- **Capillary HPLC**

Liquid chromatography/mass spectrometry, LC/MS, is a revolutionary tool in the chemical and life sciences. LC/MS is accelerating chemical research by providing a robust separations and identification tool for chemists and biologists in diverse fields. LC/MS is best done with capillary HPLC. Capillary HPLC uses smaller column internal diameters than conventional HPLC. Smaller ID columns, for fixed amounts of injected material, produce taller peaks. Taller peaks provide better detection limits for mass spectrometry and other concentration sensitive detectors. For the same amount of material injected, the peak height is inversely proportional to the cross sectional area of the column. The use of smaller ID columns requires careful planning if you are used to normal 4.6 mm columns.

   This General Introduction to capillary HPLC is designed to provide a practical survey of the day-to-day issues in solving chemical problems using HPLC and to give you the necessary information to use the Agilent 1100 Capillary HPLC and Ion Trap SL. Sufficient theory is provided so that you will know <u>why</u> you are doing the things you do. You may not personally use all the material in this tutorial, but knowledge of what others are doing with the instrument will be helpful in solving problems that may arise due to previous users.

   Electrospray mass spectrometry places particular requirements on HPLC separations. We begin with a short introduction to electrospray mass spectrometry so that the requirements for MS detection are clear at the outset.

- **Electrospray Ion Source**

The most common detector for HPLC is a UV detector. However, a mass spectrometer provides a means of identifying the components in different peaks. MS is a very powerful tool, but your HPLC method and sample preparation must be carefully designed to achieve good detection limits. The key to using MS as a detector is the ability to transfer your analytes into the vacuum of the mass spectrometer as ionic species. This process is handled by an electrospray ion source in our instrument. Electrospray ion sources are soft ionization sources, that is they produce mostly protonated molecular ions, MH^+. The proton transfer can occur in your solution or in the droplets produced by the electrospray source.

   $M + H^+ \rightarrow MH^+$
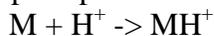
pH control for the HPLC mobile phase can have a strong effect on the ionization. As a consequence, HPLC eluants for electrospray MS usually are buffered or have added acids to enhance and control the formation of ions.

   Buffer Additives: Because of the need to control ion formation, buffers are very common in LC/MS. However, standard buffers like Tris, HEPES, and phosphate buffers are non-volatile and can clog the MS inlet capillary. Therefore, non-volatile eluant additives are necessary. The most common buffer components are formic acid, trifluoroacetic acid, acetic acid, ammonium formate, ammonium acetate, and heptafluorobutyric acid (in that order). For more basic buffers, 10-20 mM tetraethylammonium formate or bicarbonate is common. Often compromises in buffering capacity are made by choosing one of these volatile buffer components. Trifluoroacetic acid can suppress ion formation in electrospray, so its concentration is usually kept <0.1%.

- **MS and MS/MS**

Electrospray is a soft ionization technique. Electrospray spectra are very simple and molecular weights are easy to determine from the MH^+ parent peaks. Electron ionization, EI, produces fragment rich spectra. The fragment ions are useful to help determine the structure of the compound. On the other hand, in EI some classes of compounds don't produce intense parent peaks, so the molecular weight is difficult to determine. While the ease of molecular weight determination is a strength for electrospray, the lack of structural information from fragment ions can be a draw back. MS/MS techniques can solve this problem.

In MS/MS analysis, the MH^+ ions formed from the electrospray source are fragmented by adding extra collisional energy. MS/MS spectra are very similar to EI spectra and can be interpreted in the same way.

The important parameter for MS/MS based analysis in LC/MS, is that MS/MS takes extra time. If the eluting peaks are too narrow, then there won't be time for MS/MS analysis. As a consequence, some compromise in resolution and retention time may be necessary to do auto MS/MS analysis. Strangely, this means that the best efficiency isn't always best for MS/MS detection, which is a strange circumstance for most chromatographers.

Capillary HPLC

The following sections discuss reversed phase HPLC in general terms and focus on the issues that are particularly important for capillary HPLC/MS. Please see a text on HPLC for more complete discussions of chromatography principles. A separate tutorial is available for ion exchange, HILIC, HIC, and size exclusion modes.

- **Reversed Phase Chromatography, RP**

In reversed phase separations the analyte partitions between a hydrophobic stationary phase and a polar mobile phase. Typical reversed phase stationary phases are based on C18 hydrocarbon chains attached to silica particles through silyl-ether bonds: $Si-O-CH_2-R$. The mobile phase usually consists of acetonitrile-water mixtures or methanol-water mixtures. Reversed phase chromatography is useful for a range of analytes from moderately polar to rather hydrophobic. Reversed phase is the most common mode for HPLC, because it is the most versatile and the easiest to do.

- **Eluants**

Acetonitrile-water and methanol-water mixtures are the most common eluants for reversed phase HPLC. The non-aqueous component is called the organic modifier. The Solvent Properties table below is very useful for selecting the organic modifier.

Reversed phase columns always need some water, so water miscibility is necessary. The other solvents may be useful for wash solvents. The table gives the solvent strength for many of the common solvents used in HPLC. The polarity and the solvent strength parameter, $\in_p$, can be used to pick the organic component for your mobile phase. Lower $\in_p$ values correspond to stronger solvents for reversed phase chromatography. For example, acetonitrile is a stronger solvent for non-polar organics than methanol. Acetonitrile is therefore the most common organic

modifier, followed by methanol. 2-Propanol can also be used as a modifer, however, its much higher viscosity produces higher backpressures and requires the use of shorter columns. The UV cut off determines the wavelength range usable for UV/Visible detectors; see the discussion under Detectors below. You also minimize baseline changes by matching the modifier refractive index with that of water. Big mismatches make the baseline more composition and temperature sensitive.

