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REVIEW

Necessary Analytical Skills and Knowledge for Identifying, Understanding, and Performing HPLC Troubleshooting

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Abstract This review paper summarizes the main topics discussed by John Dolan in the LC troubleshooting series published over the last 16 years in LCGC North America. The paper is directed toward non-expert HPLC users to learn from challenges other HPLC users across the world faced. The topics covered here help the reader not only to understand and perform system troubleshooting but also to learn tips and essentials for data analysis, method development, and system testing (for example: proper integration, establishing best calibration curve, enhancing signal-to-noise ratio, improving selectivity, testing pump performance, etc.). The last section of the paper shows helpful tips, shortcuts, and equations that would be very helpful to remember, for example: (1) simplified equations for calculating dead volume and column efficiency, (2) easy-to-remember rules on how to set detector constant and maximum injection volume, (3) helpful tips for method development, such as choosing gradient or isocratic conditions, as well as (4) recommendations on how to take care of the HPLC system and columns. This review paper can also be useful guide for HPLC users to find references of particular interest from the LC troubleshooting series.

Keywords LC troubleshooting · Data analysis · John Dolan LC troubleshooting · Method development · LC tips and tricks · HPLC system tests

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Introduction

Chromatography is a widely used analytical technique in industry for quantitative and qualitative analyses. The main components of the high-performance liquid chromatography (HPLC) system are the same regardless of where the system is being used, which analyst is using the system, and what application it is being used for. Therefore, the real life problems encountered by HPLC users are similar and it is important to learn from others' experience as their past HPLC problems/mistakes may be our future ones.

Regardless of whether the HPLC system is in well-maintained condition or not, it is impossible not to run into issues, such as leaks, pressure ripple, artifact peaks, peak shape distortion, etc. Some of the origins of the problems encountered with HPLC usage may be due to poor technique, lack of training, or need for maintenance. Other type of problems may arise when analyzing chromatograms or when doing data analysis, for example: proper integration and differentiation between carryover and contamination. This review collects stories, recommendations, tips and tricks, and advices from the LC troubleshooting series published by John Dolan.

John Dolan has been the author of the LCGC North America magazine troubleshooting series for over the last 30 years. These LC troubleshooting articles are also archived at http://www.lcresources.com/tsbible/. John Dolan is considered to be amongst the top experts in the field with extensive practical experience. The LC troubleshooting papers are written in an easy-to-read format, with simplified explanations of general theory for non-expert users, and represent a collection of real life problems from different parts of the world. The content of this review paper covers a wide range of topics that have been discussed in the LC troubleshooting issues, with focus of the

articles published in the past 16 years. The paper is divided into five sections that focus on HPLC method development, understanding the data analysis process, HPLC tips and tricks, LC troubleshooting, and a final section that provides helpful information and shortcuts.

Rather than showing strategies solely on how to troubleshoot problems, this review is directed towards the growing population of the early stage HPLC users and/or users who have taken a deeper interest toward understanding the sources of the problems they face. This review is based upon an overview of real life HPLC problems that an analyst may encounter on a daily basis along with details on how to solve them.

Method Development

Isocratic or Gradient

Regardless of whether your final method will be gradient or isocratic, it is preferable to start with a simple scouting gradient as this is informative about the purity of the sample, the number of components, and their range of polarities and retention. This information helps speed up the method development and decisions in selecting a gradient or an isocratic run [1–3]. If all the peaks elute within 25% of the gradient time, an isocratic run is recommended. On the contrary, a gradient run is recommended when the peaks occupy >40% of the run time. Isocratic or gradient methods can be used when the occupancy window is ≥25 and ≤40% of the gradient time. Table 1 shows a comparison between the performance and instrument setup of isocratic and gradient chromatography [4].

Table 1 Comparison of isocratic and gradient

	Isocratic	Gradient
Method development and optimization	+	−−
Method transfer between systems and columns of different dimensions	++	−−
Peak shape	–	++
Baseline noise	++	–
Peak width	–	+
Limit of detection	–	++
Run time	–	+
Ability to alter selectivity	–	++
Suitable for	Analytes of narrow k' range	Analytes of wide k' range
Resolution	–	+

(+) indicates easy or good, (++) indicates very easy or excellent, (−) indicates fair, and (−−) indicates hard or poor

The instrument design in isocratic separations is simple and the method development is straightforward. Methods can be easily transferred between HPLC systems of different dwell volumes and columns of different dimensions. Rules that govern isocratic separations are: (1) peaks elute earlier as the volume fraction of the strong solvent (ϕ) increases, (2) the longer the peaks stay in the column, the broader and shorter they get, (3) the overall resolution increases significantly as the retention factor (k') reaches 5 and minimally improves thereafter, as shown in the general resolution equation plot. There are exceptions to this rule when the log k' versus ϕ plots cross over, i.e., when selectivity changes with changing % organic (%B). Section "Solvent-strength selectivity [29]" explains the log k' versus ϕ relationship in more detail.

Compared to isocratic separations, gradient separations are faster, give narrower peaks resulting in lower detection limits, and are more flexible [2]. Gradients are employed when the samples have peaks with wide range of polarities (i.e., wide range of k' values) [5]. If isocratic separation is chosen instead, peak width and run time will be sacrificed. The peak widths of analytes eluting under steep gradient conditions are approximately the same [6]. Under shallow gradients, however, the peaks widths increase with increasing retention time, in a less pronounced fashion compared to isocratic conditions. The sharper peaks obtained under gradient chromatography compared to isocratic separations are due to two reasons: (1) the peaks travel at faster velocity compared to isocratic counterpart and (2) the peak undergoes gradient compression due to the faster travel of the peak tail (which is at a higher %B) than the peak front [5].

While some experimental parameters (mobile phase composition, stationary-phase type, and temperature) affect retention factor under both isocratic and gradient conditions, certain column parameters (column size and flow rate) affect the retention factor only under gradient conditions. Retention coefficients can be kept constant under isocratic conditions by keeping the same linear velocity while changing variables, such as mobile phase composition or column parameters. In gradient separation, the retention coefficient can be kept constant by keeping the average retention coefficient during gradient elution (k^*) constant. This can be achieved by adjusting parameters, such as flow rate, gradient time, change in % organic, and dead volume of the column, as shown in Eqs. (1) and (2) below [7–16]. These equations can also be used to scale methods down from wide to narrow diameter columns to be compatible with mass spectrometry (MS). In this case, the gradient slope and the linear velocity should be kept constant [17]

$$k^* = \frac{t_G \times F}{1.15 \times \Delta\phi \times V_M \times S}, \quad (1)$$

$$k^* = \text{Constant} \times \frac{t_G \times F}{V_M}, \quad (2)$$

where t_G is the gradient time, $\Delta\phi$ is the change in the volume fraction (ϕ) of the mobile phase B solvent during the gradient, F is the flow rate, V_m is the column dead volume, and S is the slope of the linear relationship ln k' with ϕ.

How to Develop the Method

Goal [18]

Developing an analytical method gets easier as we know more about the requirements of the candidate method. Some of the requirements may include number of samples, run time, number of analytes, matrix, sensitivity, reproducibility, precision and accuracy, concentration range, equipment limitations, and validation requirements. For example, in a stability indicating method, the number of peaks can be up to 30 with some of the analytes at very low concentrations. For a dissolution method, there may be only one or two analytes, but a large number of samples will need to be analyzed. Therefore, a long method may be necessary for the first case but not the second. There are quantitative parameters used to assess the performance of method, such as retention factor (k), tailing factor (TF), resolution (R_s), and column plate count (N) [18].

Where to Start [19]

Choosing the appropriate chromatography mode is the first step. Choices include normal phase, hydrophilic interaction, ion exchange, chiral, size-exclusion, and reversed-phase liquid chromatography (RPLC). RPLC is the best option to start with, unless there is a good reason not to. RPLC provides the separation power for the majority of the sample problems. Some applications require specific separation modes (e.g., maintaining the biological activity of a biomolecule or using ion chromatography when separating ionic compounds), such that RPLC is not the best option.

Next is a list of recommended conditions/choices when developing a method: (1) Column size, particle size, and flow—100 mm × 4.6 mm, 3 µm at 1–2 mL/min (for LCMS applications, 50 mm × 2.1 mm, 3 µm at 0.2–0.5 mL/min). (2) Column temperature should be above room temperature (30–35 °C). (3) New high purity type B silica columns are recommended. (4) Column choice is a very effective way to change selectivity. It is recommended to start with C18 or C8 and then an alternative column can be selected. (4) Recommended pH range for most columns is 2–8, and 2 < pH < 3 is a good default choice. This low pH will suppress the ionization of the silanol groups. A good starting point is 0.1% formic acid. (5) pH should be at least one unit away from the pKa of the analyte(s). (6) Acetonitrile is a good first choice for organic solvent, as it is transparent in the low UV region down to 200 nm. Methanol is a good substitute that affects selectivity; however, it has higher absorbance at wavelengths less than 220 nm. Tetrahydrofuran is less commonly used [19]. (6) For stability indicating methods, impurities at 0.1% level of the main peak should be quantitated. Thus, the UV detector should be suitable to determine concentration over a 1000-fold concentration range. A mass spectrometry detector is very useful in impurities identification analysis [20].

Superficially porous particles (SPP), also named as solid core, fused core, or core–shell particles, are becoming preferred choice for method development due to excellent performance compared to fully porous particles. SPP particles were first introduced as 2.7 µm particles with performance comparable to sub 2 µm fully porous particles, with a much lower back pressure. The size of the SPP was suitable to use with both HPLC and UHPLC because of their high efficiency along with the low back pressure they generate. Several other sizes ranging between 1.6 and 5 µm have been brought to market. The solid core was initially introduced for faster mass transfer but later found to also decrease Eddy dispersion and longitudinal diffusion. Thus, the overall performance of the SPP surpasses that of the fully porous particles. The efficiency of the particles increases as the thickness of the porous layer decreases due to faster mass transfer through the porous layer. However, the loading capacity on the stationary phase is adversely affected as the thickness decreases [6, 21].

Adjusting Retention [22]

It is recommended to target a k' range between 2 and 10, because a low k' value diminishes the resolution and value above 10 does not significantly improve the resolution. In addition, setting a minimum retention factor of 2 helps minimize the interference from the solvent front peak with the peaks of interest and enhance quantitation of early eluting peaks, and decreases the susceptibility to retention variability from small deviations in % organic [22]. A single-step gradient scouting run at the onset of method development saves time, as it is informative about the complexity of the sample. It also helps to identify whether an isocratic or gradient method is a better choice, as explained previously in "Isocratic or gradient" [23].

Controlling Peak Spacing

After adjusting the retention factor to obtain reasonable separation, the peak spacing can be tuned to maximize resolution. For samples containing analytes of different

functional groups, peak spacing can be changed dramatically by changing % organic. However, changing % organic has a negligible effect on peak spacing for compounds of similar structure, such as homologs, due to the similar log k' versus % organic plots for such molecules. A resolution map is a good visual aid to optimize peak spacing and can be generated by plotting resolution versus % organic, which can be determined from the general resolution equation and solvent-strength relationship [24]. "Selectivity in RPLC" explains more in depth on how to alter the selectivity and, therefore, peak spacing of an analytical method.

Changing Column Selectivity

Selectivity is dependent on the chemistry of the analyte, the mobile phase composition, and the column bonded phase type. Except for ionic compounds, no changes in the chemistry of the analyte can be introduced to change selectivity. Improving the selectivity by changing the mobile phase composition has already been addressed in the previous section. The chemistry of the stationary phase has a major impact on selectivity. Changing the column chemistry from C18 to cyano or polar embedded group dramatically affects the selectivity due to the introduction of new types of interactions, such as π–π interactions (in cyano phase) or enhanced H-bonding interaction (polar embedded phase). Databases for selecting and comparing stationary phases are mentioned in "Column-type selectivity". For a larger change in selectivity, the organic solvent type can be changed simultaneously with the chromatography column. Mobile phase pH is another powerful tool to change the selectivity especially for ionizable compounds (i.e., acids and bases) [25–27].

