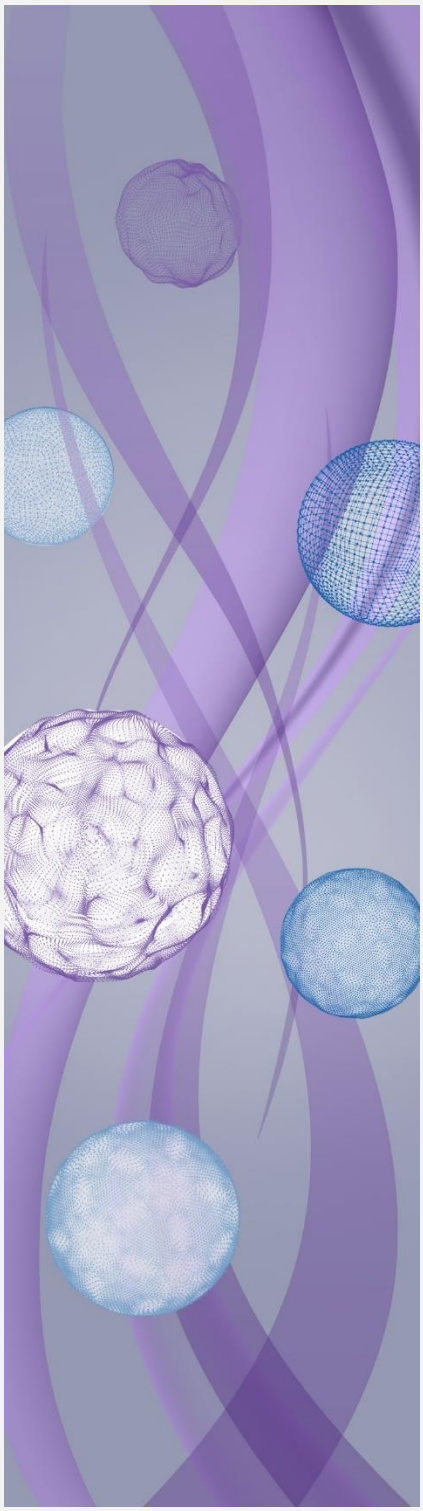
WHITE PAPER



Troubleshooting for HPLC: Recognizing and Anticipating Challenges during Reversed Phase HPLC

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Introduction

A systematic approach to HPLC troubleshooting will help to definitively address the root cause of unexpected method performance during the implementation of reversed phase HPLC methods. Most HPLC methods utilize a "C18 column" on which hydrophobic "reversed phase" retention of analytes is predicated. Diagnosing and addressing HPLC problems can seem daunting when arbitrarily changing method parameters or preparing new reagent and sample solutions. Such scattered efforts may fix the problem in the near term but undermine an opportunity to prevent or prepare for a recurrence of the problem in the future. Troubleshooting begins with a basic understanding of HPLC instrument mechanics and a few fundamentals by which chromatographic separations occur. An analysis of collective or individual analyte behavior may help to quickly determine if an oversight occurred when setting up the LC system or implementation of the method. Nuanced approaches to system maintenance and appreciating the retentive mechanisms of a method will promote column lifetime and method reproducibility.

LC Fundamentals

The fundamentals of reversed phase liquid chromatography facilitate successful troubleshooting efforts when implementing a method, for which a few concepts should be introduced. The mobile phase is the liquid that continuously flows through the column, while the stationary phase is the immobilized medium over which the liquid mobile phase flows. The retention mechanism is the set of intermolecular interactions that establish an affinity between the analyte and the stationary phase. The "strength" of the mobile phase is defined by its ability to disrupt the affinity between analytes and the stationary phase. A reversed phase method entails a primarily hydrophobic stationary phase, while the mobile phase is comprised of a "weak" aqueous solution and a "strong" organic solvent that is miscible in water. The principle is demonstrated with a Kinetex™ C18 column.