Solvent properties for Organic Solvents Commonly Used in HPLC in order of increasing polarity

Solvent	Polarity	Miscible with water?	UV cut off	Refractive Index 20°C	Solvent Strength ϵ_o	Viscosity 20°C, cp
Hexane	nonpolar	no	200	1.3750	0.00	0.33
Isooctane	↓	no	200	1.3910	0.01	0.50
Carbon tetrachloride		no	263	1.4595	0.14	0.97
Chloroform		no	245	1.4460	0.31	0.57
Methylene chloride		no	235	1.4240	0.32	0.44
Tetrahydrofuran		no	215	1.4070	0.35	0.55
Diethyl ether		poorly	215	1.3530	0.29	0.23
Acetone		yes	330	1.3590	0.43	0.32
Ethyl acetate		yes	260	1.3720	0.45	0.45
Dioxane		yes	215	1.4220	0.49	1.54
Acetonitrile		yes	190	1.3440	0.5	0.37
2-Propanol		yes	210	1.3770	0.63	2.30
Methanol	↓	yes	205	1.3290	0.73	0.60
Water	polar	yes	-	1.3328	>0.73	1.00

   In summary, always start with acetonitrile as the modifier. Test your sample to find the range of acetonitirle-water compositions over which your sample is soluble. If a more polar modifier or a modifier that can hydrogen bond is useful, next try methanol.
   Many analytes are acids and bases. The charge state of acids and bases are strongly dependent on pH. A neutral molecule typically has much longer RP retention times than an anion or cation, therefore the reproducibility of retention times of acids and bases depend strongly on pH. It is then recommended to always buffer the mobile phase.
   Eluants with ammonium acetate or phosphates are like ringing the dinner bell for bacteria. These buffers can go bad in just a few days if they have less than 20% acetonitrile or methanol. Therefore, highly aqueous mobile phases should always be made up fresh, just before you start. Always refrigerate acetate and phosphate aqueous buffers.
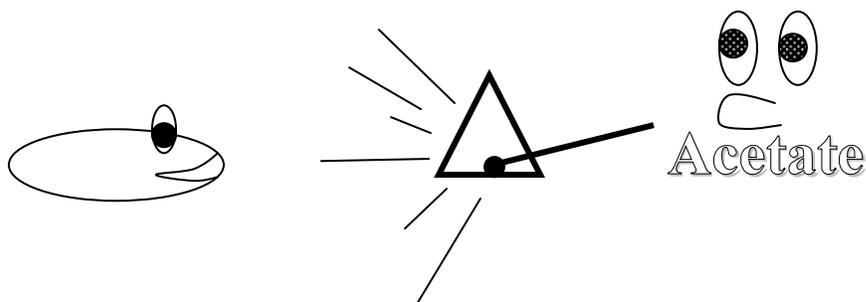
Ringing the dinner bell for bacteria!

- **Isocratic verses Gradient Elution**

In an isocratic separation the composition of the mobile phase remains constant. It is often difficult to determine the best mobile phase composition for all the components in a complex sample. The non-polar components may separate well in 70:30 methanol and water, but then the polar components often elute together in the void volume. Using a highly aqueous mobile phase may separate the polar components well, but then the non-polar compounds have long retention times or remain at the top of the column and don't elute at all. In a gradient separation the concentrations of the mobile phase components are varied during the separation. For example, a typical gradient is to start at 10% acetontitrile in water and then increase the concentration of acetonitrile linearly over a period of 20 minutes to 90%. The purpose of the gradient is to provide a better separation for all the components in your sample in a shorter period of time than possible in an isocratic run.

   In constructing a gradient, you start with a weak solvent. In reversed phase chromatography, the starting solvent is usually water. Water is a good solvent for ionic and polar compounds. Water also interacts very weakly with the solid phase. The second component is always a stronger solvent for your sample, and/or a solvent that interacts more strongly with the packing. For reversed phase work, the second component is usually acetonitrile or methanol. The polarity and the solvent strength parameter, $\in_p$, and sample solubility are used to pick the organic modifier. Acetonitrile is the most common organic component for gradient formation, although acetonitrile and methanol mixtures are also being used. The physical basis for the action of a gradient is that as the organic composition increases, non-polar analytes have a greater affinity for the eluant and are carried faster through the column. In addition, solvents like acetonitrile interact strongly with the alkyl chains of the RP packing and can displace analytes from the surface of the column.

- **Separation Variables**

   Injection Volume: The injection volume to use is a critical issue. The larger the injection volume the higher is the sensitivity, up to a point. For best resolution, the injection volume should be 1/5th of the peak volume. See the Column Characteristics table in the appendix for peak volumes for different ID columns. An injection volume of 0.25 μL will do fine for 0.5 mm columns. Any larger and you will start to see band broadening. In isocratic operation, the detected peak volume can't be any smaller than the injection volume. However, the choice of injection volume is more complicated than this for gradient operation.

   Precolumn Concentration: If the beginning composition of the gradient has a weak elution strength for your sample, then your sample will interact strongly with the column packing and the sample will be held at the top of the column or guard column. This effect focuses your sample into a narrow band. The sample will remain stationary until the elution strength of the gradient increases sufficiently. Under these circumstances, you can inject significantly larger volumes of your sample without any additional peak broadening. The maximum injection volume in this mode is determined by the amount of sample that saturates the capacity of the column packing. This is best determined experimentally. In practical terms, you might be able to inject as much as 5 μL on a 0.5 ID column.

   Injection Loading Solvent: For isocratic elution, it is customary to dissolve your sample in the mobile phase or a solvent that has a similar elution strength. For gradient work you should

choose a solvent with as low an elution strength as possible, without running into solubility problems. The low elution strength of the loading solvent helps to focus your sample on the top of the column during the initial portions of the gradient, as discussed above for precolumn concentration.

   Injection Volume and MS: Mass spectrometrists often overload their columns. Putting too much sample on the column increases band broadening and under extreme circumstances will cause tailing on the leading edge of the peak. From a MS perspective, band broadening can actually be useful since broader peaks are easier to sample than very narrow ones. So wider peaks are fine as long as the peaks are well enough separated and as long as the detection limits are exceeded. So for LC/MS we often break the $1/5^{th}$ of the peak volume rule. However, for best detection limits with limited amounts of material, it is best to follow the $1/5^{th}$ rule.

