HPLC tech tips from RESTEK

For **Free** Technical Support Call

800-356-1688, ext. 4 or your local Restek representative

System Setup Essentials

Changing Mobile Phases

1. Verify the mobile phase is miscible with the previous mobile phase. Flush the HPLC system with 10–20 column volumes of the new mobile phase.
2. Start the pump and increase the flow to the desired rate over a period of about 60 seconds. Check for leaks.
3. Allow the system to equilibrate until the baseline is stable.

Retrieving a Column from Storage

1. Verify the mobile phase is miscible with the column storage solvent. Flush the HPLC system with 10–20 column volumes of the new mobile phase.
2. Attach the column to the system. Verify the ferrules are properly seated in the column, to minimize dead volume.
3. Start the pump and increase the flow to the desired rate over a period of about 60 seconds. Check for leaks.
4. Allow the system to equilibrate until the baseline is stable.

Switching HPLC System: Normal Phase to/from Reversed Phase

1. Remove the column and install a zero dead volume connector.
2. If necessary, flush the system with 10–20 column volumes of water to remove buffer from the previous mobile phase. If going from normal phase to reversed phase, go to Step 4.
3. Make 3 injections of water at the maximum capacity of the sample loop.
4. Flush the system with 10–20 column volumes of isopropanol (IPA) or ethanol (EtOH).
5. Make 3 injections of IPA or EtOH at the maximum capacity of the sample loop.
6. Make 3 injections of the new mobile phase at the maximum capacity of the sample loop.

Column and HPLC System Storage Essentials

1. Failure to prepare and store a column properly can damage the column and shorten its life, and possibly damage other HPLC system components.
2. Rinse the system and column with buffer-free mobile phase, to remove all traces of buffer.

   Buffer precipitation within a column can cause microfractures in the packing bed. Precipitated buffer is difficult to remove.

   For reversed phase columns, maintain at least 10% organic solvent in the mobile phase during the rinse.
3. Rinse the system and column with the strong mobile phase solvent until the baseline stabilizes. Recommended storage solvents are methanol, acetonitrile, or a buffer-free aqueous mobile phase containing at least 10% organic solvent (to prevent bacterial growth in the column).
4. Remove the column from the system. Make sure the column is properly sealed with end plugs. Aluminum foil, paraffin film sheeting, etc. are not substitutes for column plugs.

   The packing bed in a poorly sealed column can dry out, creating microfractures and channeling in the bed.
5. Maintain a log for each column, and for the system. This information will assist in troubleshooting if a problem occurs. Dates used, mobile phases, theoretical plates, pressure, compounds separated, and unusual observations (e.g., peak tailing), are valuable items of information.

   Periodically check column performance with a standard test mix.
6. Once ion pair reagents are introduced into a column, most manufacturers recommend that the column be dedicated to that separation.

Mobile Phase Preparation Basics

1. Know the miscibility of all mobile phase components before mixing. If necessary, test the combination in a vial.
2. Filter all buffer solutions before use. Small particles, often present in buffer solutions; can plug a column or damage equipment.
3. Use separate glassware to measure each mobile phase component. Combining solvents in the same container will create volume displacement errors.
4. Adjust the pH of the buffer BEFORE adding the buffer to the organic phase. pH is not defined in a non-aqueous system.
5. Verify the buffer is correct for the pH range. Verify the strength of the buffer.
6. Whenever the aqueous component of a mobile phase includes a buffer, add the organic component to the aqueous component. Introducing the buffer into the organic component could cause the buffer to precipitate.
7. Be especially alert to buffer precipitation, especially at high concentration, when using gradient elution. When the organic solvent exceeds 50% of the mix, the buffer could precipitate.
8. Allow the mobile phase to thermally equilibrate before beginning an analysis. Isolate mobile phase reservoirs from locations that could cause uncontrolled temperature fluctuations.