The Kinetex C18 column features C18 hydrocarbon ligands bonded to the surface of core-shell silica particles (**Figure 1**). The C18 ligands are nonpolar and serve to establish the hydrophobic retention mechanism towards analytes. Polar analytes have low affinity and little retention along a C18 phase, while nonpolar analytes exhibit a much higher retention. **Figure 2** shows four analytes with increasing hydrophobicity from left to right, along with corresponding LogP values (partition coefficient).

Figure 1. C18 Hydrocarbon Ligands

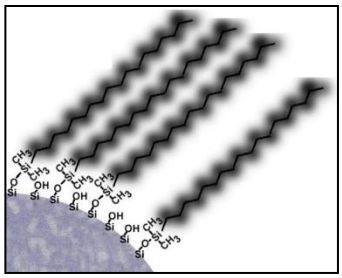
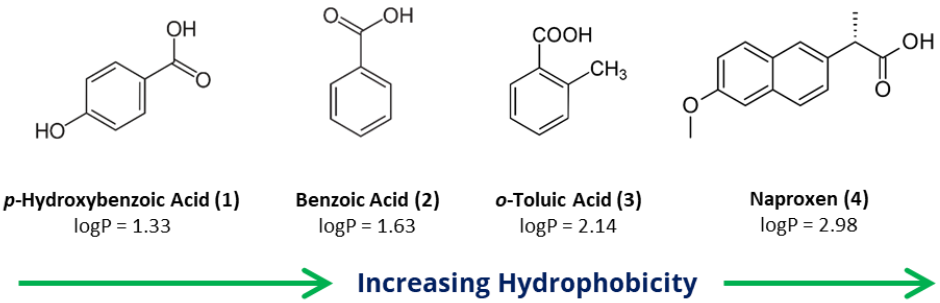


Figure 2. Analytes with Increasing Hydrophobicity



_p_-Hydroxybenzoic Acid (1) logP = 1.33

Benzoic Acid (2) logP = 1.63

_o_-Toluic Acid (3) logP = 2.14

Naproxen (4) logP = 2.98

Increasing Hydrophobicity

Have questions or want more details on implementing this method? We would love to help! Visit www.phenomenex.com/Chat to get in touch with one of our Technical Specialists

These four analytes were analyzed using the Kinetex™ 5 µm C18 column under the conditions listed in **Figure 3**. Two different ratios of mobile phase A and B were used to show how the strength of the mobile phase affects the affinity and retention of the analytes depending on their degree of polarity. Chromatogram 1 uses a "stronger" solvent containing more Acetonitrile to disrupt retention. This is evidenced by the short retention times of the analytes. When a higher ratio of "weak" solvent is used (chromatogram 2), there is less disruption of the affinity of the analytes to the stationary phase, encouraging longer retention times.

Troubleshooting is facilitated by an awareness of the retention mechanisms during reversed phase HPLC. The myriad of complications that may be addressed include ghost peaks, potential carry-over, discrepancies during sample preparation, and causes for an accumulation of impurities that may affect peak shape or column backpressure. Analyte retention may also be dependent on the pH of the mobile phase and diluent, which will be addressed when troubleshooting peak shape.

Routine Column Cleaning

One way to help prevent HPLC issues is through routine column cleaning, which may be either a manual or automated process. The process of cleaning a reversed phase C18 column will generally entail the use of Water and either Methanol or Acetonitrile. Cleaning begins with a sustained (isocratic) solvent ratio of Water / Acetonitrile (90:10) (or Water / Methanol) to flush out any salts, mobile phase modifiers, and residual hydrophilic impurities. A "strong" wash may then be used without the risk of salts and hydrophilic impurities precipitating within the column. Next, an isocratic ratio of Water / Acetonitrile (5:95) (or Water / Methanol) is used to purge most hydrophobic impurities from the C18 column, knowing that hydrophobic retention is being disrupted. Finally, the column is equilibrated for storage with a ratio of Water / Acetonitrile (50:50) (or Water / Methanol).