● **Detection**

The most common detector for HPLC is a UV detector. Our system uses a diode array detector, which allows complete spectra to be acquired during the elution of each peak. Complete spectra can be useful in identifying the compounds that are in your sample. The critical parameters for the detector are the cell path length and volume. The longer the path length is the more sensitive the detector. However, longer path lengths mean larger cell volume. The detector cell volume should be at least 1/5 of the peak volume to avoid band broadening (i.e.10% increase in band width). In other words, you can spend a lot of time getting really good separations, but if your detector cell is too large you won't see the difference. The detector cell broadens the peaks for the MS also. So, having the detector cell in-line decreases the sensitivity of the MS.

   A table of Column Characteristics is given in the appendix, including approximate peak volumes for different ID columns. Smaller ID columns are used because they give smaller peak volumes. But as you choose smaller columns, you must also decrease the size of the detector cell. The detector cell on the Agilent 1100 diode array detector has a volume of 500 nL. The peak volume for a 0.5 mm ID column is 1.6 µL, which is ~3x the detector cell volume. Therefore, columns less than 0.5 mm will show significant band broadening, and you should consider bypassing the diode array if you want maximum MS sensitivity. The connection tubing also adds another 1.6 µL, which also should be considered.

   Wavelength limits: Your choice of mobile phase solvents and additives will limit the shortest wavelength that you can use. 2-Propanol, for example, absorbs strongly down to 210 nm. So if you are using an eluant with 2-propanol, you should choose 215 nm as the bluest wavelength for your analysis. A table of cut off wavelengths for common HPLC solvents is given is the Solvent Properties table, above. This table shows that water and acetonitrile are the solvents that give the broadest range of acceptable wavelengths for reversed phase chromatography.

   The reason for this limitation is the dynamic range of the detector. Typical UV/Visible spectrophotometers have a linear range up to about 2. At higher absorbances, stray light in the monochromator dominates the signal. This is the reason that absorbance bands appear to have a flat, noisy top when the absorbance exceeds 2. If the absorbance of your mobile phase is near 2, the detector will be insensitive to your sample peaks. This is the basis of the cut off wavelengths in the Solvent Properties table, above. Acetate salts, acetic acid, and formic acid absorb strongly down to 230 nm. So if you are using an eluant with these additives, you often choose 230 nm as the bluest wavelength for your analysis.

Intermediate cases are also common. For example, 0.1% formic acid has an absorbtivity of 0.920 in our instrument. This leaves 2.0-0.920 ≈ 1 left for your peaks. So if the peaks in your sample have absorbances less than one, they will be accurately recorded. Often for MS work, we don't need to know the absorbances accurately; we just need to know when the peak enters the MS. Under these circumstances we can get away with using 210 nm as a wavelength even in mobile phases with considerable absorbtion. We also often "overload" the column to increase the MS peak intensity, so flat, noisy top peaks peaks are common in LC/MS work.

Balance or Baseline Subtraction: If common eluants like acetate and formate buffers have strong absorption, then why don't you see these as peaks in the spectrum? The reason is that we use baseline subtraction to enhance the visibility of the spectrum of the components in our samples. At the beginning of a separation we take a spectrum to use as a baseline. This baseline spectrum is then subtracted from all subsequent spectra. When your sample elutes into the detector, the recorded spectrum is then the difference spectrum, which records the change in absorbance caused by your sample.

Baseline subtraction causes several artifacts that you will notice. Often the spectrum will show negative absorbances. Negative absorbances just mean that the current eluant absorbs less than the eluant at the beginning of the run. If you run gradient separations, the baseline of your chromatogram will usually show a gradually increasing or decreasing baseline. This is caused by the gradual change in the composition of the mobile phase, which can give rise to negative absorption in the recorded difference spectrum. For this reason, we usually try to keep the concentration of any additives the same in both eluants that are mixed to form the gradient.

Another artifact of baseline subtraction is that the spectrum at low wavelengths can be very noisy. This noise in the short wavelength UV is caused by the limited dynamic range of the detector. Remember that range of UV/Visible detectors extends only to about 2. If your mobile phase has a large absorbance, then baseline subtraction corresponds to subtracting two large numbers, giving a small result. The uncertainty in this small result is large, which is visible as baseline noise. If you see a lot of baseline noise, then you should know not to trust those wavelengths for monitoring your separation.

● **Degassing**

A significant amount of air can dissolve in eluants. This dissolved air can outgas and form bubbles in the head of the HPLC pump and stop the pump from working properly. As the pressure drops after the column, dissolved air can also form bubbles. Bubbles are often difficult to remove from the detector cell. There are several methods to remove dissolved gases from mobile phases. Pumping on the solutions after filtering can help. Sparging with helium is very effective, where sparging means bubbling helium through the solution. Our system uses a vacuum degasser, which is a very convenient and efficient way to degas. Teflon tubing is permeable to air. The eluant is run through a piece of Teflon tubing that is held in a vacuum chamber. The dissolved air diffuses through the Teflon and is pumped away.

The practical issue in vacuum degassing is that the Teflon tubing can have a considerable hold-up volume. This extra volume must be considered when purging the pump after a solvent change. You must purge long enough to ensure that the old eluant is washed out of the system. The hold-up volume for our degasser is about 2 mL, which requires at least 10 mL to purge the tubing inside. You also need to add at least as much for the tubing leading to the degasser and between the degasser and the pump.

● Pump

There are three ways to achieve the low flow rates necessary for capillary chromatography. The first is to use a syringe type pump. This option provides stable flows, but the system needs to be taken off line periodically to refill the syringe. The second choice is to use a standard style reciprocating HPLC pump with a small piston. These pumps can have large pressure fluctuations and reliability problems. Most capillary HPLC systems use a standard HPLC with a flow splitter. The split ratio on a flow splitter can be very pressure dependent, so setting the desired flow rate can be difficult. The Agilent 1100 pump uses a flow splitter to achieve the low flow rates for capillary columns, but the system also uses active flow control using a flow sensor. Active flow control is a very reliable method to achieve low flow rates. The disadvantage of any flow-splitting scheme is that you don't achieve the same solvent consumption savings that you would with a syringe pump. The main pump flow is still 0.2-0.5 mL/min, depending on the pump flow settings in the More Pump, Auxiliary… control panel.