Trident™ Guard Columns

Chromatography Formulae

Parameter	Unit	Formulae	
		Kirkland et al.*	USP**
Retention time of an unretained solute	sec.	t_o	t_a
Retention time, measured from the start	sec.	t_R	t
Reduced retention time	sec.	$t'_R = t_R - t_o$	—
Peak width measured at baseline	sec.	w	w
Peak width at 1/2 height	sec.	$w_{1/2}$	$w_{h/2}$
Capacity factor	—	$k = \frac{t'_R}{t_o}$	$k' = \frac{t}{t_a} - 1$
Selectivity	—	$\alpha = \frac{k'_2}{k'_1} = \frac{t'_{R2}}{t'_{R1}}$	$\alpha = \frac{t_2 - t_a}{t_1 - t_a}$
Resolution	—	$R_S = 2\left(\frac{t'_{R2} - t'_{R1}}{w_2 + w_1}\right)$	$R = \frac{2(t_2 - t_1)}{w_2 + w_1}$
Resolution (general equation)	—	$R_S = {}^1/_4\left(\frac{\alpha - 1}{\alpha}\right)\sqrt{N}\left(\frac{k}{1+k}\right)$	
Number of theoretical plates	—	$N = 16\left(\frac{t_R}{w}\right)^2$	$N = 16\left(\frac{t}{w}\right)^2$
Number of theoretical plates (peak width at 1/2 height)	—	$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$	$N = 5.54\left(\frac{t}{w_{h/2}}\right)^2$
Column length	cm	L	—
Height equivalent of a theoretical plate (plate height)	cm	$H = \frac{L}{N}$	—

* *Modern Practice of Liquid Chromatography* J.J. Kirkland, ed., Wiley, New York, 1971.
** *United States Pharmacopoeia,* USP 26/NF 21, pp 2135–2136, 2003.

Optimum flow rates

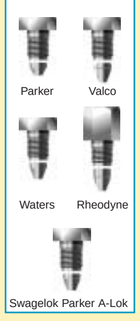
Column ID (mm)	5µm Particles Flow Rate (mL/min.)	3µm Particles Flow Rate (mL/min.)
4.6	1.00	1.5
3.2	0.50	0.73
2.1	0.20	0.31
1.0	0.05	0.07

HPLC Pump Pressure Conversion Factors

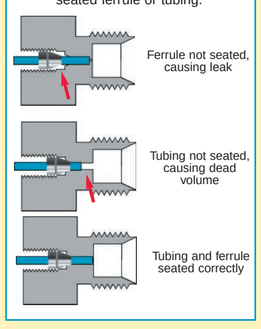
Multiply units in the left-most column by the conversion factors listed to the right.
e.g., 10PSI x 0.068 = 0.68atm or 10 bar x 29.5300 = 295.300 inches Hg

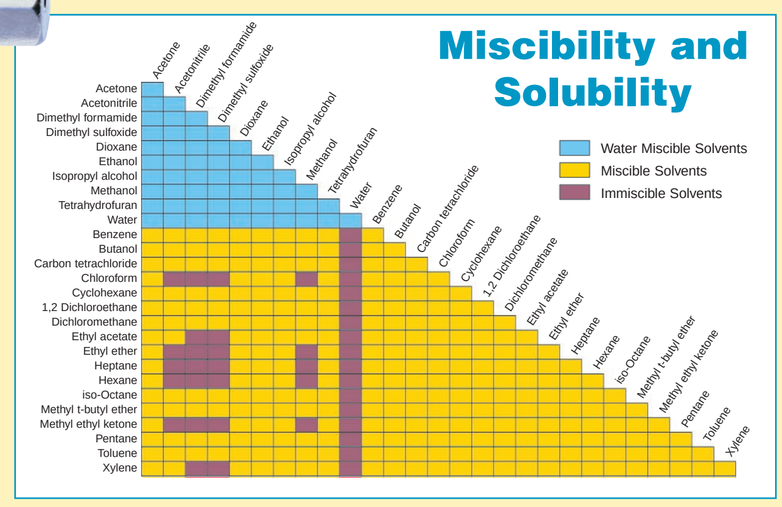
Pressure	PSI	atm	kg/cm²	torr	kPa	bar	inches Hg
PSI =	1	0.068	0.0703	51.713	6.8948	0.06895	2.0359
atm =	14.696	1	1.0332	760	101.32	1.0133	29.921
kg/cm² =	14.223	0.967	1	735.5	98.06	0.9806	28.958
torr =	0.0193	0.00132	0.00136	1	0.1330	0.00133	0.0394
kP*a* =	0.1450	0.00987	0.0102	7.52	1	0.0100	0.2962
bar =	14.5038	0.9869	1.0197	751.88	100	1	29.5300
in Hg =	0.49612	0.0334	0.0345	25.400	3.376	0.03376	1

Fitting Styles
Fitting styles differ among various manufacturers.