A cleaning method may be automated, for which two examples are presented in **Figure 4** for common column dimensions. Each isocratic wash step above should be sustained ideally for 10 column solvent volumes, while the final equilibration step is only necessary for 5 solvent volumes. Pure solvents should be used to avoid storing the column under deleterious acidic or basic equilibration.

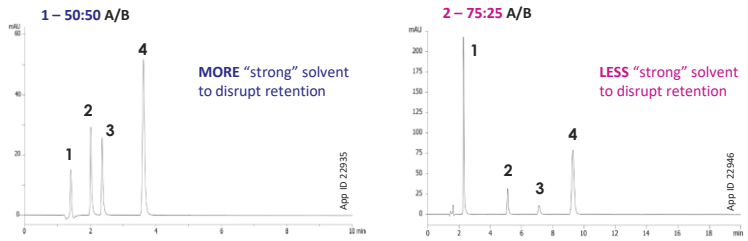
Diagnosing Problems

There is an important distinction between a method problem, system problem, and combination of the two when beginning to diagnose problems during reversed phase HPLC. Individual and collective peak behavior can be an indicator as to where the issue resides. Tailing, fronting, splitting, shifting retention times, and peak area response can all be used as indicators to help begin diagnosing problems. Examples can be found in **Figure 5**. Other potential indicators include abnormal backpressure, extra peaks, and detector abnormalities. An understanding of reversed phase retention mechanisms and fluid dynamics provides a foundation on which to diagnose and resolve problems.

Retention Time Shifts

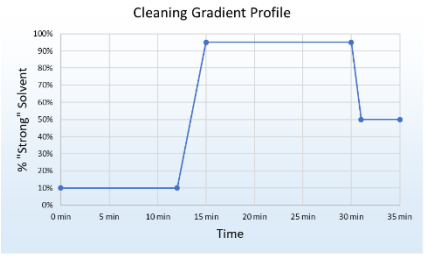
The column "dwell volume" is the volume of solvent within the column that must be displaced before any unretained analytes from an injected sample will reach the detector. The time needed for this dwell volume displacement (depicted as t_0 in **Figure 6**) is dependent on the flow rate and is commonly referred to as either the column "dead time," "dwell time," or "void time." The behavior of t_0 can help determine the extent to which shifting retention times may be caused by either the mechanical performance or setup of the HPLC system, or a chemical discrepancy within the method parameters or implementation of the method.

Figure 3. Isocratic Analysis of Increasingly Hydrophobic Analytes



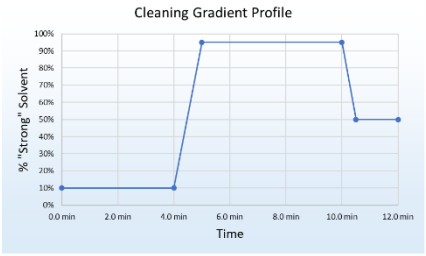
Column:	Kinetex 5 µm C18
Dimension:	150 x 4.6 mm
Part No.:	00D-4790-AN
Mobile Phase:	A: 0.1 % Phosphoric Acid in Water (weak) B: Acetonitrile (strong)
Flow Rate:	1 mL/min (Isocratic)
Temperature:	Ambient
Detection:	UV @ 254 nm

Figure 4. Examples of Gradient Profile for Cleaning and Storing a Kinetex C18 Column