Flow splitting also means that there are two places where the mobile phase goes. Most of the mobile phase goes to a waste reservoir attached to the pump. Only a small fraction of the mobile phase goes through the column and detector, ending in the column waste reservoir. It is often difficult to remember to empty the single waste reservoir in a normal HPLC. With flow splitting, without MS detection, there are two waste reservoirs to check. Spilling acetonitrile or methanol on the floor is very bad form.

Setting up the flow splitting also requires one more step. You need to set two flows: the micro-flow rate for the column and the flow rate for the pump before splitting. The main pump flow is 0.2-0.5 mL/min. Choose 0.2 mL/min if you want the best solvent consumption savings. Choose higher flows if you are running fast gradients. Faster main pump flows decrease the gradient delay time.

● Autosampler

Autosamplers are useful since multiple samples can be analyzed with unattended operation. However, several practical issues attend the use of autosamplers. First, each run requires 30-45 minutes, so your samples may be sitting in the autosampler for extended times. Therefore, for sensitive samples, you should choose to cool the sample tray. Secondly, some autosamplers require the sample vials to be capped. However, the Agilent 1100 Micro-Autosampler does not. Use a cap if you wish to avoid evaporation or you wish to store the residual sample in the sample vial.

The injection needle vertical position needs to be carefully controlled. For the flat bottom sample vials, a needle position of 0 mm should be chosen, if you want to access the full depth of the sample. The flat bottom vials require about 0.5 mL of sample. Conical bottom inserts are available for small volume samples. However, these inserts and the one-piece conical vials require a higher draw position to avoid having the injection needle damaged by hitting the bottom of the vial. Injection needles are expensive.

Sample carry-over is always a concern with autosamplers. Agilent autosamplers use a unique design that flushes the needle, sampling valve, and all the connecting tubing during each injection. However, a small amount of sample can still be carried on the exterior of the needle. You will probably want to use a needle wash to clean the outside of the injection needle to avoid

carryover to the next sample. A vial of a good wash solvent is placed in the autosampler tray, and the injector dips the needle in this vial after withdrawing your sample and before injection. You should choose a wash solvent that is a good solvent for your sample. For many samples, methanol or isopropanol are good wash solvents. If you wish to save time and aren't concerned about the small amount of carryover, just use a standard injection.

Chromatography Packings

- **Particle Size and Column Length**

The most common particle size for reversed phase chromatography is 5 μm. Ion exchange and size exclusion packings can be larger, 7-12 μm. However, the advent of LC/MS has intensified the search for higher resolution and shorter separation times. Shorter separation times give narrower peaks, because there is less time for diffusive broadening. Narrower peaks provide lower MS detection limits. The key to obtaining shorter separation times is to use shorter columns, but without much loss of resolution. Shorter, high resolution columns are possible with smaller particle sizes. It has been said that "the smarter the chromatographer the shorter the column." Newer commercial packings are available in sizes from 3 μm down to 1 μm.

Most LC/MS reversed phase columns use 3 μm packings. A 3 μm packing column is approximately equivalent to a column 50% longer. So a 3 μm 100 mm column is equivalent to a 150 mm column with conventional packing. The trade off is that smaller solid phases cause much higher back pressure. So a 3 μm 100 mm column requires as much or slightly more pressure as a 150 mm column with 5 μm packing. Smaller particle size columns are also more difficult to pack, however 3 μm and 5 μm columns typically cost the same.

Another disadvantage is that 3 μm columns require smaller pore size inlet and outlet frits, which are easier to clog. The use of a guard column or an inlet filter is mandatory for small particle size columns. Sample filtering is also mandatory.

A trend in LC/MS is to use the discrimination power of MS to resolve the components of poorly resolved chromatographic peaks. In other words, the use of very selective detectors like MS means that you can get away with incompletely separated peaks. The discrimination ability of MS allows you to purposely choose columns that are too short. The separation times are then very short, the detection limits are improved, and the throughput of the instrument is increased. You may be using 30 mm length columns or shorter in some of your analyses. Smaller particle sizes are necessary to maintain resolution in these very short columns.

Chromatography Modes

- **Reversed Phase, RP**

Chain Size: The alkyl chains for the stationary phase range from C1, that is methyl, to C30. C1 packings are used for very non-polar compounds and C30 packings are for strong retention of relatively polar compounds. C18 is the most popular for small molecule separations, while C8 is common for non-polar organics where C18 provides too much retention. In biological applications, C18 is good for peptides, but C4 is used for large proteins. Oligonucleotides are often run on C18 columns, although small oligos separate well on C8.

End Capping: Reversed phase silica based packings still have active silanols left after the alkyl chains have been attached. End capping reagents are often applied to block these sites. A common end-capping reagent is dimethyldichlorosilane, which provides dimethyl groups on the surface. Many types of end-capping reagents are used including butyl groups. Residual silanols on non-endcapped packings cause excessive peak tailing for amines and other basic compounds. End-capping reduces this peak tailing. End-capping is often called base deactivation. Brand names for these columns often include a DB: e.g. C8-DB.

Aqueous Compatibility: Reversed phase columns often are subject to "phase collapse" when used with highly aqueous eluants. A column suffering from phase collapse shows very poor retention. There are three types of reversed phase packing in reference to their use in aqueous solvents: compatible, tolerant, and incompatible. Compatible stationary phases can be used with 100% aqueous mobile phases. Such columns often have an AQ in their brand name. Aqueous compatible columns are not very common. Aqueous tolerant columns do show phase collapse, but they recover quickly when the mobile phase organic composition rises above 70-80%. Aqueous intolerant phases may require overnight contact with 70-90% organic eluant to recover.

One explanation of phase collapse is that the long alkyl chains on the surface tangle and collapse onto the surface of the particle, which precludes interactions with the analyte. Recent research offers a better explanation. The micropores of the solid phase are lined with hydrophobic alkyl chains that are not easily wet by aqueous solutions. As the aqueous concentration increases the surface tension of the mobile phase increases. As the surface tension increases, the interior of the micropores are no longer wet by the mobile phase and the mobile phase cannot enter the micropores. The polar groups that are inserted in aqueous compatible packings allow for hydrogen bonding with water that provides a stronger interaction with aqueous mobile phases.

