	0.9	0.2	0.4	2.1	1.9
C	60	0.012	0.024	0.020	0.038	0.7	0.6	0.1	0.4	0.1	0.2	1.2	1.1

Table 10 B

Contribution to the computed retention precision from each variable and the resulting cumulative precision (min), 0.25 °C change in T .													
Solute set	t_G , min	Precision due to $T(\text{min})^{2a}$		Total t_R , precision (min) ^f		Precision due to ϕ_0^b relative to T		Precision due to ϕ_f^c relative to T		Precision due to t_G^d relative to T		Precision due to F^e relative to T	
		Ave	Worst	Ave	Worst	Ave	Worst	Ave	Worst	Ave	Worst	Ave	Worst
A	10	0.0058	0.0078	0.0083	0.011	0.4	0.4	0.2	0.3	0.4	0.7	0.8	0.8
B	10	0.0022	0.0035	0.0058	0.0091	1.1	0.8	0.4	0.7	1.0	1.6	1.9	1.4
C	10	0.0073	0.011	0.0095	0.015	0.2	0.2	0.1	0.3	0.3	0.6	0.7	0.6
A	30	0.013	0.020	0.017	0.025	0.5	0.4	0.1	0.2	0.1	0.2	0.7	0.6
B	30	0.0070	0.012	0.013	0.019	1.0	0.7	0.2	0.4	0.2	0.4	1.1	0.8
C	30	0.018	0.031	0.021	0.036	0.2	0.2	0.1	0.2	0.1	0.2	0.5	0.5
A	60	0.021	0.037	0.027	0.046	0.6	0.4	0.1	0.2	0.0	0.1	0.7	0.6
B	60	0.013	0.025	0.022	0.037	1.0	0.6	0.1	0.3	0.1	0.1	0.9	0.8
C	60	0.029	0.059	0.033	0.067	0.3	0.2	0.1	0.2	0.0	0.1	0.5	0.4

^aChange in t_R (min) due to a 0.25 °C change in T .

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^b Change in t_R (min) due to a 0.0002 change in ϕ_0 relative to changes in temperature.

^c Change in t_R (min) due to a 0.0002 change in ϕ_0' relative to changes in temperature.

^d Change in t_R (min) due to a 0.01 min change in t_G relative to changes in temperature.

^e Change in t_R (min) due to a 2 $\mu\text{L}/\text{min}$ change in F relative to changes in temperature.

^f Total change in t_R (min) due to the sum of the indicated change in all variables from Eq. (6).

Table 11

Effect of changes in temperature in isocratic elution

Solute Sets	t_G , min	Average b_s^a	Average b_w^b	$\phi^{0.5c}$	$(b_w \phi^{0.5} b_s)^d \times 10^{-2} \text{ } ^\circ\text{C}^{-1}$
A	10	0.0031	-0.013	0.31	1.40
	30			0.25	1.38
	60			0.21	1.40
Average				0.26	1.38
B	10	-0.054	-0.024	0.32	0.69
	30			0.24	1.13
	60			0.19	1.40
Average				0.25	1.07
C	10	-0.027	-0.025	0.32	1.64
	30			0.24	1.86
	60			0.19	2.0
Average				0.25	1.83
Overall Average				0.25	1.43

$\% \sigma_I R / R = \%(q_I - q_W) / I \phi^{0.5} b_s T$

^a See Eq.(4)
^b See Eq.(5)
^c See Eq.(34)
^d See Eq.(31)