Example of a (150 x 4.6 mm) cleaning method

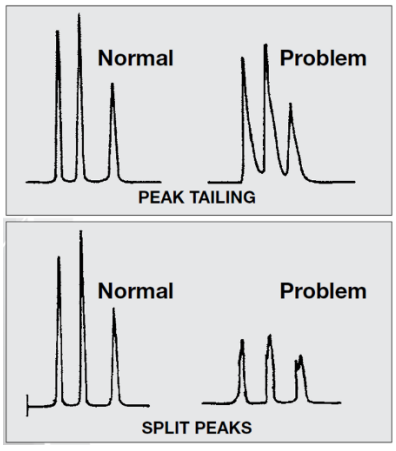
Column:	Kinetex 5 µm C18		
Dimension:	150 x 4.6 mm		
Part No.:	00F-4601-E0		
Mobile Phase:	A: Water B: Acetonitrile or Methanol		
Temperature:	ambient		
Gradient:	Time (min)	% A	% B
	0	90	10
	12	90	10
	15	5	95
	30	5	95
	31	50	50
	35	50	50



Example of a (50 x 2.1 mm) cleaning method

Column:	Kinetex 2.6 µm C18		
Dimension:	50 x 2.1 mm		
Part No.:	00B-4462-AN		
Mobile Phase:	A: Water B: Acetonitrile or Methanol		
Temperature:	ambient		
Gradient:	Time (min)	% A	% B
	0	90	10
	4	90	10
	5	5	95
	10	5	95
	10.5	50	50
	12	50	50

Figure 5. Examples of Problematic Peaks



If the value of t_0 differs from the expected value for a method, or shifts during consecutive runs of the same method, then problems with the instrument performance or setup are likely. A consistent but incorrect t_0 often results from either an incorrect flow rate or an incorrect column. An erratic t_0 may be the result of a leaking connection, a bubble within the solvent lines, or a blocked frit (pump frit or solvent line frit).

Solvent lines and any proportioning valves within the mobile phase mixer on the instrument may be cleaned periodically by first opening the purge valve on the pump. Each solvent line would then be flushed with a sequence of 100 % Water to rid the lines of salts, 100 % Isopropanol to rid the lines of hydrophobic impurities, once more with 100 % Water to flush out the Isopropanol, and finally with the appropriate mobile phase for each solvent line. It should be noted that this recommended sequence is intended for reversed phase systems. After completing this sequence, the purge valve should be closed and the pump frit within the purge valve may need to be replaced.

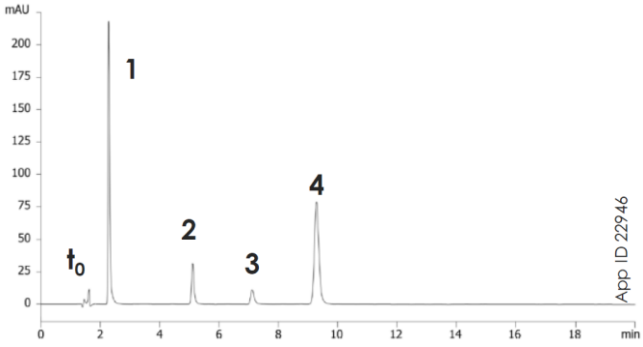
If analyte retention times shift while the value of t_0 remains correct and consistent, then there may be a problem with the method parameters or method implementation. To address this, the installation of the correct stationary phase column should be confirmed, and that the proper temperature has been set. The placement of mobile phases on the correct solvent lines should be checked and that they are assigned correctly within the method parameters on the LC workstation software. Finally, the composition of the mobile phases and diluent with respect to solvents, concentration, and pH value should be examined. An aqueous mobile phase with a high pH is susceptible to the dissolution of Carbon Dioxide, which dissolves as Carbonic Acid and lowers the pH of the mobile phase. Mobile phases that simply feature a percentage of Formic Acid are susceptible to the evaporation of additive from solution within a few days of preparation.

The temperature at which a method is performed should be considered. Ambient, or room temperature, methods are susceptible to fluctuating retention times based on the temperature of the column. Specifically, the column temperature will affect the rate at which analytes interact with the column stationary phase, as well as the viscosity of the mobile phases. An established temperature just above room temperature is one means of assuring consistent method performance when a method temperature is not specified.