Strongly base deactivated, or end-capped, packings are never aqueous tolerant. Aqueous tolerant packings often have a polar linkage, such as amide, carbamate, or ether groups, inserted into the C18 chain close to the packing surface. Very polar molecules often elute in the void volume on normal C18 columns. Aqueous compatible columns are used to provide some retention for these very polar substances. Aqueous compatible columns are popular for peptide separations, since many peptides are very polar and require gradients starting at 0% organic for good separation.

The aqueous tolerance of your column is important, because it determines what the gradient range can be for your separation.

Gradient Range: Most reversed phase columns are restricted to a gradient range of 10%-95% organic. Too much water causes phase collapse. Too much organic can also cause problems. Reversed phase materials have a monolayer or so of adsorbed mobile phase on their surface. Some authors claim that the water in this adsorbed layer dominates the mechanism for the separation. Running your column in 100% organic removes the water from this layer and ruins your separation. Running the column in 40-50% organic for an hour or so will recover the column, however. Aqueous compatible columns, like Aquasil C18, can safely be run with a 0%-95% gradient.

pH Range: Silica based packing have a pH range of 2-8. Basic solutions slowly dissolve the silica in the packing. If much silica dissolves, the bed will shrink and produce a void at the top of the column. This void will cause band broadening, and in extreme cases can cause double peaks. For normal use, pH=7.5 is a good practical limit.

Low pH causes the hydrolysis of the silyl-ether bonds to the hydrophobic chains on the surface of the packing. This hydrolysis produces free silanols, Si-OH groups that can interact strongly with analytes and cause band broadening. Therefore, you should not let a reversed phase column sit in acid overnight. See the table in the appendix for some approximate pH values for common eluants.

In some cases, very basic proteins for example, better separations are possible at pH>8. Columns with extended pH range are available for these separations. Many of these columns are based on polymeric supports instead of silica (e.g. divinylbenzene-polysytrene copolymers, DVB/PS). Such columns can be used over the pH=0-14 range. New extended range silica columns are also available that use end-capping methods to protect the silica surface and can be used over the pH=1-10 range.

To be safe, always assume a 2<pH<7.5 range for your column.

Pore Size: Chromatographic supports, silica and polymeric, have pores in a range of sizes. Pores can be macropores and micropores. Most commonly used packings are macroporous. That is they have large pores that allow analytes to diffuse into the particle interior. Macropores are important because they increase the available surface area. LC supports also have micropores and the size of these pores has an important effect on the selectivity of the separation for different sized molecules. The micropore size typically ranges from 80 μm to 300 μm.

Small pore sizes favor small molecules and exclude large molecules. Small molecule columns use pores in the 80-100 μm range. Columns for peptide separations use 100 μm – 300 μm micropores. Protein separations require 300 μm packings. Aquasil C18 columns are useful for small molecules and peptides and have 100 μm pores. Zorbax SB columns have a pore size of 80 μm.

The surface area of the support increases with decreasing pore size. Therefore, to enhance retention of small moderately polar molecules the use of small pore size packings is necessary. 300 μm packings don't provide sufficient retention for small molecules.

● Ion Pairing

The formation of ion pairs allows the use of reversed phase columns for very polar substances. An ion pair is a loosely associated cation and anion. For example if your analyte is a cation, adding an anionic ion paring reagent, P^-, corresponds to:

$M^+ + P^- \rightarrow [M^+ P^-]$

The ion pairing reagent often has a long hydrophobic chain to provide strong interactions with a reversed phase packing. Basic compounds, such as amines, form cations in acidic and neutral solution. Acidic compounds, such as carboxylic acids, form anions in neutral and basic solution. Typical ion pairing reagents for analyzing basic compounds are: sodium docecylsulfate (SDS) and sodium decane, heptane and octanesulfonates. Ion pairing reagents for acidic compounds are often quaternary amines like hexadecyltrimethyl ammonium bromide, and tetraethyl, tetrabutyl, tertrahexyl, and tetraoctyl ammonium salts.

Large concentrations of ion paring reagents are usually not used in electrospray mass spectrometry, because of potential clogging of the inlet capillary and high intensity background ions caused by the ion pairing reagent. However, trifluoroacetic acid, TFA, is a very common ion pairing reagent used for basic compounds, especially proteins and peptides. TFA is very volatile, which causes no problems with electrospray ion sources. Acetic acid, formic acid, and phosphoric acid are also used as ion pairing reagents, but these small anions don't improve

retention as much as the reagents with hydrophobic side chains. Phosphoric acid is not compatible with electrospray.

Trifluoroacetic Acid: TFA acts in two ways. Chromatographic retention is improved by the ion pairing mechanism. Chromatographic resolution is improved by blocking the residual active silanols on the solid phase surface. Reversed phase silica based packings have a small concentration of active silanols. End capping reagents are often applied to block these sites, but a few still remain. These active –OH groups can ionize to give –O$^-$ sites that interact very strongly with proteins. TFA is a strong enough acid to protonate the silanol –O$^-$ groups, which decreases protein-solid phase interactions. Less solid phase silanol interaction gives better resolution (less band broadening) and better total protein recoveries.

TFA and MS: Unfortunately, TFA is very effective at suppressing ionization in the electrospray source. The reason is that it is difficult to separate the analyte-reagent ion pair to produce ions for the MS. New low-TFA columns have been developed specifically for protein and peptide separations that require much smaller concentrations of TFA. Conventional columns use 0.1% TFA. The new MS compatible reversed phases require as little as 0.02% TFA. In some cases, TFA may be replaced entirely by acetic or formic acid at 1.0%-5.0%, but in practical applications it still appears better to stick with some TFA.

TFA Mobile Phases: TFA has a higher molar absorbtivity in acetonitrile solution than in water. Therefore, it is traditional to make up an acetonitrile mobile phase with 80% of the concentration for TFA in the aqueous eluant. A ommon example is 0.02% TFA in water and 0.016% in acetonitrile. Aquasil C18 columns are designed for low TFA use and are aqueous compatible. This column works well at 0.02% TFA.

Storage: Reversed phase columns have a limited pH range. For pH≤ 2, long exposure will hydrolyze the silyl-ether bonds to the hydrophobic chains on the surface of the packing. Therefore, you should not let a reversed phase column sit in acid overnight. See the table in the appendix for some approximate pH values for common eluants. The pH of even 0.02% TFA is 2.7. It is advisable not to let your column sit in 0.02% TFA or 0.1% acetic acid for long periods.