The effect of selectivity (α), retention coefficient (k'), and column efficiency (N) on resolution can be summarized in the general resolution equation shown below. Figure 1 is a visual interpretation of the general resolution equation that helps identifying the suitable parameter to change for a maximum change in resolution. The increase in resolution as a function of column efficiency and retention coefficient reaches a diminishing value at some point. However, this trend is not observed for the resolution–selectivity relationship which is almost a linear relationship. Therefore, the most efficient way of improving resolution is by altering selectivity by changing column, mobile phase [28–30]

$$\mathrm{Rs} \approx \frac{\sqrt{N}}{4} \times \frac{(\alpha - 1)}{\alpha} \times \frac{k'}{1 + k'}. \tag{3}$$

Speeding Up an Isocratic Method [31]

With sufficient spacing between peaks, the method can be made faster by sacrificing some resolution. This can be done

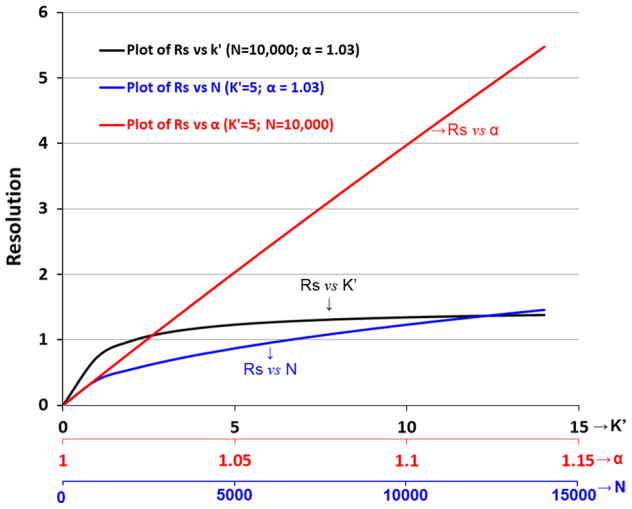

Fig. 1 Effect of efficiency (N), retention coefficient (k'), and selectivity (α) on resolution (Rs), calculated using Eq. (3)

by changing the column parameters, i.e., the parameters that influence column efficiency but not selectivity. These parameters include flow rate, column length, and particle size. Temperature is an example of a non-column parameter that can change both selectivity and efficiency. Analysis time can be shortened by increasing the flow rate. However, as known from the Van Deemter plot, an increase in flow rate beyond the optimum value is accompanied with a drop in efficiency, especially for large particles.

Using a shorter column is another way to speed the analysis. The change in column efficiency, pressure, and analysis time is directly proportional to the change in the length of the column. Changing the particle size is the third way of reducing run time. The accompanied changes in column efficiency, pressure, and resolution are not straight forward as in the case of column length change. Column efficiency changes in proportion to the particle size change and resolution changes as a function of the square root of the particle size, while pressure changes with the square of the particle size change. Some of the aforementioned suggestions to cut the analysis time should be carefully done to prevent a loss in the separation efficiency. For example, injection volume and extra-column volume should be adjusted when using small columns [31].

Precautions for Successful Gradient Method [32]

Gradient methods are subjected to more problems than isocratic methods. Therefore, some precaution steps are recommended to minimize the problems. These tips include: (1) using high purity reagents, (2) maintaining the HPLC system cleanliness by washing the system and the column, replacing mobile phase bottles rather than topping off,

leaving the system at low flow rather than shutting it off, and using seal wash, especially with buffer mobile phases, (3) pretreating sample, such as centrifuging or filtration to clean up any particulate matter, (4) degassing the mobile phase, (5) running blank injections to check for the presence of baseline noise, drift, carryover, etc, (6) dedicating columns for applications rather than sharing columns between different methods, (7) checking the performance of the LC system before running a test, (8) performing priming injections to equilibrate the column (refer to "Helpful tips and tricks, shortcuts, and information"), (9) ignoring the first few injections, especially on brand new columns, (10) running a system suitability test to confirm that the system and the method will produce consistent results, (11) running a standard of know concentration, and (12) re-equilibrating after each gradient with approximately ten column volumes of mobile phase at the initial condition [32]. The re-equilibration volume can be reduced by gradually shortening the re-equilibration time while tracking the retention time variability of the early eluting peaks [33].

How Fast Can a Gradient be Run [10]?

To be able to successfully run fast gradient, the dwell volume of the system should be known to take into consideration the gradient delay, mixer flush-out, and gradient distortion. This issue becomes more significant as the gradient time gets shorter or as the chromatography column gets smaller. It is recommended that the gradient time should be at least double the dwell time. The conventional UHPLC systems are capable of running fast LC gradients reliably due to their low dwell volume, small mixer volume, ability to operate at high pressure, and low extra-column volume. Longer gradients are less problematic and less susceptible to gradient distortion [10].

Testing Method Performance of LC Methods [34]

There are four parameters used to check the performance of a separation method: retention factor, resolution, tailing factor, and column plate count. It is key to check the robustness while developing a method. This can be done by checking the effect of deliberate changes to the method, such as: ±0.1 pH unit, ±2% of the B solvent, ±3 °C, ±10% in flow rate, and different column lots [34–36].

While the recommended retention factor range is 2–10, it is acceptable to extend it range to 1–20. Knowing the retention factor is useful as it is a thermodynamic parameter that is independent of column dimensions and flow rate [34]. Retention factor is defined as

$$k' = \frac{t_R - t_0}{t_0}, \tag{4}$$

where t_R is the retention time of the analyte and t_0 is the dead time of the column.

Resolution, defined below, is the second recommended check for method performance. For peaks of equal height, a resolution value of 1.5 is acceptable, but a value of 2 is recommended. As the difference between the peak heights increases, the recommended resolution value should increase. The numeric value of resolution becomes less meaningful for small peaks eluting after large and tailing peaks. In such a case, larger values of resolution are needed [37, 38]

$$\mathrm{Rs} = \frac{t_{R,2} - t_{R,1}}{0.5(w_1 + w_2)}, \tag{5}$$

where 1 and 2 refer to the first peak and second peak, respectively. w is the peak width at baseline which is determined by drawing tangents to the sides of the peaks and measuring the distance between the tangents at the baseline.

Tailing factor (T_F) values of less than 1.5–1.7 should be targeted for methods developed on high purity silica columns. Starting the method in this range helps avoiding problems as tailing factor tends to increase, and thus reducing resolution, as column ages. Tailing factor is the peak width to twice the front portion of the peak, all at 5% peak height [39, 40]

$$T_F = \frac{AC}{2AB}, \tag{6}$$

where AC is the retention width of the peak at 5% of its height and AB is the front half width at the same height [34].

Column plate count, i.e., the efficiency of a column defined in Eq. (7), is a good tool to track column performance. As a rough estimate, a 100 mm length, 3 µm column generates ~10,000 plates. The plate number increases as particle decreases. When a drop in plate number of 25–30% is observed, it is recommended to change the column

$$N = 16\left(\frac{t_R}{w_b}\right)^2 = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2, \tag{7}$$

where t_R is the retention time of the peak, w_b is the peak width measured between tangents to a chromatographic peak, and $w_{0.5}$ is the peak width at half height [41].

Selectivity in RPLC

Selectivity in chromatography is the ability of a method to separate two analytes from each other. It is defined as the ratio of the retention coefficient of the more retained peak to that of the less retained peak. Selectivity can be altered by changing percentage of organic solvent in the mobile

phase, the solvent type, the column, and the pH. The rank of the effect of these changes on the separation power is as follows: $\%B \approx t_G \approx$ °C < solvent type ≈ column type ≪ pH [42]. To check experimentally whether any of these changes would separate two co-eluting peaks, the following changes are recommended: (1) change ±10%*B*, (2) substitute 5–10% less acetonitrile for methanol, or 25–30% acetonitrile for 35% methanol, (3) find a column of different selectivity (F_s comparison factor from hydrophobic subtraction model >100), and (4) change pH by 4–5 units [42].

Solvent Type Selectivity [43]

The main solvent properties driving the selectivity are the acidic, basic, and dipole properties. The solvent selectivity triangles are means of showing the relative significance of each of these properties for a solvent. In such a triangle, the corner points are three solvents with 100% basicity, 100% acidity, and 100% dipolarity. If such solvents exist, we would be able to tailor any property we need based on blending different proportions of these solvents. However, these solvents do not exist. Because the solvents closest to ideal properties are not miscible (such as carboxylic acid, amines, and chlorinated solvents which have dipole properties), the three solvents we work with and try to mix are alcohols, nitriles, and ethers (e.g., methanol, acetonitrile, and tetrahydrofuran). The solvent selectivity triangle helps in making wise solvent selection choices and solving difficult-to-separate peaks [43].

Solvent-Strength Selectivity [29]

Changing solvent strength is an easier way to change peak retention, compared to changing solvent type. It is usually a sufficient way of separating peaks. However, it has less dramatic effect on selectivity compared to changing solvent type. Therefore, changing the solvent type is suggested when peaks are not resolved after the solvent strength of the mobile phase has been varied. The change in retention factor as a function of %*B* is defined by the following equation:

$$\log k' = \log k_w - S(\phi), \tag{8}$$

where k_w is the retention coefficient in pure water.

This linear relation can be established with just two data points. The slope is dependent on the molecular weight. Therefore, as the difference in molecular weight between two analytes decreases, the resolution between the two analytes does not dramatically change as %*B* changes [29].

When developing isocratic separations, it is recommended to start with a high percentage of organic solvent and drop the percentage composition by increments of 10%*B*. Tracking the resolution between the critical pairs along with the aid of the rule of three (i.e., 10% change in %*B*, results in approximately a threefold change in retention time) helps predict the optimum conditions. It is important to keep in mind that peaks may crossover as ϕ changes due to dissimilar *S* values of the analytes [44].