Peak Shape and Retention as Affected by Solvent Strength and pH Value

The nuanced ramifications of solvent strength and pH values towards method performance are evident when peaks exhibit shifting retention times, amorphous peak shape, and unexpected area recoveries.
The "strength" and pH of the mobile phases and diluent will affect the consistency with which an analyte establishes retention onto the stationary phase. The retention mechanism during a reversed phase method on a C18 stationary phase is hydrophobic retention. An unexpectedly high ratio of organic solvent within the overall mobile phase will induce faster elution of the analytes by disrupting hydrophobic interactions more readily, generating faster retention times. More importantly, if the ratio of organic solvent within the sample diluent is high, the analytes may have difficulty establishing initial retention onto the stationary phase as the diluent dissipates from the immediate surroundings of the analyte. Conversely, a high ratio of aqueous solvent within the diluent will promote the initial establishment of retention when analytes first reach the column.

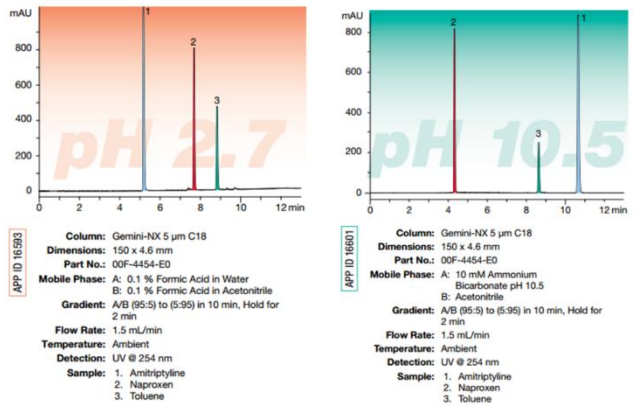
Figure 6. t_0 on a Chromatogram



The pH of the mobile phase and diluent is another tool with which to control and promote consistent retention. The absence or presence of an ionic charge on the analyte will affect the hydrophobicity of the analyte and the extent to which it can establish hydrophobic retention. The acidic and basic functional groups within the chemical structure of an analyte each have a pKa value that represents the pH at which the analyte molecules are equally distributed between a particular protonated and deprotonated ionic form. Acidic groups will be neutral when protonated at pH values below their pKa and will be negatively charged when deprotonated at pH values above their pKa. Bases will primarily be positively charged at pH values below the pKa of their conjugate acid form and will be neutral at high pH above the pKa of their conjugate acid.

Generally, the pH of the mobile phases and diluent are both aligned and distant from the pKa values of the targeted analytes to promote a consistent ionic form of the analyte molecules. If the pH of a mobile phase or diluent is too close to the pKa of an analyte, the analyte molecules will waver between ionic states and result in shifting retention times and amorphous peak shape (tailing, split, and fronting peaks). Discrepant pH values between the diluent and mobile phases will also generate inconsistent peak shape and retention, as the injected analytes must rapidly re-equilibrate from one ionic state to another. Finally, an incorrect pH value of the mobile phases may generate different retention times by altering the protonated state of an analyte, as demonstrated in **Figure 7**. The basic analyte Amitriptyline (pKa = 9.76) and acidic analyte Naproxen (pKa = 4.19) each exhibit different retention times upon altering the pH of the mobile phase without changing any other method parameters. The analyte Toluene remains in place steadfast since it does not contain an acidic or basic functional group.

Figure 7. The Dependance of Analyte Selectivity Towards Mobile Phase pH



Additional Causes of Abnormal Peak Shape

Abnormal peak shape may result from other causes attributable to an HPLC system or setup. Tailing peaks may result from "silanol activity" along the stationary phase of a column or simply from a bad tubing connection. The surface of the functionalized C18 silica media will always have a few remaining silanol groups, which are acidic. Deprotonated silanol groups possess a negative charge that will bind with protonated basic analytes to generate tailing peaks that compromise recovery during quantitative methods. Old columns or methods that run at moderate and high pH are susceptible to inconsistent silanol activity and should be evaluated for column lifetime. Bad tubing connections create an extra pocket of volume in which flowing mobile phase and analytes may become briefly trapped while the bulk of an analyte band migrates onward, resulting in tailing peaks.