Flushing: Ion pairing reagents, by their nature, interact strongly with the stationary phase. Therefore, it takes a long time to wash ion pairing reagents from a column when you want to change eluants or flush for overnight storage. The normal 5-column volume rule for flushing usually won't be sufficient. Let's assume that you are using a 0.5mmx150mm column. The table in the appendix lists the column volume as 30 µL. Therefore, 15 column volumes at 10 µL/min will require 45 minutes. Remember that it is your responsibility to make sure the column and system is in good shape for the next user. So you should allow sufficient time to make sure your column is well-flushed and the system is ready for the next user.

● **Other Chromatography Modes**

Reversed phase is the most common mode for separations. However, for polar molecules, ions, and very hydrophobic analytes, reversed phase separations may not work. Normal phase chromatography uses unmodified silica for the packing and works well for small, polar molecules. However, biomolecules tend to bind to normal phase silica packings irreversibly. Ion Exchange, IEX, is used for separations of ionic compounds. Size Exclusion, SEC, separates molecules on the basis of molecular weight. Hydrophilic Interaction Chromatography, HILIC, is useful for polar molecules. HILIC is like normal phase chromatography, but works well for

biomolecules. Very hydrophobic compounds can be separated by Hydrophobic Interaction Chromatography, HIC.

Most of these modes require mobile phases with high salt concentration, so extra caution is required to protect the pump from damage. These alternative chromatography modes are covered in a separate tutorial. You must be separately licensed to use these modes on our system. Please don't try these other types of columns unless you have been licensed to use them.

Eluant preparation

There are two enemies when preparing HPLC eluants. The first are chemical contaminants and the second are small particles. Small particles will clog your expensive HPLC column and make it useless. Always follow the procedures below to avoid contamination of the HPLC and MS. Contaminates introduced from bad solvents and dirty glassware can take days or weeks to remove from the system. Good citizenship is required to keep from harming your research and the research of others. Be constantly aware of your work to be sure you don't introduce contaminants or particles into your samples and HPLC eluants.

● **Solvent Purity**

Always use HPLC grade solvents. Use the highest purity available for any buffer components or ion pairing reagents. Acetic and formic acids are best purchased in plastic containers. Trifluoroacetic acid should be purchased in snap-cap glass vials. Never stick a pipetter or pipet into a bottle of solvent. Pour the solvent directly into your graduated cylinder or use a clean beaker. Don't use solvents that have an unknown use history, the previous user may not have been careful.

Use filtered, reagent grade water stored in glass containers or directly from the Milli-Q system. Don't store water in plastic. Wash bottles are OK if they have been used for a long time, but avoid their use if possible. The plastic containers have plasticizers in them that can provide persistent background ions (391 Da is common for phthalate plasticizers).

● **Glassware**

Keep your glassware for making up eluants separate from other general use glassware. Use glassware that has only been used for HPLC eluant preparation. If you must use glassware with an unknown history, use the following cleaning procedure.

Cleaning Procedure for HPLC glassware.
   Wash with <u>lab grade</u> detergent and a clean brush. Rinse three times with very hot water. Rinse with 20% acetic acid. Rinse with 25:25:50 cyclohexane/acetonitrile/isopropanol. Rinse twice with HPLC grade methanol and three times with reagent grade water. Use reagent grade water stored in glass containers or directly from the Milli-Q system. Cover with plastic film

Rinsing Glassware
   HPLC grade solvents are expensive, and reagent grade water is also expensive to produce. You will be using HPLC grade solvents for your rinsing procedures. Several small portions

of rinse are much more effective than one big rinse. So, using three small rinses instead of one big rinse saves on expensive solvent and produces better results. Even 1-L bottles require less than 5 mL per rinse, so rinsing does not require large amounts of solvents. In rinsing, add a small amount of solvent and then swirl the bottle to wet the bottom, and then tilt the bottle on its side and rotate to wet the entire interior surface. Don't forget to rinse the bottle cap, too. Organic solvents should not be poured down the drain. You should collect your organic rinses for proper waste disposal. Cutting down on rinse volume saves a lot of money on disposal costs.

Teflon Bottle Cap Liners
Be careful to notice the liner of your bottle caps. Paper liners can be a source of contaminants that are difficult to track down. Teflon or polypropylene liners are best. Some plastic liners contain plasticizers that leach into solution. If you aren't sure of your bottle cap liner—don't use it.

Keep all particle free glassware covered with plastic film. Don't use plastic film for non-particle free glassware to avoid confusion. If you use your clean glassware with HPLC grade solvents, just recover with plastic film. Glassware used for TFA, acetic acid, formic acid, and ammonium salts should be rinsed three times with reagent grade water and then recovered with plastic film. Use of other mobile phase additives may require more extensive cleaning procedures, consult with your advisor. Lets complete this section with a short caution on plastic film.

Plastic Film
Don't use parafilm above solutions that contain an organic solvent. Parafilm is soluble in acetonitrile. Dissolved parafilm can be very difficult to clean from the system. Also, rubber stoppers and rubber septa can also cause gross contamination of your samples and the HPLC.

● **Filtering**

All HPLC mobile phase eluants and samples should be filtered through 0.2 μm membrane filters. There are several types of materials for membrane filters. All of the standard solvents and TFA, acetic acid, formic acid, ammonium formate, and ammonium acetate based eluants should be filtered through <u>regenerated</u> cellulose or polypropylene membrane filters. Some more aggressive mobile phases may require Teflon membrane filters. Consult with the Solvent Compatibility chart in the appendix and your advisor if you use an unusual eluant <u>before</u> you begin filtering. Do not use hexafluoroisopropanol with nylon or standard Teflon membranes.

Don't use membrane filters from unlabeled boxes; verify that the filter is made from the proper material and has the correct pore size. Notice in the Solvent Compatibility chart that the different kinds of cellulose have very different properties. Cellulose acetate and polycarbonate based membrane filters are common in laboratories and are not suitable for HPLC eluant and sample preparation. Handle membranes with forceps.