Column-Type Selectivity [45]

Changing column chemistry is a powerful tool to change selectivity. The difference in selectivity may arise from the silica support material, the chemistry of the bonding phase, or both. When trying columns of different bonding phases from the same manufacturer on the same silica type, the difference in selectivity is based on the difference in the bonded phase chemistry, as the silica particle chemistry should be the same. For larger difference in selectivity, both changes in silica support type and bonded phase should be targeted, by changing manufacturer and bonded phase. A more systematic way to choose columns of similar or different selectivity is by comparing column properties available on databases, such as (http://www.hplccolumns.org/database/index.php and http://apps.usp.org/app/USPNF/columnsDB.html). These databases contain almost 700 stationary phases and provide a method to compare the selectivity of the stationary phases based on the F_S factor which is the vector distance between columns in five-dimensional space. The F_S factor for two columns is calculated according to Eq. (9). The constants in the equation are weighing factors determined for a 67-component sample of "average" composition. These constants describe the relative importance of each of the column parameters for an average sample. As the value of F_S factor value increases, the difference in selectivity between the two columns increases [45, 46]. Examples of calculated F_S factor for four columns are shown in Table 2. Columns with F_S value of 3 or less are considered equivalent columns and are considered excellent matches. As the F_S value increases, the difference in the selectivity of the two columns increases until they are considered orthogonal when the F_S value reaches 50. A visual aid to understand the column comparison in five-dimensional space is the spider plot shown in Fig. 2 which shows the basis of the difference in selectivity of columns. For example, the F_S value of Acquity BEH Shield RP18 and XBridge BEH C18 to indicates that the two columns have different selectivity, but no further details are given on which parameters are different. However, the radar plot shows that the difference in selectivity is mainly due to difference in the hydrogen-bond basicity parameter. In contrary to the aforementioned comparison, the difference in selectivity between XBridge BEH C18 and Kinetex Biphenyl is driven by difference in more than one column parameter. Such plot is helpful for choosing the right column when the analyst knows the physio–chemical properties of

Table 2 Calculated *Fs* factor from the hydrophobic subtraction model, based on Eq. (9)

F_S	XBridge BEH C18 (Waters)	Acquity BEH shield RP18 (Waters)	Zorbax bonus RP (Agilent)	Kinetex biphenyl (Phenomenex)
XBridge BEH C18 (Waters)	0	26	268	25
Acquity BEH Shield RP18 (Waters)	26	0	247	32
Zorbax Bonus RP (Agilent)	268	247	0	263
Kinetex Biphenyl (Phenomenex)	25	32	263	0

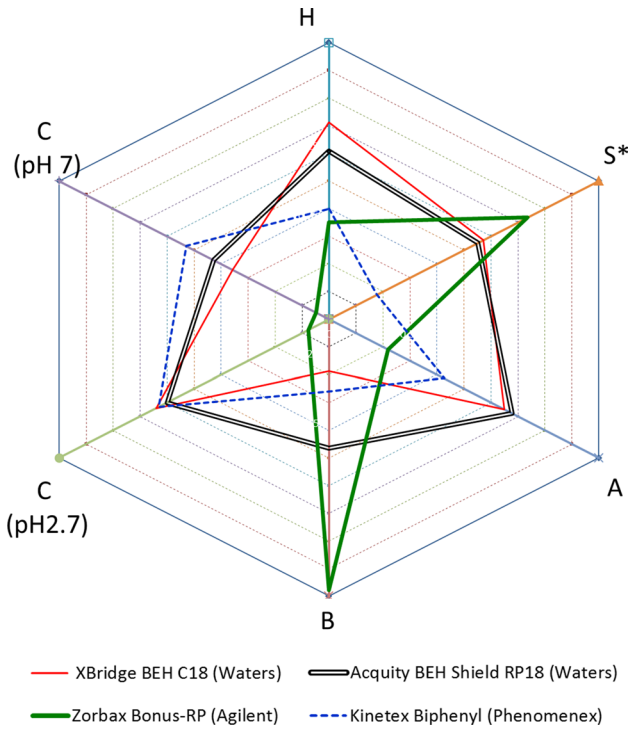

Fig. 2 Radar plot comparing selectivity of four chromatography columns based on hydrophobic subtraction model parameter. The *center* of the spider plot denotes the minimum value observed for a column in the database, while the *corner point* corresponds to the highest possible value of that parameter

the critical-pair separation [i.e., if one of the critical-pair analytes has hydrogen-bond donation sites, a column of high hydrogen-bond acceptance (B) capability, such as the Zorbax Bonus RP, should be chosen)]

$$F_s = \sqrt{\begin{aligned}&(13 \times (H_1 - H_2))^2 + (100 \times (S_1^* - S_2^*))^2 + (30 \times (A_1 - A_2))^2 \\ &+ (143 \times (B_1 - B_2))^2 + (83 \times (C_1 - C_2))^2,\end{aligned}} \quad (9)$$

where *H* refers to column hydrophobicity, *S** refers to steric resistance, *A* refers to hydrogen-bond acidity, *B* refers to hydrogen-bond basicity, and *C* refers to cation-exchange activity. The subscripts 1 and 2 refer to the two columns being compared.

Pressure Selectivity [47]

Effect of pressure on selectivity can be observed when running equivalent gradients at different flow rates (i.e., same flow rate × time). Under such equivalent gradient, a shift in peak relative retention is indicative of a change in selectivity due to change in pressure. The interpretation of the effect of pressure on selectivity is not straightforward as the increase in flow rate is accompanied by an increase in frictional heating. Thus, there is a temperature effect on selectivity. In RPLC, the retention increases as pressure increases for all molecules, with polar and ionized molecule being affected more than others. The effect of pressure on selectivity increases as the chain length of the bonded phase increases. The water/organic ratio of the mobile phase affects the magnitude of the pressure-selectivity effect. The observations are consistent with the change in solute molecular volume when pressure is changed. It is important to note that changing pressure to tailor selectivity is not practical and not as straightforward as changing column type, solvent strength, or solvent type [47].

Temperature Selectivity [48, 49]

Temperature is an effective way to change selectivity, especially for ionic compounds. The change in temperature results in a change in pH and thus a change in the degree of ionization. Therefore, changing the temperature has the same effect as changing pH. Small adjustment in temperature (1–2 °C) of the column can be used to adjust small errors in buffer preparation. A change in temperature may result in a change in retention time, selectivity, or peak shape. Combined effects may be seen [50]. Increasing temperature gives shorter run time and narrower peaks [2].

Issues When Scaling Isocratic Methods Down [51]

Transferring methods from large to small columns is usually for the purpose of saving solvent and/or running faster separations. The analyst should make sure that the chemistry of the column is the same (i.e., same manufacturer, silica, bonding, end capping, etc). If the purpose of the method is to save solvent, then the flow rate is adjusted to

give the same linear velocity. The method can be further adjusted by increasing the flow rate. This can usually be done without sacrificing efficiency, especially with small particles. However, this will lead to higher backpressure. To prevent loss in efficiency, the injection volume should be adjusted and the extra-column volume should be taken into consideration. The injection volume should be at most 15% of the volume of the first peak of interest. The extra-column volume effects refer to peak dispersion that occurs outside the column, such as tubing, injector, detector cell, etc. Because the peaks get smaller as the method is translated into a smaller column, extra-column volume effects become more pronounced. The data collection rate should be increased to collect at least 10–20 data points across the peak. More data points need to be collected as the peak gets smaller. Therefore, extra-column volume should be minimized to prevent a loss in efficiency. This includes adjusting detector constant, data collection rate, tubing volume, injection volume, and strength of sample solvent [52, 53].

Mobile Phase Buffers

Buffer Selection and Preparation [54, 55]

The role of buffer in mobile phase is to resist any changes in pH. Buffers are used when the sample or the column has acidic or basic components. To get a precise control over pH, the buffer should be used at its maximum buffering capacity, i.e., within ±1 pH units of its p*K*a. The buffering capacity drops 25 times at 2 pH units away from the buffer p*K*a. Common approaches to prepare buffer include: (1) using buffer calculator program, such as (https://www.zirchrom.com/pass.asp), (2) using the Henderson–Hasselbalch equation, and (3) by titration until the targeted pH is achieved [54, 55].

Phosphate buffers are easy to prepare, can be obtained at high purity, cover suitable pH range, and have low UV absorbance. However, they are not mass spectrometry (MS) friendly buffers. Trifluoroacetic acid is MS friendly and is suitable for work at pH ~2. Phosphate buffers are suitable for the following pH ranges 2–3.1 and 6.2–8.2. Due to their volatility, acetate buffers are MS friendly, but care should be taken to prevent selective evaporation. It is recommended to use organic buffers at high pH rather than phosphate buffers to minimize dissolving silica. Examples of buffers that can be used at high pH are tris[hydroxymethyl] aminomethane, pyrrolidine, trimethylamine, and glycine, If possible, it is advised to have the same buffer concentration in lines A (aqueous) and B (organic) [56, 57].

Buffer in Aqueous-Organic Mixtures [58]

The interpretation of pH value in partially aqueous solutions is difficult and different than aqueous solutions. The change in pH associated with the presence of organic solvent is due to four reasons: (1) change in the junction potential, the small current established across the frit between the pH sensing electrode and reference electrode, due to the presence of organic solvent. This change results in a difference in the junction potentials for the calibration standards and the sample. (2) Change in autoprotolysis constant ($K_W = 10^{-14}$). In aqueous solution, neutral is defined as when $[H]^+=[OH]^-$, while in methanol, the neutral state is when $[H]^+=[CH_3O]^-$. Therefore, pH = 7 is no longer the neutral state for methanol, but it is rather 8.3. (3) Change in pH scale due to change in solution properties, such as solvent composition or temperature. (4) Change in buffer p*K*a. Compared to water, organic solvents dissociate ions differently due to their lower dielectric constants. The difference in pH between aqueous and aqueous-organic solvents is minimum if the organic component constitutes less than 50% of the composition. Nonetheless, the difference may be up to two pH units or more when comparing the pH in organic solvent to that in aqueous solvent.

Ion Pairing Advantages and Challenges [59]

The primary choice for analysts is to operate at low pH (2–3) where numerous column choices are available. At low pH, acidic and neutral samples are not ionized and are well retained. On the other hand, basic samples will be protonated and retained (if they have a major non-polar chain) or unretained if the molecule is polar. The ion pairing reagent has an ionic end and a non-polar tail, and its addition to the mobile phase improves the retention of the polar molecules. The mechanism of retention enhancement is as follows. First, the non-polar tail of the ion pairing reagent is held strongly by the non-polar chain in the stationary phase, while the ionic part is directed to the mobile phase. Then, the ionic part of the ion-pairing reagent will retain ionic compounds of opposite charge. For positively charged ionic bases, a negatively charged ionic pairing reagent, such as alkyl sulfonic acid, can be used. Negatively charged ionic acids are retained using a positively charged ionic pairing reagent, such as tetrabutyl ammonium chloride [59].

The robustness of a methods decreases by adding ion pairing reagent. Yet, sometimes, adding the ion pairing reagent is the solution for the separation problem when some of the ionized peaks elute in the dead volume. It is important to keep in mind that the retention time of neutral compounds will decrease when adding an ion pairing reagent, as the ion pairing reagent occupies some of the stationary phase. When picking the optimum concentration of the ion pairing reagent for an isocratic method, it is recommended to have two mobile phases, one with an ion pairing reagent and one without. The optimum concentration of the ion

pairing reagent can be found by gradual increase of the ion pairing concentration in step wise fashion by blending the two mobile phases until the retention factor of the first peak is >1. Retention can be fine-tuned by changing temperature, pH, or percent methanol [59].

Some precautions are needed when working with ion pairing reagents: (1) the concentration of the ion pairing reagent in the stationary phase is dependent on the organic content and temperature, (2) the equilibrium of the concentration of the ion pairing reagent in the stationary phase is slow, i.e., 20–50 column volumes are needed for equilibration, (3) some ion pairing reagents have substantial UV absorbance, (4) ion-pairing reagents cannot be washed easily off the column, and (5) trace level of ion-pair reagent can change selectivity when used [59]. Retention time may vary in methods using ion-pairing due to temperature change, pH change, pH mismatch between mobile phase and sample solvent, insufficient ion pair concentration, and use of a pH adjusted mobile phase rather than a true buffer [60].

Alternatives to ion pairing reagents include using: (1) trifluoroacetic acid as it acts as an ion pair for large biomolecules and it equilibrates quickly, (2) polar embedded phase columns or columns that are suitable with 100% aqueous, (3) ion exchange instead of ion pairing if all the samples are ionized, and (4) mixed-mode stationary phases which have a charge near the base of the alkyl station phase chain [59].

How to Increase the Retention of Polar Compounds

The retention of polar compounds in RPLC can be increased to separate from the void volume. This can be very useful if mass spectrometry analysis is needed to be done on the sample. Increasing the retention can be achieved by: (1) adjusting the pH if the analyte is basic or acidic. Low pH suppresses the ionization of acids, while high pH suppresses the ionization of bases; (2) using polar embedded phase column or a more retentive column; (3) decreasing the strength of the mobile phase; (4) adding an ion pairing reagent; (5) using normal phase chromatography, or (6) selecting specialty columns (such as carbon columns) [61, 62].