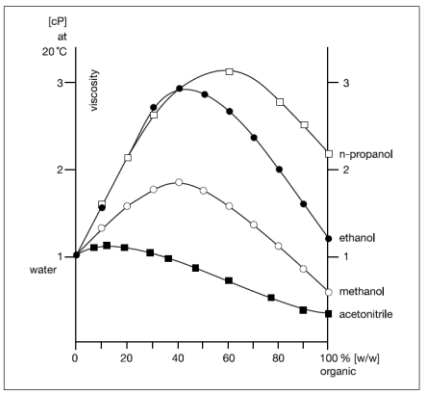
Fronting peak shape is not as common as tailing and split peaks but can arise from diluent discrepancies or when concentrated samples are injected during a reversed phase method. A "strong" diluent that features a high ratio of organic solvent may generate fronting peaks, in addition to splitting and tailing peaks. More interestingly is the prospect of analytes within a highly concentrated sample to become bound to one another upon establishing retention along the stationary phase. The resulting band of analyte molecules during such an injection would be distributed in the typical "Gaussian" form associated with symmetrical peaks, since the bulk of the analyte molecules elute from the column later within the peak.

Abnormal Backpressure

An uncharacteristically low or high backpressure is less complex to navigate. Low backpressure values may simply be the result of either a leak or a slow flow rate. A low backpressure may also result from air bubbles within the solvent lines. Degassing the mobile phases prior to placement onto the LC instrument, or the use of an LC instrument that features a degasser within the solvent flow path can address this. Priming the solvent lines is one means of ridding the lines and instrument degasser of suspected bubbles.

High backpressure is often caused by a gradual or sudden blockage at the entry of the column but solvent viscosity among the mobile phases should also be considered. Methanol is particularly viscous when blended with Water, as shown on the graph in **Figure 8**. Other common protic solvents have a naturally high viscosity, namely Isopropanol.

Figure 8. Viscosity of Solvent Mixtures as a Function of Composition



Clogs at the column entry may result from inadequate sample preparation, such as "dilute and shoot" of complex sample matrices. Hydrophobic impurities within the sample will precipitate within the LC tubing leading to the column once the sample begins mixing with the initial mobile phase conditions of the method, which often favor the aqueous mobile phase. Thorough sample preparation and the regular application of column cleaning methods will prolong the lifetime of a column.

If a clog is suspected, then an attempt to clean the column should be made with a slower flow rate using a sequence of Water / Acetonitrile (80:20), Water / Acetonitrile (5:95), either 100% Isopropanol or 100% Tetrahydrofuran, Water / Acetonitrile (5:95), and finally Water / Acetonitrile (50:50). Water / Methanol is suitable in place of Water / Acetonitrile. The intent of the Isopropanol or Tetrahydrofuran is to apply a "stronger" solvent towards the reversed phase column while maintaining water miscibility.