Use a vacuum filtration apparatus that has been cleaned using the procedure listed above in the glassware section. The filter bed should be made from fritted glass or stainless steel screen. Büchner funnels are never acceptable. Polypropylene is hydrophobic, which means that a small amount of methanol or acetonitrile might be necessary to wet the filter before you can filter

100% aqueous samples. If you are filtering water, acetonitrile, or methanol mobile phases with no additives you may reuse the membrane filter; just leave the filter in the filtration apparatus.

If you are filtering just HPLC grade solvents, you don't need to wash the filtration apparatus after use, just recover the vessel and side-arm with plastic film. After filtering mobile phases with TFA, formic, or acetic acid, and their ammonium salts, just rinse three times with reagent grade water and recover with plastic film. If your mobile phase additive has an amine or other component that is not very water soluble, rinse the apparatus with several small quantities of a compatible solvent (e.g. isopropanol), followed by two small methanol rinses. When rinsing use the aspirator to pull your washes through the fritted glass or stainless steel screen.

Column Conditions

- **Flow rate**

The Column Characteristics table in the appendix lists flow ranges and optimum flows for HPLC columns with different diameters. Good starting flow rates are 50 µL/min for 1-mm columns, 10 µL/min for 0.5-mm columns, and 5 µL/min for 0.3-mm columns. In terms of resolution, there is less of a penalty for running too fast than too slow. For example, for 0.5-mm columns, don't go below 10 µL/min and do try 12-16 µL/min to optimize the detection limits for the MS.

The upper flow limit is determined by the back pressure. Please don't go above 140 bar (2000 psi). Connections may begin to leak above this pressure, unless you are very careful and haven't over- or under-tightened the tubing connections.

- **Temperature**

In general increasing the temperature of the column increases resolution. As a consequence, separations are often run at 40-50°C. Running the column in a thermostated column compartment also provides better reproducibility of retention times; you get consistent separations from day to day. However, care must be taken not to exceed the maximum temperature for your column. Silica reversed phase columns can have maximum temperatures of 90°C. Higher temperatures increase the rate of hydrolysis of the silyl-ether bonds to the alkyl chains on the surface of the stationary phase. This hydrolysis is accelerated by low pH. Peptide separations, which are often done with 0.02% trifluoroacetic acid, TFA, are at pH 2-3, and therefore 50°C is the normal operating temperature.

The mechanism for the effect of temperature has two parts. The first is thermodynamic, in that the Gibbs Free Energy of solvation of the mobile phase for your analyte and the partition coefficient of the stationary phase for your analyte both vary with temperature. The second is kinetic, in that the analyte has enhanced diffusion in the pores of the stationary phase with increasing temperature. The net effect is usually that retention times are shorter and the resolution is increased with increasing temperature.

- **Gradients**

Gradients can be linear or non-linear, however, linear gradients are most common. Gradients can also have multiple segments. The most typical solvent program is in four segments. The first is a

short isocratic hold at the starting composition, to attempt to separate the polar molecules well. The gradient is usually formed in segments two and three, with segment two a shallow gradient and segment three a steep gradient. The shallow gradient optimizes the separation of complex mixtures and the steep gradient rapidly elutes any highly retentive contaminants. The fourth segment is a final isocratic hold, which is designed to clean the column before returning to the initial composition.

It is advisable to end your gradient at high organic, typically 80-95%. In doing so, your column will be kept cleaner. Otherwise, very non-polar compounds may foul the column and decrease the resolution of your separation. The starting and ending compositions will depend on the column chemistry; please see the section below for the discussion of the different types of reversed phase columns.

The two components of the gradient are often called Buffer A and Buffer B, where Buffer A is mostly aqueous and Buffer B is mostly organic. There are two choices for setting up Buffer A and Buffer B. The most convenient is to make Buffer A just water and Buffer B just organic, i.e. acetonitrile. The second choice is to make up Buffer A at the starting composition of the gradient, e.g. 10% acetonitrile, and Buffer B the final concentration, e.g. 90% acetonitrile. This second choice has three advantages. The first is that all your gradients will run from 0% B to 100% B; you don't need separate method files for different gradients. The second advantage is that bubble formation is minimized. When the two eluants are mixed in the pump, out gassing is likely, leading to bubbles that can cause erratic baselines in the detector cell. Starting with the solvents at least partially mixed makes out gassing less likely. The third advantage is that keeping some organic in Buffer A discourages bacterial growth that may foul the pre-column or pre-column filter. As a consequence, many literature procedures use mixed A and B buffers. However, with vacuum degassing, bubble formation is minimized and as a consequence 0% B and 100% B are now quite common. You will need to insure that the aqueous phase is fresh however, especially if buffer salts are added.

Gradient Delay Time: The mobile phase takes awhile to travel from the flow splitter to the top of the column. This delay is called the gradient delay. The volume of the tubing, the injector needle, the injector valve, and the column-switching valve determine the gradient delay. Even if the gradient program has no isocratic hold at the beginning, the gradient delay produces an isocratic hold. Therefore, as you are planning your gradient you need to add the gradient delay to any isocratic hold that you specify to find the total effective isocratic hold. The gradient delay is inversely proportional to the flow rate. Slower flow rates produce longer gradient delays, so gradient delays are much more important for capillary HPLC than normal HPLC. The gradient delay using a 10 µL/min flow rate on our system is about 4 minutes.

You may not want an initial isocratic hold, or at least you may want a hold shorter than the gradient delay. To get around this problem, the autoinjector may easily be programmed to avoid the problem. The easiest way is to delay injection of your sample for several minutes after the gradient starts. A more elegant way is to bypass the injection syringe after the sample is injected. Most of the delay volume is in the injection syringe, so this method greatly decreases the system gradient delay. The disadvantage of bypassing is that you must be careful about timing the bypass. For our system with 0.5 mm columns and larger, it is best to just delay the injection. Several trial runs will be necessary to set the timing for bypassing if you are using 0.3-mm columns.

Post-Gradient Equilibration: After the last segment is complete, the composition of the mobile phase must be returned to the starting composition. This is usually done using a quick linear

ramp. Sudden changes in the composition of the mobile phase can cause large pressure fluctuations. When you set up your method, you specify a maximum pressure to avoid loosening tubing connections or worse damaging the system. When the pressure exceeds this limit, the pump is turned off. Returning the compositon of the gradient with a ramp avoids exceeding the pressure limit. A 2-5 minute ramp works well.