Data Analysis: Understanding Chromatogram Troubleshooting

Calibration Curves

Types, Construction, and y Intercept [63]

Calibration curves are very common with UV detection, because the detector signal is linear over at least four orders of magnitude. There are three types of calibration curves: (1) single-point calibration is used where the calibration standard is injected at a single-point concentration around which the concentration of the analyte is tightly clustered (±10%), (2) two-point calibration: is commonly used when the concentration of the analyte is narrowly dispersed. The calibration standards are injected at two concentration levels bracketing the concentration of the analyte, (3) multipoint calibration is usually used for samples that cover wide concentration range. In such calibration, standards of different concentration are used to establish the response factor–concentration relationship [63].

To determine linearity of a method, a minimum of five concentrations is suggested by regulatory guidelines. Two different dilution schemes can be used: (1) linear dilution where the concentrations of the standards are linearly apart from each other, (2) exponential dilution where the samples are prepared to follow an exponential increase ($y = a \times e^{bx}$). In such a curve, more data points are available on the low end of the concentration range rather than the high end [63].

The intercept of a calibration curve can be set to zero (i.e., force the calibration curve to go through zero and using $y = mx$ model instead of $y = mx + b$) when the y intercept is less than one standard deviation from zero. If the calibration curve is forced inappropriately through zero, it can generate large errors especially at the low concentration end. On the contrary, if the calibration curve is not forced through zero, when it should be, large errors arise, especially at the high end of the concentration range [63, 64].

The Limits: S/N, LOD, and LLOQ [65]

The signal to noise (*S/N*) can be measured manually by drawing lines bracketing the noise (this is denoted as the noise value) and then dropping a perpendicular from the peak apex to the midpoint between the two lines bracketing the noise (this is denoted as the signal). The signal to noise increases as the peak gets larger and the percent relative standard deviation (%RSD) of the peak area can be estimated by Eq. (10). As the *S/N* exceeds 100, the %RSD becomes negligible. The signal-to-noise ratio can be enhanced by injecting higher concentration, injecting larger volumes, or by optimizing the detector time constant and collection rate [65]

$$\%\mathrm{RSD} \approx \frac{50}{S/N}. \tag{10}$$

Limit of detection (LOD) is the smallest concentration that can be detected with confidence, at which the analyst can state that the analyte is present. Three methods can be used to determine LOD: (1) visual evaluation, (2) signal to

noise, and (3) standard deviation of the response and the slope. The latter two techniques are more reliable than the visual assessment. Because measuring *S/N* is accompanied with many challenges, it is recommended that the *S/N* value is supported by measuring the %RSD of the peak area. A signal-to-noise value of 3 and %RSD value of 17 are commonly used as LOD. The last technique is the standard deviation of the response and the slope which relies on the overall performance of the calibration curve rather than a single concentration. LOD is calculated according to the following equation:

$$\mathrm{LOD} = \frac{3.3\sigma}{S'}, \tag{11}$$

where σ is the standard deviation for a calibration curve and S' is its slope.

The LOD can be optimized according to Eq. (12) [66]

$$\mathrm{LOD} = f\left\{\frac{M_\mathrm{w} V_\mathrm{m}(1+k')(S/N)}{\mathrm{CV}N^{0.5}L_\mathrm{fc}\times\varepsilon}\right\}, \tag{12}$$

where M_w is the molecular weight, V_m is the column volume, k' is the retention factor, CV is the desired precision, N is the column plate number, L_fc is the length of the detector flow cell, and ε is the extinction coefficient.

The LOD increases as the breadth of the peak increases which may result from (1) low diffusion coefficient as the molecular weight of the sample increases, (2) the column volume (mainly diameter) increase, (3) retention time increase (especially in isocratic), (4) *S/N* decrease, and (5) large detector cell volume, especially for applications with sub 2 µm particles. Shortening the detector cell is one option to reduce the cell volume, but this results in a decrease in detector response. A better approach is to use the new cell designs where the volume of the cell is decreased, but the path length is kept constant using total internal reflection techniques. LOD can be improved by increasing the extinction coefficient of a molecule through derivatizing it or by selecting a wavelength where there is minimum interference from the mobile phase. LOD can also be improved by thermostating the column and washing it regularly.

Lower limit of quantitation (LLOQ) is the limit at which the analyst can report the concentration of the sample with confidence. It is determined to be at an *S/N* level of 10 or %RSD area value of 5%. It can also be determined using the standard deviation of the response and the slope, as shown in Eq. (13) [65]. Issues with determining LOD arise when using a wide range of concentration (20 fold or so). The concentration at the higher end of the plot may affect the error at the low end of the calibration curve, because the detector is no longer linear at that point. Values of R^2 can be misleading about how good the data set is [67]

$$\mathrm{LOQ} = \frac{10\,\sigma}{S'}, \tag{13}$$

where σ is the standard deviation of the response and S' is the slope of the calibration curve.

Visualizing Problems with Calibration Curves [11, 68]

The conventional way of plotting the calibration curve (response factor versus concentration) is not the best way to evaluate the abnormal behavior of the plot, because the lower concentration points are aggregated in a small region. A better way to look at the data is by plotting the concentration (x axis) on a logarithmic scale and to convert the y axis values to % error rather than response factor. The % error is the percent difference between from the actual response factor and the value obtained from the best fit line. The points are expected to have an approximately even distribution above and below the x axis. A positive bias in the plot indicates a mistakenly added response to the point which may, for example, result from a contamination in the sample diluent or glassware. On the other side, a negative bias may indicate the presence of adsorptive losses such as to the glassware or filtration membrane [68].

Choosing Appropriate Calibration Model [69]

There are three types of calibration models: external standardization, internal standardization, and standard addition. The use of external standard is the simplest and most commonly used model. The concentration of an unknown sample is determined by comparing its response to that of a known sample. When the sample preparation involves many steps, internal standards can improve the accuracy and precision of the method. The internal standard analyte is not the analyte of interest but a closely related compound, while the external standard is the analyte itself. The internal analyte should be carefully chosen as it should mimic any changes that happen to the sample (sample loss, incomplete extraction, error in injection volume, etc.). If the choice of external versus internal standard is not clear for the analyst, an empirical study comparing results for known samples using the two models is a good way to approach the selection. When a blank sample is not available (i.e. analyte-free matrix) and the matrix had a big impact on the analyte signal, the method of standard addition is used. In this method, a series of calibration standards are prepared at several concentrations and spiked into the sample. The concentration of the analyte in the matrix is determined from the x intercept [69].

Curve Weighing [70]

Curve weighing is the process of weighing the data inversely with concentration. This is done to prevent the data at the higher concentration from dominating the linear regression calculation, which results in large errors at the

low concentration. Weighed the calibration curves should be considered if the %RSD is fairly constant throughout the calibration range. Curve weighing decreases the total error and improves the accuracy of the LC method.

Integration Problem [71]

The types of integration discussed here are the valley-to-valley, perpendicular line drop, tangent skim, and manual integration. It is suitable to use the valley-to-valley technique if a known baseline disturbance is present under a set of eluted peaks. However, this is seldom the case, and more often, this technique misses some peaks, especially the ones that look like a shoulder, and greatly underestimate peak areas. Dropping a perpendicular at the valley between two overlapping peaks along with proper integration of the baseline is a better approach. The error in peak area is minimum when the two peaks are of equal height. As a larger peak tends to dominate over a smaller one, the integrated area of the larger peaks is more accurate than the smaller one. Manual integration is appropriate when doing trace analysis where the peaks are small and the *S/N* is low, even though this is not preferred by analysts in quality control and can be subjective [71, 72]. In such cases, it may be difficult to set auto-integration parameters that work for all injections in a sequence. When a small peak (i.e., <10% of the larger peak) is on the shoulder of another one, a linear or curved skim is a suitable integration method. If the peak on the shoulder is >10% of the main peak, a perpendicular drop is the better option. After finalizing the integration method, it is recommended to check every chromatogram in the sequence to make sure that the integration is set properly [71].

Compared to peak height, peak area is a better option for quantitation as it is less affected by peak shape distortion. Nonetheless, peak height is a better option to quantitate overlapping peaks. In such a case, there is a little overlap of the peaks at the apexes. The best way to determine the best integration method is to confirm by running a set of known samples [71, 73].

Peak Area Variability

Variability in the peak area may occur when one of the active interaction sites on the stationary phase has a slower equilibrium with the analyte than other sites. This problem can be solved by either a large-mass injection to saturate the active sites or by several small injections in series without running the entire method, both prior to actual analysis sequence [73, 74].

A source of variability in the peak area (or height) from on LC system to another can be the accuracy of the autoinjectors. Autoinjectors have very good precision, but the accuracy is not as great as the precision. The variability in accuracy is worst when injecting the full volume of a fixed loop due to the laminar flow profile of the sample in the loop. In such a case, the loop should be filled up three times its volume to make sure that it is fully flushed. It is best to have a robustness check for the method during development to make sure that it is can be transferred with minimal problems [75]. Variability at low injection volumes can also be at the precision of the injector, as shown in Fig. 3. In this plot, the relative standard deviation of the area of six caffeine injections is shown for five injection volumes ranging between 0.1 and 20 µL. Therefore, it is advised to inject at least 0.5 µL to keep the area precision under 1% in this case.

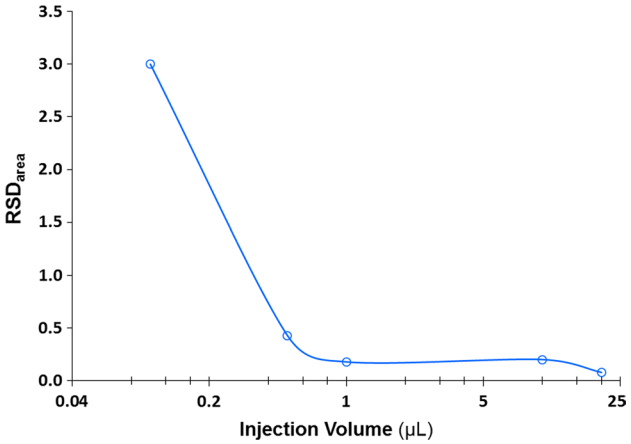

Fig. 3 Area RSD as a function of injection volume. Agilent 1290 I, flow through needle design, loop volume 25 µL. *X axis* is plotted on a logarithmic scale with base 5

A partially blocked needle (for example, part of a vial septum is cut and drawn into the needle) may result in an injection volume lower than expected [76, 77]. Another cause for lower injection volume is due to needle cavitation when sampling from a viscous sample. The syringe speed should be adjusted to accommodate the sample viscosity [76]. In such cases, checking the autosampler performance is a useful test. Other sources area variability may be due to a loose metering drive or a leak in the injector rotor seal [77]. Leaks can usually be fixed by tightening a fitting or a component. Finger tightening should be enough for low pressure fittings [78].

Artifact Peaks

Artifact peaks can be due to LC system mechanical problems or contamination from glassware, filtration of reagents, chromatography column, sample, mobile phase(s), etc. To check whether the source of the artifact peak is the LC system, it is recommended to (1) clean the LC system

thoroughly with a mixture of water, isopropanol, methanol, and acetonitrile, (2) install a new in-line filter or guard column, (3) clean and purge the injector, (4) clean and wash the UV detector cell, and (5) confirm that the UV lamp is not aging. It is advisable to run blanks using different mobile phase bottles and to check whether the artifact peak grows after the mobile phase is sonicated (due to more extraction of contaminant from the bottle). Artifact peaks can originate from impurities in mobile phase solvent or additives. The easiest way to isolate whether the source of the artifact peak is from mobile phase is to use different batches of solvents and/or additives used while tracking the changes in the area of artifact peak [79]. If the source of contamination is the presence of trace organic contaminant in the aqueous mobile phase component, a cleanup C18 column can be installed between the pump and the injector to remove the organic contaminant. When the column is saturated, it can be cleaned with strong organic solvent and reused again [80].