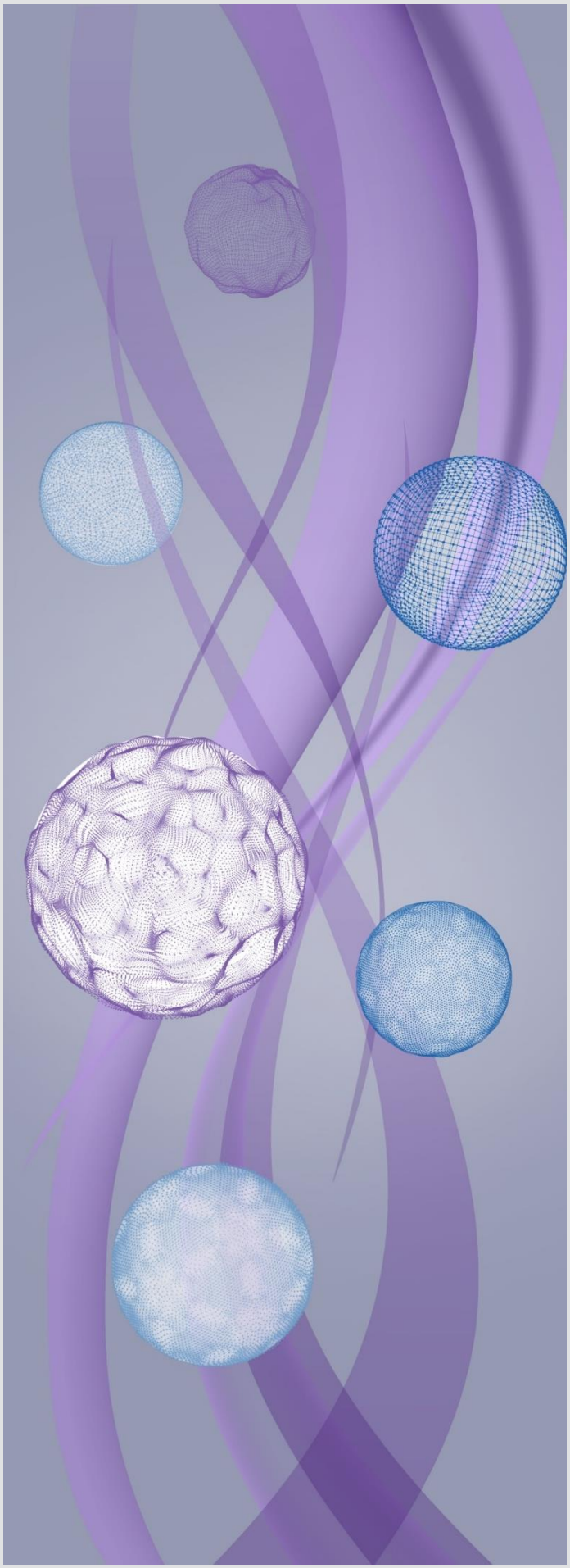
Ghost Peaks

Unexpected "ghost" peaks may result from either mobile phase contamination or the presence of hydrophobic impurities that do not adequately elute from the column during a previous sample analysis. Residual dust or hydrophobic impurities within the mobile phase solutions may precipitate upon mixing and then establish retention along the hydrophobic stationary phase. The impurity would then elute as the mobile phase progressively disrupts the retention of the impurity. The mobile phase reservoirs should be covered, and the mobile phases filtered prior to placing them onto the LC instrument.

Ghost peaks may also result from an impurity that is inherent to an injected sample. An isocratic method (constant ratio of mobile phase) may not feature a long enough duration to elute the impurity, only for the impurity to elute during the subsequent sample injection. Similarly, a gradient method may not reach a high enough ratio of organic solvent to elute the impurity during the method, but rather the impurity gradually migrates through the column to elute during a subsequent sample injection. The duration and strength of a method may be prolonged or increased if ghost peaks are suspected.

Summary

A systematic approach towards diagnosing and addressing HPLC problems will help to stave off any ambiguity that may otherwise arise during troubleshooting. An initial assessment of the collective or individual behavior of analyte peaks will guide decisions to address either the HPLC system setup or method design and implementation. An understanding of the fundamental retention mechanisms during reversed phase HPLC is key when considering mobile phases and sample diluent with respect to "weak" and "strong" solvents. Proper pH values necessary for a method and their possible effect on the ionic state of targeted analytes is also important. Regular column maintenance and an awareness of reversed phase chemistry will ultimately promote column longevity and method reproducibility.



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