Proper Connections
Problems can arise from an incompatible fitting or improperly seated ferrule or tubing.





Buffers for HPLC

1. Always prepare buffers and take pH measurements in 100% aqueous solutions.
2. When preparing a buffer of known absolute molarity, prepare the acidic and basic components individually, to the same ionic strength. Adjust the final pH simply by adding a portion of the one component to the other.
3. Always adjust pH with an appropriate counterion (e.g., ammonium hydroxide and phosphoric acid are suitable for adjusting the pH of ammonium phosphate, but hydrochloric acid or acetic acid are not).

Buffer Type		pKa	Buffer pH Range	Typical Examples	Empirical Formula	Anhydrous ● Formula Mass	g needed to prepare ● one liter at 10mM
Phosphate	Note A Note B	2.1	1.1 - 3.1	Potassium phosphate monobasic,	KH_2PO_4	136.09	1.3609
				Sodium phosphate monobasic	NaH_2PO_4	120.00	1.2000
		7.2	6.2 - 8.2	Potassium phosphate dibasic,	K_2HPO_4	174.18	1.7418
				Sodium phosphate dibasic	Na_2HPO_4	142.00	1.4200
		12.3	11.3 -13.3	Sodium phosphate tribasic,	Na_3PO_4	188.10	1.8810
				Potassium phosphate tribasic	K_3PO_4	212.30	2.1230
				Phosphoric acid	H_3PO_4	98.00	0.9800
Citrate	Note C Note A	3.1	2.1-4.1	Trisodium citrate,	$C_6H_5O_7Na_3$	258.10	2.5810
				Tripotassium citrate	$C_6H_5O_7K_3$	306.40	3.0640
		4.7	3.7-5.7	Diammonium citrate (dibasic)	$C_6H_8O_7(NH_3)_2$	226.20	2.2620
		5.4	4.4-6.4	Triammonium citrate (tribasic)	$C_6H_{17}N_3O_7$	243.20	2.4320
				Citric acid	$C_6H_8O_7$	192.20	1.9220
Formate		3.8	2.8-4.8	Ammonium formate,	$CH_2O_2(NH_3)$	63.06	0.6306
				Sodium formate,	CHO_2Na	68.01	0.6801
				Potassium formate	CHO_2K	84.12	0.8412
				Formic acid	CH_2O_2	46.03	0.4603
Acetate		4.8	3.8-5.8	Ammonium acetate,	$(NH_4)C_2H_3O_2$	77.08	0.7708
				Potassium acetate,	$KC_2H_3O_2$	98.14	0.9814
				Sodium acetate	$C_2H_3O_2Na$	82.03	0.8203
				Acetic acid	CH_3COOH	60.05	0.6005
Borate		9.2	8.2-10.2	Sodium borate	$Na_2B_4O_7$	381.40	3.8140
				Boric acid	H_3BO_3	61.83	0.6183
Ammonia		9.2	8.2-10.2	Ammonium phosphate monobasic,	$(NH_4)H_2PO_4$	115.00	1.1500
				Ammonium phosphate dibasic	$(NH_4)_2HPO_4$	132.10	1.3210
				Ammonium carbonate	$(NH_4)_2CO_3$	~96*	~0.96*
				Ammonium hydroxide	$(NH_4)OH$	35.05	0.3505
Carbonate		10.2	9.2–11.2	Sodium carbonate	Na_2CO_3	105.99	1.0599
				Ammonium carbonate	$(NH_4)_2CO_3$	~96*	~0.96*
				Potassium bicarbonate	$KHCO_3$	100.12	1.0012
				Sodium bicarbonate	$NaHCO_3$	84.01	0.8402
				Ammonium bicarbonate	$(NH_4)HCO_3$	79.06	0.7906

● When using a hydrated form of the salt, substitute the formula mass of the hydrated form and divide by 100.
● To increase/decrease the buffer strength, divide the desired millimolar buffer strength by 10 to determine the correction factor. To prepare one liter of buffer, multiply the correction factor by the mass listed for a 10mM concentration. When using a material that is less than 100% pure, divide the final adjusted mass by the decimal value for the purity of the material.
A Generally, only first pKa value is significant for ion exchange.
B Low UV absorbance.
C Prolonged use will corrode stainless steel.
*Varies—consult manufacturer's label.