A clue to the source of the extra peak can sometimes be obtained from the chromatogram. For example, a broad peak among narrow neighboring peaks indicates that it is a carryover late eluting peak from the prior injection, while a peak appearing in a blank injection at the same retention time as the peak(s) of interest is indicative of injection carryover (e.g., autosampler needle was not washed thoroughly) [81–84].

In gradient elution chromatography, the peaks have approximately similar width, while in isocratic separation, the peak width increases as the retention time increases. An unexpected peak in the middle of a chromatogram having a width that does not align with the pre-mentioned peak–width rule is a late eluting peak carried from a previous injection. To fix this problem, the run time should be extended and flushing with a strong solvent is recommended [85, 86]. In addition to being aberrant peaks, late eluting peaks can also manifest as irregular baseline and as co-eluting peaks with peaks of interest [87].

A reduction in the area of the peak seen in subsequent blank injection is a confirmation that injector carryover is the cause of the artifact peak. The percentage drop in the area of the peak from the initial injection to the second blank should be similar to the drop from the second blank to the third blank. If the peak area does not change from the first injection to the second injection, then it is probably a contamination problem rather than a carryover problem [88, 89]. To confirm that, it is a contamination problem, the blank should be replaced with a freshly prepared one. If replacing the blank does not solve the problem, the injection volume should be changed (doubled or halved) while tracking the change in the carryover. If the carryover change matches the change in the injection volume, then it is a contaminated blank. Other steps to solve the carryover problem include: (1) tightening fitting at injection valve and downstream to eliminate any extra void volume, (2) replacing wash solvent, (3) increasing wash volume and/or wash cycles, (4) increasing wash solvent strength, acidifying, or basifying, if needed, with volatile buffers only, (5) adding some organic solvent to the sample, in case the sample solvent is pure water to minimize adsorption, (6) looking for a chemical solution if carryover is specific to one analyte only, and (7) changing hardware, such as needle seat, injection loop, or valve [89]. In summary, cleanliness is the key to overcoming carryover problems. Most carryover problems can be eliminated by running extra wash cycles and suitable rinse solution containing high percentage of organic solvent and some acid to solubilize the sample [88].

Some sources of artifact peaks are easy to find, such as preparing fresh mobile phases, replacing the column, adding thorough autosampler needle wash cycle, and running the sample on another instrument. When the source of contamination is the instrument, a harsh acid cleaning may be needed, especially when algae has grown. The acid wash consists of cleaning the system inlet lines and parts by successive rinsing of water, 30% phosphoric acid, and then water [81].

Artifact peaks can be due to on-column concentration of contamination from the aqueous mobile phase (usually termed as ghost peaks). To confirm this, longer re-equilibration times can be used to check whether the signal of the artifact peak increases or not. Guard columns installed before the mixer on high pressure mixing pump can solve this problem [90]. Sources of ghost peaks can be any lab equipment or material that contacts the prepared samples or solutions, such as pH probe or solid-phase cartridge [90, 91]. Artifact peaks from sample degradation can be due to heat, light, or on-column degradation [92]. System peaks due to disturbance in refractive index originating from a change in pressure, while sample is being injected can be mistakenly categorized as artifact peaks. Such peaks are easy to identify as they show up at the start of the run and their UV spectra look like a solvent spectra.

Peak Distortion

In the case of a distorted peak, a first check is to confirm whether the analyst is using the correct column, vial position, temperature, mobile phase, etc. [93]. To further check if a problem is in the LC system or in the hardware, a new column is installed and a well-retained sample ($k \geq 2$) is injected while comparing the tailing factor to the manufacturer specifications [94]. The same technique can be used to check for retention time reproducibility [93]. If the LC system is not the source of tailing, then other parameters should be tested as detailed below [94].

Column Dimensions

As a general rule, the larger a column is, the less it is prone to problems. With columns of small volume, extra-column effects should not be ignored as they are a major source of tailing peaks. For column with smaller particle diameters (such as sub 2 μm), the frits can easily clog, leading to tailing or split peaks [95, 96]. Column aging can be another source of tailing [94].

Column Type

Even though they are unlikely to be used in new methods, type A silica columns contain some metal impurities which can result in distorted peak shape. The advancement in making type B silica is impressive. Nowadays, the new type B silica is purer than the old type B silica. Another check is to make sure that the pH of the separation is within the tolerance window of the column. For most silica phases, the silica hydrolyzes at pH <2 and dissolves at pH >8. Both of these conditions will lead to peak distortion [94].

Mobile Phase

Most common at high pH, some silanol groups may deprotonate and become ionized, generating ion exchange sites on the surface of silica. This secondary ion exchange interaction results in extra tailing. The buffer prepared should be within 1 pH unit of the pK_a. For volatile buffers, it is recommended to prepare them freshly and not to use them for a long time to minimize evaporation that may lead to tailing due to pH shift [94, 97].

Column void, blocked frit, and other Sources

Collapse of the column bed mainly leads to peak fronting of all peaks in a chromatogram [39, 95, 98, 99]. Fronting can also be due to use of aggressive temperatures or mobile phases for new columns or due to poor packing techniques for old columns [39, 98]. Other sources of fronting include pH mismatch between the sample solvent and the mobile phase [98, 100], or running the column at a high pH that dissolves the silica and creates a void [95]. On the other hand, peak tailing can be due to undesired interactions with silanol groups. This usually occurs between the basic, positively charged molecules, and the acidic silanol groups [98].

When all the peaks tail or split, then the sample is not introduced into the column in a homogenous manner. A partially blocked frit due to particulate matter from the sample or from a worn seal can cause such a problem. The blocked frit distorts the sample stream arriving at the head of the column. An easy way to check is to replace the column and check whether the problem is solved [39, 101, 102]. If all the peaks are broadened, this may be due to a tubing end not firmly seated onto the column nut [103]. Such loose connections, especially at the end of a chromatography column, result in broad peaks due a small void volume created. This problem is usually observed with polyether ether ketone (PEEK) fittings. To fix this, it is recommended to shut the flow off, push the tubing tightly against the column, and then tighten the fitting [104].

Sometimes, the source of tailing can be difficult to identify. In the next example, column load is shown to adversely affect tailing for one molecule and favorably for the other. The effect of injection volume and interaction with stationary phase on the peak shape is shown for amitriptyline and mefenamic acid. At pH 2.8, the mefenamic acid is nearly fully protonated, because the pH of the mobile phase is well below the p*K*a of the compound. At low injection volume, the peaks tail due to different retention processes occurring simultaneously. There are two types of column interaction sites: one type equilibrated slowly, while the other equilibrated quickly. At higher injection volume, the retention mechanism is driven by the sites that equilibrate quickly resulting in a better peak shape. For amitriptyline, the peak tailing increases as the injection volume increases and the retention time starts shifting earlier. The cause of the tailing in this case is due to ion exclusion when amitriptyline molecules, which are positively charged at pH 2.8, reside in the pores, blocking active sites, and repelling other amitriptyline molecules to the next available column site to interact with [39].

Retention Time Variability [75]

It is hard to track differences in instruments between different laboratories, and it is not unusual to see differences when transferring methods between instruments. Temperature may be the source of deviation in retention time from one system to another. To test this, the first step is to premix the mobile phase and to compare the retention time using the same column, mobile phase, sample, etc. Observed deviation can be due to temperature fluctuations. As a general guidance, a 2% deviation in retention is observed when there is a deviation in temperature by 1 °C [75]. The second step is to compare the hand mixed to the online mixed mobile phase to determine whether the pump is accurately mixing the correct proportions of the mobile phases. Online mixing can be complicated by not accounting for the compressibility of the solvents. Dwell volume differences may be another source of retention time deviation between instruments. Retention time may increase slowly due to a leak from the injection valve resulting from a scratch in the valve rotor. Such a scratch may be due to debris from tubing or sample [104].

When methods are transferred between instruments of different dwell volumes, there are some considerations to remember: (1) the gradient reaches the column at different

times, (2) the retention time difference between the two systems will approximately equal the difference in dwell volume, (3) early and late eluting peaks are affected in a different way, (4) differences in resolution or selectivity may be observed, especially for the first eluting peaks, (5) a shift in retention may result in peak misidentification as peaks in some methods are expected to elute in a certain retention time window [12, 73, 105] or may result in shifting the ion suppression region [106].

Approaches to minimize problems when transferring methods between instruments of different dwell volumes include: (1) having enough resolution that can tolerate differences in dwell volume (i.e., without adjusting the method); (2) building the method based on the largest dwell volume available, then an isocratic step is added to the beginning of the gradient for systems with a smaller dwell volume. The length of the isocratic step is determined by dividing the difference in dwell volume by the flow rate ($\Delta t_D/F$), (3) having a zero dwell volume by programing the autosampler to inject the sample when the gradient reaches the head of the column [105].

Baseline Noise and Drift Problems

Sources of Noise [107]

Baseline noise can originate from mobile phase mixing, insufficient degassing, lack of system maintenance and tune-up, detector lamp aging, uncleanliness of any system part or reagent, and electronic filtration. Certain steps can be taken to decrease noise as follows: (1) hand-mixing the mobile phase in case of isocratic separations, (2) Partial pre-mixing of mobile phases (i.e., small percentage of organic solvent in the aqueous mobile phase and vice-versa) as such a process provides better overall mixing, (3) regularly check and replacement of pump seals and check valves, (4) additional degassing, (5) using higher purity reagent and solvent, (6) cleaning the column on regular basis and retire column early, (7) using good laboratory practices to maintain the lab equipment clean, (8) pretreatment of sample to minimize contamination, and (9) optimizing the detector constant and data collection rate [107].

Baseline Drift

Baseline drift under gradient conditions is usually due to the different extinction coefficients of the two mobile phases at the chosen wavelength of analysis. Therefore, the baseline will drift as the *%A*/*%B* changes during the gradient. For example, a rise of 1 absorbance unit is expected when running a water–methanol gradient 100% water–100% methanol while monitoring the baseline at 215 nm. That is why acetonitrile is widely used when detection is needed at a low wavelength (<220 nm) [108]. To minimize the baseline drift problem, the absorbance of water is adjusted to match that of methanol by adding buffer or a UV absorbing agent to water. For example, adding 10 mM of potassium phosphate decreases the drift in base line by tenfold. Replacing water with buffer (such as 25 mM ammonium acetate) can result in a negative baseline that can be problematic. In such a case, adding the buffer to both mobile phase A and B may minimize the negative baseline issue [108].

Baseline Goes Through a Minimum or Maximum

An interesting example of baseline drift is seen with gradient of *A*: 0.1% trifluoroacetic acid in water and *B*: 0.1% trifluoroacetic acid in acetonitrile. The curvature of the baseline depends on the detection wavelength. At 200 nm, the absorbance goes through a maximum at 50%*B*. At higher wavelengths, the absorbance as a function of *%B* starts flattening out until reaching $\lambda \sim 215$ nm. At 220 nm, the baseline has a positive slope. An approach to minimize the baseline drift is to detect at a higher wavelength, due to the lower absorbance of organic solvents. This approach is also successful with the negative and positive baseline drifts [108].

Noise that appears in the form of a sine wave function can be due to electrical noise or due to pump malfunctioning. The frequency of the cycles helps determine whether it is electronic noise or pump related problem. If the frequency is, for example, 70 cycles/min, then it cannot be pump related as the cycle is faster than the piston cycle. Noise cycle frequency related to pump problems changes with flow rate. Electronic noise can be due to malfunctioning resistance–capacitance filter, from fluorescent light instruments, or nearby equipment [109, 110].

Electronic noise can be due to external electronic equipment drawing large current. Relocating the instrument to another electrical circuit or shutting down the nearby instruments can confirm whether the noise is electrical. Such an investigation may take a lot of time and effort, as in the case study in this Ref. [111]. Baseline noise related to lamp failure shows up in the form of large pseudo-peaks that can be mistaken as a bubble in the system. To confirm that it is a lamp failure, the large pseudo-peaks should be observed even when setting the flow rate to zero [109].

Enhancing Signal to Noise [112, 113]

The signal-to-noise ratio (*S*/*N*) can be improved using several approaches. It may be as simple as using a more concentrated sample, injecting larger sample volume, giving the detector longer time to warm up, or optimizing the

detector time constant and data collection rate. A value larger than the optimum one for the detector time constant will decrease baseline noise, but also decrease the peak height, and vice versa. A value larger than the optimum one for collection rate will increase baseline noise and peak height, and vice versa. It is possible to increase the *S*/*N* by changing the wavelength of detection, because the optimum wavelength for detector response may be different that the one optimum for wavelength selectivity (i.e., where more species absorbs preferentially over the other). Compound derivatization, such as attaching a fluorophore to the compound, and using a fluorescence detector may enhance *S*/*N*. However, this is time consuming and inconvenient, and may introduce new sources of error.

A lower k' value will result in a sharper peak because an analyte will spend less time in the column. A smaller column will result in better *S*/*N* ratio, while keeping the same linear velocity when adjusting column diameter. Using a column with smaller particle size will also increase column plate count and increase *S*/*N* ratio.

Further wavelength is from the mobile phase UV cutoff, the lower is the noise. Signal-to-noise can sometimes be enhanced by trying a new UV detector. As a lamp ages, the intensity test starts to fail at lower wavelengths before failing at higher wavelength [77]. One may also try a different type of detector: fluorescence, CAD, evaporative light scattering detector, etc. [96, 112].

Other ways of improving *S*/*N* are to better control the temperature of the column by making sure that the column compartment is functioning properly and that the column is not operating at ambient temperature. Fluctuations in temperature result in fluctuations in refractive index and thus detector noise. Pressure fluctuations due to worn part in the pump (such as check valve) may also result in increased noise. Another way to increase *S*/*N* is to improve mixing using higher purity reagents, premixing the mobile phases (for isocratic only), or using larger mixer volume [112, 113].

HPLC Tips and Tests

Stainless Steel Surfaces Corrosion [114, 115]

Stainless steel is used in some parts of HPLC due to properties, such as hardness, machinability, strength, low cost, etc. Stainless steel can corrode in LC systems due to: (1) low pH from corrosive acid, (2) presence of reducing reagents, chelating agents, or strong ligands, and (3) salts, such as chloride or lithium. Corrosion may be continuously taking place under the protective oxide passivation layer. Materials that are individually inert to stainless steel can be corrosive. For example, acids are inactive in aqueous solutions but corrosive in acetonitrile-aqueous solutions. Stainless steel is vulnerable to nitric acid. Therefore, LC systems with nitric acid should be the last resort. If cleaning with nitric acid is needed, it is recommended to clean with concentrated nitric acid (50–100%) rather than more dilute concentration (10–30%) as the latter is more corrosive.

Leading Bubble Technique

Leading bubble technique is an injection method in which the autosampler will draw an air gap into the tip of the needle after drawing the sample and before leaving the vial. This technique minimizes band broadening and dispersion of the sample. However, this technique results in injecting higher volumes if extra layer of liquid adheres to the neck of the vial, a case that may happen when working aqueous buffers with high surface tension. To overcome this problem, the organic strength of the sample solvent should be increased to break the surface tension and a higher injection volume is recommended minimize the error [116].

UHPLC Tips [117]

Ultrahigh high-performance liquid chromatography systems were designed to take advantage of the sub 2 μm particles, which generate high-efficiency separations in shorter times, compared to HPLC. To be able to take full benefit of the UHPLC capabilities when using sub 2 μm particles packed in small columns, dispersion should be minimized. Other precautions include: (1) filtering the samples with 0.2 μm filters to prevent clogging the 0.2 μm frits on sub 2 μm columns, (2) using freshly prepared mobile phases in clean bottles rather than replenishing the old bottles to minimize the chances of bacteria growth which may clog the column, and (3) increasing the sampling frequency and decreasing the response time to collect enough data points to prevent band broadening. Improper selection of detector response time may result in tailed or distorted peaks [118]. Besides generating band broadening, low data collection may be also related to hidden noise filtering algorithms that software run simultaneously with data acquisition [119].

Column Dead Time as Diagnostic Tool [120]

The column dead volume is the volume of the mobile phase inside the column. The dead time (t_0) can be estimated according to Eq. (20) and used as a diagnostic tool for potential LC problems. It is advisable to compare the experimental and theoretical dead time values and investigate whenever the difference between the two is larger than 20%. If the experimental t_0 is larger than theoretical, this may be indicative of a leak in the system. Conversely, an

issue in the flow rate or exclusion from the pores due to size or chemical repulsion between the stationary phase and analyte results in experimental t_0 values lower than estimated ones.

Testing the Accuracy of Mixing

A gradient step test is run to check the mixing accuracy. The column is replaced with a capillary tubing to generate enough back pressure. Line *A* is placed in HPLC grade water and line *B* is placed in 0.1% acetone in water. A gradient of 100%*A* to 100%*B* is ran in form of 5 min multisteps, where at each step, the %*B* is increased by 10%, with more points near 50%*B*. This test is useful for checking the mixing accuracy and gradient linearity. In a quaternary pump, the test is also done for lines *B* and *D*. The flow rate is set at 1 mL/min, wavelength at 265 nm, and total gradient run is 15 min. The detector signal at each individual step is plotted against %*B* to check the linearity of the gradient, and thus accuracy of mixing [121–123].

If a pump does not deliver the correct composition, a shift in retention time of the analyte is observed. After estimating the *S* factor of the analyte according to Eq. (14), the shift in retention can be estimated from the linear solvent-strength relationship shown in "Solvent-strength selectivity", which can be rearranged as follows [123]:

$$S = 0.25\sqrt{\mathrm{Mw}} \tag{14}$$

$$\Delta \log k = -S\Delta\phi, \tag{15}$$

Where Mw is the molecular weight of the analyte in Dalton.

Issues with LC Pumps

The original reciprocating single piston design gave a lot of pressure ripple that resulted in noisy baseline and short column lifetimes. For this design, pulse dampeners of large volumes were needed to reduce pressure pulses. The dual-piston design was a big improvement as it minimized pressure ripple due to the continuous flow of solvent through the column. This is achieved by having two pistons working in reciprocating fashion, i.e., one piston will be filling, while the other is infusing, and vice versa. A further improvement in the design was the accumulator-piston design, or the tandem-piston design, where two pistons of different stroke volumes are connected in series. The piston with the higher stroke volume delivers double the targeted flow rate and infuses half the flow to fill the lower stroke volume piston. Next, the low volume piston will infuse and high stroke volume piston will fill up. The accumulator-piston design has only three (can be minimized to two) check valves compared to four check valves on the dual-piston pump. Both designs are capable of generating highly precise and accurate flow rates and organic/aqueous ratios [124, 125].

The weak points in the pump design are the seal and the check valves. The seals wears out with time, especially when using buffer due to the formation of abrasive layer of buffer crystals behind the seals [124, 126]. The inlet check valves can become sticky, especially ball type ones to the sapphire seat. This is commonly observed when using acetonitrile–water mobile phases and can sometimes be solved by sonicating the check valve in alcohol. The stickiness of the check valve is due to the strong surface tension of the thin layer between the ball and the seat. The problem can be solved by either breaking the surface tension between the ball and the seat or by allowing the check valve to dry out. The change in the surface of the sapphire check-valve seat is due to a polymer buildup when using acetonitrile. This buildup results in smooth surface that enhances the stickiness of the ball to the seat due to surface tension [127, 128].

Gradient Tests

Gradient Proportioning Valve (GPV) Test [129]

This test is done only for the low pressure mixing systems as high pressure mixing systems do not have a GPV [129, 130]. This test checks whether accurate delivery is obtained using all the combinations of the proportioning valves. Lines *A* and *B* are placed in water, while lines *C* and *D* are placed in 0.1% acetone in water mixture. A series of 2-min steps of all the possible combinations of water and acetone in water mixture is run as follows: 50%*A*:50%*B*, 90%*A*:10%*C*, 50%*A*:50%*B*, 90%*A*:10%*D*, 50%*A*:50%*B*, 90%*B*:10%*C*, 50%*A*:50%*B*, 90%*B*:10%*D*, and 50%*A*:50%*B*. The 50%*A*:50%*B* functions as the baseline, while the other combinations serve as plateaus. A deviation between the maximum and minimum plateau should not exceed 5% (preferably 1%) of the average. Three sources may contribute to the failure of the GPV test: (1) poor pump performance due to improper mobile phase degassing, check-valve issues, or pump seals issues; (2) blockage of inlet line frits which can be tested by disconnecting the lines from the proportioning valve and verify that the flow rate is approximately 10 mL/min should flow through the line; (3) proportioning-valve failure due to either mechanical malfunction or improper control of the valve cycles [129].

Dwell Volume Measurement

Dwell volume (V_D) is the volume of liquid contained between where the solvents are mixed and the head of the column [131]. In high pressure mixing LC systems, this mainly includes the mixer, the tubing, and the autosample

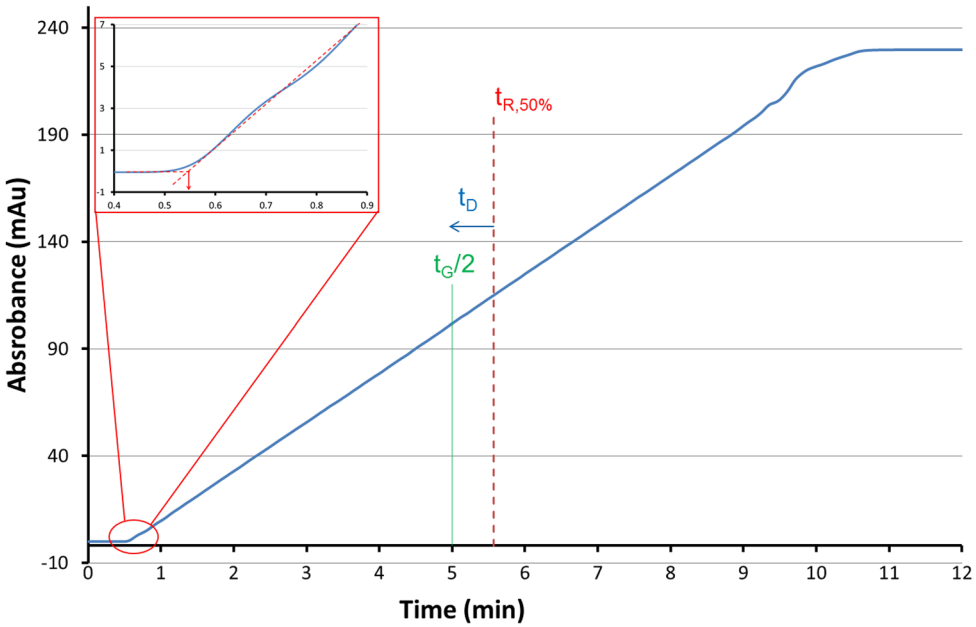

Fig. 4 Plot showing the two methods to determine dwell time for an Agilent 1260 pump. $F = 1$ mL/min

loop. Due to the mixing of mobile phase before the pump, additional components increase the dwell volume, such as the volume of the pump head and the tubing from the mixer to the pump head [105]. The traditional way of measuring the dwell volume is by running a one-step gradient 0–100%B where the weak solvent is water and the strong solvent is 0.1% acetone in water and the detector is set at 265 nm. A long and narrow tubing is put in place of the column to generate enough back pressure for the system to operate [105, 131]. For easy calculation of V_D, the flow rate and gradient time are chosen, so that the gradient volume is ten times larger than the dwell volume. For example, for the conventional HPLC (where the expected V_D is 1–2 mL), $F = 2$ mL and $t_G = 20$ min, while for ultrahigh pressure liquid chromatography (where dwell volume is ~200 µL), $F = 0.5$ mL/min and $t_G = 5$ min. As shown in Fig. 4, the dwell volume is determined by (1) extending the baseline to meet the best fit line through the gradient, or (2) finding the retention time that corresponds to 50%B ($t_{R,50\%B}$), and subtracting $t_G/2$ from $t_{R,50\%B}$ to give the dwell time which can be converted to V_D using the flow rate. In this figure, the dwell time determined based on the first method results in V_D value of 1.1 mL, while the value obtained based on the second method is 1.15 mL. It was easier to determine the value from the first method as it was difficult to draw the tangent for the first method due to baseline fluctuations, as shown in the inset in Fig. 4

$$t_D = t_{50\%} - (t_G/2), \tag{16}$$

where t_D is the dwell time, $t_{50\%}$ is the time corresponding to half maximum signal, and t_G is the gradient time.