System Optimization

Chemistry

1. Sample components must be at least partially soluble in the mobile phase.
2. Do not exceed the manufacturer's recommended pH range for the column.
3. If possible, perform the separation at least 2 pH units above or below analyte pKa values.
4. When using ion exchange or ion pairing mode, reduce the pH below the pKa (acidic) to protonate bases, raise the pH above the pKa (basic) to ionize acids. When using reversed phase mode, prevent ionization by adjusting the pH above the pKa of bases or below the pKa of acids (if possible).
5. Some separations can be improved by changing solvents and adjusting the isoelutropic strength.
6. To avoid equilibration problems, use ion pair agents only with isocratic methods.
7. Temperature and pH must be controlled during ion pair separations.

Plumbing

1. When doing gradient analyses, determine the delay volume of the system. The larger the delay volume, the slower the gradient change.
   To determine tubing volume: volume (cm^3) = length x π x (diameter/2)^2
   Length and diameter in cm; 1 inch = 2.54cm
2. Minimize volume between injector and detector, to increase resolution and theoretical plate values. Restrict the diameter of all injector-to-detector tubing to 0.007" or less.
3. Tubing volume between the mixing chamber and the injector will contribute to the delay volume.
4. Minimize the length of all plumbing lines.

Pump

1. Verify miscibility of all solvents before use.
2. Degas all mobile phases before use (helium sparging) or in line (vacuum degasser). Gas bubbles can reduce sensitivity, obstruct check valves, and outgas in the detector flow cell. Use only helium for sparging; helium molecules are small enough to displace other atmospheric gases.
3. Maintain a sparge of about 25cc/min., to prevent re-entry of atmospheric gases.
4. Over-sparging can change the mobile phase composition, through evaporation.
5. Sonication under vacuum is an effective method of degassing.

Injector

1. For greatest precision use the smallest syringe capable of providing the needed sample volume.
2. The elution strength of the sample solvent should be equal to or weaker than that of the mobile phase. Dissolving a sample in a solution stronger than the mobile phase can cause peak fronting.

Detector Quick Tips

Select flow cell volume according to column internal diameter.
If column internal diameter is 3mm or less, use a 3µL flow cell, or smaller.

Refractive Index

1. To increase sensitivity, exploit differences in solvent refractive indices by changing solvents and adjusting isoelutropic strength.
2. Do not use refractive index detection with gradient methods.
3. Control of column and detector temperature is critical.
4. Reduce detector noise by using a backpressure regulator to reduce outgassing in the flow cell.

Fluorescence Detection

1. Determine maximum wavelengths of absorbance for the analyte(s), using a UV spectrophotometer. Remember: Not all compounds fluoresce.
2. Set the fluorescence detector to the greatest absorption excitation wavelength, then perform a scan to determine the greatest emission wavelength.

Ultraviolet Detection

1. Adjust the wavelength to or near the absorbance maximum for the analyte(s).
2. Absorbance maxima can vary with the sample diluent and, sometimes, pH.
3. Determine if a secondary absorbance allows detection at a higher signal-to-noise ratio.
4. For greatest selectivity, the signal bandwidth should not exceed 2x the slit bandwidth.
5. Inorganic buffers, such as potassium phosphate, increase the range of an analysis and contribute little UV background noise.

Evaporative Light Scattering

1. Evaporative light scattering can be used with gradient methods.
2. Mobile phase must be more volatile than all analytes. Avoid using nonvolatile buffers.
3. Use the lowest temperature required to vaporize the mobile phase.

Mass Spectrometry

1. Use only volatile buffers for LC/MS. Ammoniated species also stabilize ions.
2. For compounds already ionized, an electrospray interface (ESI) works well.
3. To improve sensitivity for ESI, select stationary phases for optimum retention to maximize the amount of volatile organic solvent in the mobile phase.

Universal 10-32 PEEK® Column Connectors and Plugs, cat.# 25324

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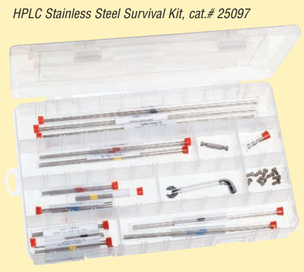

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