Gradient Linearity and Accuracy Tests

The gradient linearity test is a single-step gradient, the same as the one used for determining dwell volume. Deviation of the ramp from linearity is indicative of pump mixing accuracy problems. The accuracy test is a step test that consists of series of 4 min steps in 10% increments with more points at 50%B where pump usually fails. The acceptance criterion for the step test is 1% deviation [129].

Extra-Column Volume, When Can be Neglected

Using short columns to improve the separation can give worse results due to the contribution of extra-column broadening. The effect of extra-column volume gets amplified as the column volume decreases (i.e., length, diameter, or both) or particle size decreases, or both [28]. Extra-column volume can be determined by plotting σ^2 as a function of $(t_R)^2$ for a non-ionic compound (such as nitroalkane) at different mobile phase strengths. As shown in Eq. (17), the y intercept is $\sigma^2_{extracolumn}$. The early eluting peaks are more affected by extra-column broadening than late eluting peaks and larger columns are less affected than small columns. For example, the effect of extra-column volume effect on two analytes ($k' = 1$ and $k' = 5$) was assessed for three columns (4.6 × 150 mm, 2.1 × 150 mm, and 1.0 × 150 mm) under the same linear velocity. The addition of a 15-µL extra-volume results in loss of resolution for peak of $K' = 1$ by 1% on 4.6 × 150 mm, 19% on 2.1 × 150 mm, and 71% on 1.0 × 150 mm, while a peak of $k' = 5$ results is a loss in

resolution by 0% on 4.6 × 150 mm, 3% on 2.1 × 150 mm, and 32% on 1.0 × 150 mm [132–134]

$$\sigma^2_{\text{obs}} = \frac{t^2_{\text{R}}}{N} + \sigma^2_{\text{extracolumn}}. \quad (17)$$

A common practical reason for increase in extra-column volume is having a gap at the inlet or outlet of the column. This happens when the tube is preassembled with a shallow setback from the ferrule and used on an instrument with insufficient setback. Finger-tight fitting made of polyetheretherketone (PEEK) has solved this problem as the ferrule recess is designed to grip on the tube while tightening to prevent leaking, while when loosened, the ferrule can be readjusted if needed. Extra-column volume can originate from column, detector cell, injector, tubing, or fitting. It is recommended to use the shortest narrow tubing possible, while keeping in mind that pressure increases and probability of clogging increases as the diameter of the tubing decreases. PEEK tubings are very convenient to use as they can be cut easily and they come in different internal diameters with color codes to prevent confusion. However, they are not compatible with solvents, such as tetrahydrofuran or chlorinated solvents as they may break or leach some chemicals [135].

Solvent Consumption [136]

Solvent consumption, for environmental and economic concerns, can be reduced several ways. Recycling the mobile phase works for isocratic separations but comes with the disadvantage of: (1) using a contaminated mobile phase with a minor amount of analyte (especially as it is more often used), (2) bacterial growth in some mobile phases, and (3) selective evaporation of the more volatile component of the mobile phase. Automated recycling decreases the buildup of contaminants in the mobile phase by selective recycling of the waste using a diverter valve. Distillation is another method of recovering the organic component of your mobile phase.

Changing the column diameter is a simple way to reduce the consumption of mobile phase. Dropping the column diameter from 4.6 to 2.1 decreases the mobile phase consumption fivefolds approximately $(4.6/2.1)^2$. Another way to save mobile phase is to transfer methods to use shorter columns packed with smaller particles. Decreasing the particle size is accompanied with an increase in efficiency. Therefore, shorter columns can be used with smaller particles while maintaining the same efficiency [136, 137].

HPLC Troubleshooting

Troubleshooting Basics

Where to Start [138]

The major categories of LC problems are pressure problems, leaks, peak shape distortion, data processing problems, and retention time shift. The basic rule when troubleshooting the problems is to follow the rule of one, the rule of two, the divide and conquer rule, the module substitution rule, put-it-back rule, and the documentation rule. The rule of one recommends to change one thing at a time while observing the result. If the change does not solve the problem, then undo the change. The rule of two is to make sure the problem is repeatable (i.e., occurs at least two times). The divide and conquer rule means finding and running the experiment that eliminates a large number of possible root causes. The module substitution rule involves changing the suspect part with a new one. The put-it-back rule indicates that if replacing/removing a part did not fix the problem, then it should be put back. The documentation rule helps in avoiding future problems.

Pressure Problems [9, 10, 139]

The pressure generated on an HPLC system is primarily dependent on the length, diameter, and particle size of the column as well as extra-column tubing (especially for narrow bore tubing). A record of normal system pressure is vital to evaluate the source of any pressure issues.

Two types of reference measurement are suggested to investigate the source of the pressure problem. The first reference is the system reference pressure, a method-independent value which acts as a record of the normal system pressure. In this test, the system pressure is recorded under specific conditions (F, T, and mobile phase) using a new column and used as a reference point. The second reference measurement is the method reference pressure, where the pressure is recorded using the normal method settings. For a comprehensive evaluation of system pressure for both of the two references, the contribution of each part of the HPLC system is recorded by working progressively backwards from the detector to the injector, disconnecting each connection fitting and measuring the pressure difference [9].

An increase in pressure is due to obstruction in the flow path. This can be due to an obstruction in a tube, frit (in-line or column frit) blockage due to the accumulation of debris, or column aging. The latter is usually observed in the form of gradual increases in pressure. Progressive

loosening of fitting is the correct way to investigate the source of the pressure rise [9, 93, 140].

A low pressure is an indication of air in the system, faulty check valve, or a leak. Purging the system is a quick way to remove bubble(s) from the system and to wet dry lines. A flow rate check can be done to confirm the pump is delivering the correct flow rate. Fittings can slip if not properly tightened or due to high pressure. Another possible pump problem is a leaky pump seal [9, 140]. Leaks usually originate from three sources: (1) wear of system parts because of moving components, such as injection valve in the autosampler or pump seals due to piston rubbing against the seal, (2) loose components, or (3) excessive pressure [141].

Pressure ripple are usually due to a defective check valve, air in the system, or piston stroke volume. This is usually fixed by changing check valve and/or pump seals and purging the system. If the problem persists, then it may be due to a faulty degasser [9, 140].

Retention Problems [142–144]

Retention time shift may originate from mobile phase, stationary phase, or system hardware problem. Improper preparation of mobile phase (such as volumetric measurement or pH adjustment), instrument errors in settings or performance (such as inaccurate in-line mixing or improper degassing), or microbial growth in aqueous mobile phases can lead to retention time shifts. Degraded columns usually show distorted peak shapes. Retention time shift can be related due to chemical changes to the stationary phase. System hardware problems can be due to pump malfunctions or leaks. The pressure signal is the best tool to track the performance of the pump [142]. A retention time that shifts to a higher value than expected may be due to a leak, presence of an air bubble in the system (usually accompanied by a drop in the flow rate), leaky check valve, or worn out pump seals resulting in a leak. Retention time drift can be due to a change in flow rate, temperature, column aging, and mobile phase composition, such as selective evaporation of a component in the mobile phase.

Two important measurements that are used to determine the source of the retention time shift include the retention factor (k') and relative retention (selectivity, α). If k' is constant, while the retention time increases, this indicates the presence of a leak in the system or a drop in the temperature of the column compartment. On the other hand, if k' remains constant and the retention time decreases, this may be due to an increase in the temperature of the column compartment. Retention drift that occurs over hundreds of injections is due to normal column aging. When a change in the value of k' is observed, it indicates a chemical change which can be confirmed by calculating α. A change in the value of α chemical change influencing the interactions between the stationary phase, mobile phase, and analytes [142].

In the case of retention time variability, a good test is to check whether pump accuracy is the source of the variability. This is done by premixing the mobile phases in the case of isocratic separation. In the case of a gradient run, some organic mobile phase is added to the aqueous mobile phase, and vice versa. The gradient program should be adjusted to deliver the same gradient. If the retention time variability decreases, then the source of the variability is the mixing accuracy. This premixing check is advantageous as it also decreases the pressure ripple [145]. The initial drift in retention time for the first few injections is due to the equilibration of the silica surface, which can be a slow process [145, 146]. Retention time variability due to deviation in mobile phase percentage composition from one injection to another or from one system to another depends on the S value of the analyte. The drift in retention time as a function of $\%B$ variability increases as the S value of the molecule increases [147].

To avoid retention time shift problems, here is a list of prevention steps: (1) maintenance of the instrument at least annually, (2) use column oven and operate at a temperature well above ambient conditions, (3) use an in-line filter to prevent particulate matter from blocking your column frit, (4) change your buffer once a week and your organic mobile phase at least once a month, (5) do not top off the mobile phases, and always use new bottles, (6) wash the column with high organic solvent at the end of your sequence. When a buffer was last used in a column, a mixture of water and organic is recommended before using strong organic solvent, (7) dedicate a column for each method [142], (8) never flush 100% aqueous mobile phase to the column as most RPLC columns will collapse when subjected to such an environment, (9) after being stored in strong organic solvent, recondition the column with the initial mobile phase for 10–20 column volumes [72, 142, 148]. (10) avoid using type A silica and weak buffer concentration (such as 0.01 mM) [149].

Column Problems

To avoid problems, (1) dedicate columns to specific methods, (2) use online filters, (3) flush columns regularly, (4) use guard columns, (5) minimize garbage by filtering the sample and/or centrifuging to remove particles, and (6) use high purity HPLC solvents [150, 151]. A buildup of particulate matter on the inlet frit or having a void at the head of the column can result in excessively tailing, split, or doubled peaks [95, 151–153]. Back-flushing the column with a strong solvent is an effective way to unclog the frit.

When doing so, the detector should be bypassed [95, 101]. Another source of retention shift and tailing, which is hard to distinguish from the previous sources, is a permanent change of the stationary phase in the column [151].

How to Take Care of the Chromatography Column?

There are few steps to follow to extend the lifetime of a chromatography column. These recommendations include: (1) keeping the sample clean by centrifuging or filtrating the sample to prevent delivering any particulate matter to the column, (2) operating the column within recommended conditions (i.e., pH and *T* range), (3) adding a guard column (this, however, may introduce disadvantages, such as peak distortion due to loose packing, loss of analytical column efficiency and resolution, and the addition of extra-column volume) [72], (4) using an in-line filter and solvent reservoir filter, (5) cleaning the column regularly with special cleaning reagents, (6) selecting solvents that are compatible with the column, (7) choosing high purity mobile phase, (8) dedicating columns to specific methods, and (9) retiring column early [99, 153–158].

Maintain the LC System [159]

The three most effective steps in preventive maintenance of LC systems are: (1) degassing the mobile phase, (2) filtering the mobile phase and sample, and (3) flushing the system. Even though the degassers built into modern systems are usually sufficient, additional steps taken for degassing the mobile phase to prevent introduction of air bubbles into system which can cause problems, such as system shutdown due to a drop in pressure, spikes in the chromatogram, or a quench in the florescence detector signal [75]. Degassing can be done by helium sparging, ultra-sonication, vacuum degassing, or refluxing [33, 160]. Having particulate matter in the system may result in increased systems back pressure or distorted peaks due to clogged online filter or the column frit. Sources of particulate matter can be mobile phase, sample, or wear from internal parts, such as valves, rotors, seals, etc. Therefore, it is recommended to filter the mobile phase especially when the solvent is not HPLC grade and when using non-volatile buffers. Filtering the sample is another way to minimize introducing particulate matter to the system. However, this is not always an option as it is expensive and requires additional steps in validation. Finally, to minimize wear of internal parts, annual or semiannual maintenance is recommended. The third preventive maintenance key is to keep the system clean by: (1) flushing the system with neat solvents, especially after running non-volatile buffer, (2) replacing the mobile phases on a regular basis rather than topping off the bottles, and (3) removing any builtup deposits or salts due to leaks [159].

Peak Distortion (Fronting and Tailing)

Large injection volume, strong sample solvent, or both can distort peaks creating fronted, tailed, or split peaks. To avoid peak distortion, the injection solvent strength and volume must be chosen carefully. It is recommended to dissolve your sample in the weakest solvent possible and for the injection volume not to exceed 15% of the volume of the first peak of interest [42, 135, 149, 161–166].

Peak distortion can be due to mass overload or volume overload on the column. To check whether peak distortion is due to mass overload, 10 fold less mass is injected on the column and the change in peak shape and retention time is monitored. An increase in retention time accompanied by improvement in peak shape indicates mass overload. To further investigate whether the volume injected is not overloading the column, 50 and 200% of the injection volume are re-injected and the peak shape is compared. As indicated before, a change in retention time and peak shape indicates the presence of overloading [167–169].

Peak distortion is measured in terms of the asymmetry or tailing factor. Asymmetry is usually determined by measure the ratio of the width of the back portion of the peak to the front portion of the peak at 10% of the peak height (*b*/*a*), while the tailing factor is the peak width to twice the front portion of the peak, all at 5% peak height [39, 40]. Acceptable values of tailing factor are less than 1.5 and values larger than 2 indicate the presence of a problem.

There are several ways to check whether the peak distortion is due to the presence of two components or due to tailing of one component, (1) comparison of the UV spectra at the different parts of the peaks can be used, although this may not be successful for co-eluting peaks that have similar chemical structure (and thus similar spectra) [167, 170]. (2) Comparison of the mass spectra across the peak, although this may not be straightforward if the method is not MS compatible. (3) Fractions collection across the peaks followed by re-analysis [167].

A special case was reported in [171] where a split peak problem was not due to overload, column frit blockage, in-line filter, or secondary interactions with the column, it was the result of a faulty proportioning valve [171].

Peak tailing can be related to unwanted secondary interactions, especially acidic or basic compounds undergoing ion exchange interaction or interaction with metal contamination in the silica. This problem is less prevalent with high

purity type B silica compared to type A silica [95, 172]. It can also be because of column deterioration at extreme pH (<2 or >7) or due to large extra-column volume [163].

Helpful Tips and Tricks, Shortcuts, and Information

1. Rule of retention change with temperature (2%/ °C): for isocratic separation, an increase in 1 °C will result in a 2% change in retention time [173].
2. Rule of one and rule of two. Rule of one: when trouble shooting a problem, make sure to change one thing at a time. Rule of two: make sure the problem is reproducible [90].
3. Rule of three, change of retention factor change with $\%B$ under isocratic separation [2, 163, 173]. On average, the retention factor will change about threefold for a 10% change in volume fraction of the strong solvent.
4. Quotes by Dolan:
   - "one of the most difficult decisions to make during method development is when to stop… it is impossible to prove that no minor peaks are hiding under a large peak" [170].
   - "As practicing chromatographers know, a perfectly symmetric peak is a rarity" [170].
   - "If I were a gambling man, I'd lay my money down on the reversed-phase bet every time, unless I had a solid reason to choose otherwise" [19].
   - "One of the reasons for the success of LC as an analytical technique is that users can be pretty sloppy about the technique and still get good results" [134].
5. Isocratic or gradient (the 25/40% rule) [1].

In a simple gradient scouting run, if the peaks occupy less than 25% of the total run time, an isocratic method can be used, while a gradient is necessary if the peaks occupy more than 40% of the run time. If the peaks occupy between 25 and 40% of the chromatogram, either gradient of isocratic can be used depending on the sample.

6. Loss of resolution: the <15% rule and the <40% rule [149, 162].

If the sample solvent is the same as the mobile phase, an injection volume of less than 15% of the volume of the first peak of interest should be used to sacrifice less than 1% of resolution. If up to 10% loss in resolution is acceptable, the injection volume can be increased to a maximum of 40% of the peak volume. When the sample solvent is stronger than the initial mobile phase composition, the injection volume should be decreased [149, 162].

7. It is recommended to work with mobile phases at two pH units lower than the pKa of acids and two pH units higher than the pKa of bases. Under such conditions, the analyte is 99% un-ionized [36].
8. Detector time constant and collection rate: set the detector time constant, which is a noise filter, to ~0.1 times the width of the narrowest peak in your chromatogram. Set the collection rate, so at least 15–20 data points are acquired per peak [104].
9. Pressure issues can be in the form of high, low, or erratic pressures. The pressure psi can be approximated using the following equation

$$P \approx \frac{2500 \times L \times \times F}{d_{\mathrm{p}}^2 \times d_{\mathrm{c}}^2}, \tag{18}$$

where η *is* the mobile phase viscosity in cP, L is the length of the column in mm, d_{p} is the particle diameter in μm, d_{c} is the column diameter in mm, and F is the flowrate in mL/min. For gradient, the viscosity changes as $\%B$ changes, so to estimate P properly, the highest viscosity should be used. Due to the pressure generated from non-column sources, actual observed pressure is closer to 150% value of the calculated pressure [9, 10].

10. Method or system [139]: when in doubt whether the method or the system is the source of an LC problem, try to repeat a manufacturer's new column test.
11. Negative peaks: negative peaks may be observed if a UV absorbing impurity is present in the mobile phase but not in the diluent. The negative peak would be observed at the retention time that the impurity would elute. Another source may be due to dissolved air in the sample solvent [152, 174].
12. Measuring N for gradient methods: measuring column efficiency in terms of number of theoretical plates is a common mistake that may result in the inconsistent measurements when transferring the method between instruments of different dwell volumes [73].
13. Why some methods use 214 nm: zinc lamps which were used in the past provide prominent 214 nm energy. A 214 nm wavelength dates from the days of using a zinc lamp in a fixed wavelength detector. If a method developed, nowadays, the analyst is expected to specify a 215 nm wavelength rather than 214 nm [57].
14. Finding scouting gradient [1, 10]: the gradient time for a scouting gradient can be calculated based on the equation below while targeting values of k^* between 2 and 10.

$$t_{\mathrm{G}} \approx \left(5 \times k^* \times V_{\mathrm{m}} \times \Delta\%B\right)/F, \tag{19}$$

where V_m is the dead volume of the column, F is the flow rate, $\%B$ is the volume fraction of the strong mobile phase, 5 is the S factor used for small molecule, and k^* is the gradient retention factor.

15. Dead volume of the column: can be approximated using the dead volume (V_m) equation [175]

$$V_m \approx (0.5 \times L \times d_c^2)/1000, \quad (20)$$

where L is the length of the column in mm and d_c is the column internal diameter in mm [1, 10, 173]. Dead time can be calculated using dead volume and flow rate.

16. S is the slope of the plot of the isocratic log k' versus $\%B$, can be estimated according to Eq. (14) [10]. A typical S value of a small molecule (<1000 Da) is 5.
17. The maximum volume to be injected on a column can be estimated from the width of the first peak of interest. It is equal to 15% of the volume of the first peak 0.15 × peak base width (in volume) [175].
18. C18 columns can be significantly different. Do not assume that all C18 columns are the same [176, 177].
19. The efficiency of a column can be estimated as follows:

$$N \approx 300L/d_p, \quad (21)$$

where L is the length of the column in mm and d_p is the particle diameter in µm [173, 175].

20. When you cut a tube (whether it is plastic or stainless steel), make sure that you always rinse it as some debris may cause serious problem, such as scratching the rotor on the injector valve [104].
21. Do not turn the LC system off for long time with buffer in it, bacteria may grow.
22. Column wash: first flush with your mobile phase followed by 100% organic (such as acetonitrile). For cleaning ion paring reagents, wash with 100 mL 50:50 aqueous:methanol where the aqueous solution is 200 mM phosphate buffer at pH 6 [74].
23. Always use temperature control and do not run at ambient temperature as the ambient conditions can change from one place to another even in the same lab if the temperature control is turned off during night to save energy [104].
24. For method development for proteins, it is recommended to (1) denature the protein before injection, (2) run at low flow rate, (3) select wide pore particles, and (4) use a shallow gradient [178].
25. The regression coefficient value can be misleading as the points on the high end of the plot dominated the calculation. To give a better consideration of the points at the low end of the curve, appropriate weighing factors that give the lowest total sum of the relative error (absolute value of 100 percent recovery) should be used [179].
26. Parallel chromatography: is a type of chromatography in which two LC systems run samples with off cycle times. This is done to speed up the analysis of LC–MS sample by the aid of a switching valve that directs the sample to the MS and the column wash to the waste [180].
27. Misconceptions/Facts [181]:

- If the pump runs out of solvent, it will not pump air into your column as it is designed to pump liquid only [181].
- The amount of air that gets into the column if the caps are not on is minimal, the challenge is how to remove air out of the column rather than how much air got into the column. It is recommended to pump the air out using a degassed organic solvent of low viscosity [181].
- The pump flow does not need to be on when making connections. The amount of air that may get into the column is minimum [181].
- Columns can be reverse flushed, except for those that are packed with sub 2 µm [95, 181].

28. Problems that existed in the past and diminished nowadays.

- Air bubbles in the pump and the detector are minimized with the proper vacuum in-line degassing of mobile phases compared to the offline vacuum or helium sparging.
- Pump seals now can last longer with the proper cleaning after using a buffer.
- Detector lamps last longer and have longer shelf time due to the better manufacturing techniques and the lamp warm-up features.
- Peak fronting with ion pairing separations can be minimized by changing the temperature on type A silica columns, this effect is no longer observed in type B silica [98].
- Much better packing and particle size technology. No more void at the top of the columns that we need to fill. Smart tags are attached to columns to allow tracking LC diagnosis.
- Fittings problems A-line fittings and finger-tight nanoViper fittings can handle high pressure.
- Columns type B silica columns have minimal metal impurity and better distribution of silanol groups on the surface of the silanol groups. Therefore, less tailing (no need for more additives to minimize tailing) and better column-to-column reproducibility.
- Smaller particles and polar embedded phases are made available (compatibility with 100% aqueous mobile phase).

- Shorter cycle times for autosamplers are available now [182].

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Compliance with Ethical Standards

No funding for this work was provided.

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Ethical approval This article does not contain any studies with human participants or animals performed by the authors.

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