   After the gradient returns to the starting composition, the column isn't ready to use again. The column must equilibrate for a short time, to ensure that you get consistent separations from run to run. A general rule is to allow at least 5 column volumes to flow through the column. Let's assume that you are using a 0.5mmx150mm column, including the length of the precolumn. The table in the appendix lists the column volume as 30 µL. Therefore, 5 column volumes at 10 µL/min will require 15 minutes. Most chromatographers (once you get some experience you will be a chromatographer, too) prefer to allow 10 column volumes, if there is enough time.

Sample Preparation

● **No SDS**

Sodium docecylsulfate, SDS, is a commonly used anionic surfactant in biochemical preparations and electrophoresis. The presence of SDS, even at trace levels, can severely depress ion formation in electrospray MS. Therefore, SDS should be completely avoided. SDS is very difficult to remove from solution and can be carried through multiple sample treatment steps. If you must use SDS, there are several ways to remove SDS from solutions (in order of preference):

> SDS removal cartridges (Michrom, PolyLC)
> Off-line HPLC using HILIC or reversed phase with an SDS trap guard column[1]
> ZIP tips (Millipore[2], Amika, and other suppliers)
> Solid phase extraction, SPE, cartridges

(The ABRF Web site has an excellent discussion of SDS removal techniques: abrf.org/ABRFNews/1997/December1997/dec97detergent.html).

   SDS is very difficult to remove from HPLC columns. Backflushing with ethanol and isopropanol are useful, but may require several days at low flow rate to achieve sufficiently low levels for use with MS.

● **No nonionic detergents**

Non-ionic detergents, NIDs, are even worse for compatibility with MS. Common NIDs, are Tween 80, Triton X-100, and ND40. NIDs are often used in the early stages of preparation including cell lysis. However, they persist through many stages of purification and can cause very large clusters of peaks in the MS that completely obscure your peaks of interest. NID residue is almost impossible to remove from C18 columns. Treatment with 10% SDS is one of the few ways to do this, but then the SDS is almost as difficult to remove. NID removal cartridges are available (Michrom), but are expensive. The best choice is to avoid NID use altogether.

● **Filter through 0.2 μm membrane**

Use a 0.2 μm membrane filter cartridge to filter your samples. Use the same care in cleaning the syringe that you use for filtering as you do for other HPLC glassware. Pull your sample into the syringe, then attach the membrane filter, and slowly expel your sample through the filter cartridge into a small beaker, sample vial, or centrifuge tube.

Filter cartridges may be reused, if you rinse them well between uses and work with similar samples at moderate concentrations. However, reusing filters will cause cross-contamination if you are working with very dilute samples or samples that are poorly soluble (like proteins).

Solvent and sample chemical compatibility with the membrane and the cartridge housing are very important. Common cartridge housings are made from polypropylene and nylon. Use the Solvent Compatibility chart in the appendix to decide which materials work well for your sample. Regenerated cellulose membranes with polyproylene housings are the best all purpose choice, followed by polypropylene membranes and housings. Polypropylene is hydrophobic, which means that a small amount of methanol or acetonitrile might be necessary to wet the filter before you can filter 100% aqueous samples. You must also be careful to choose a membrane that will not adsorb your sample.

Membrane Filter Selection

Type	Features	Hydrophilic	Solvent Resistance	Protein Binding
Regenerated Cellulose	Maximum protein recovery	Yes	High	Very low
Polypropylene	Low protein binding	No	High	Low
Polyvinylidene Fluoride	Good flowrate	Yes	High	Low
Nylon	General Filtration	Yes	Good*	Medium
Teflon, PTFE	Highest solvent resistance	No	High	High
Cellulose acetate	Aqueous only	Yes	Poor	Very low

* no chloroform, methylene chloride, hexafluoroisopropanol, DMSO, many acetates, and pH<2.

Nylon membranes have a moderate ability to adsorb proteins and a very strong ability to adsorb DNA and RNA, so be careful not to use nylon for most biochemical samples. Nylon also contains small amounts of plasticizers that can leach into your samples. The membrane you choose should not leach extractables, since these will cause peaks in the MS. Regenerated cellulose and polypropylene are very low in extractables.

Regenerated cellulose works best for proteins. Remember that there are several common types of cellulose filters in the lab; use only <u>regenerated</u> cellulose for normal use.

● **Yes, filter every sample**

You will end up destroying your column if you don't filter your sample.

● **Use a Guard column**

Guard columns are short HPLC columns filled with the same packing as your analytical column. Guard columns can use the same particle size as the main column or larger size packings- up to pellicular packings. Pellicular packings have diameters of 15-30 μm. Guard columns remove

particulates that might otherwise clog the inlet frits on your analytical column. Guard columns also prevent strongly absorptive contaminants from fouling the top of your main column. Guard columns are much cheaper to replace than analytical columns. Guard columns are highly recommended.

   The dead volume for your guard column should be small compared to the peak volume for your main column (see the table in the appendix for peak volumes), so care is required in selecting a guard column to avoid excessive band broadening. That is why guard columns are chosen to have the same ID as the main column. The guard column length can vary from 1mm to 30 mm. Sometimes guard columns are built into the analytical column. Usually guard columns screw directly into the analytical column or are attached with a short narrow–bore piece of connecting tubing.

● **Or Use a Pre-Filter**

Pre-column filters can be used if guard columns are not available. They prevent particles from reaching the analytical column, but they don't provide chemical protection. The hold-up volume for your guard filter should be small compared to the peak volume for your main column (see the table in the appendix for peak volumes), so care is required in selecting a pre-column filter to avoid excessive band broadening.

Column Care

• **New Columns**

When you receive a new column, before running anything else, you should always run a reference chromatogram. This reference chromatogram is useful for verifying the performance of the new column. But more importantly, you should test your column periodically to ensure it is still working well. If the performance of the column does deteriorate, use the cleaning methods suggested below to renew the column. It is also a good idea to run reference chromatograms for the guard column and the main column separately. In this fashion you can isolate problems quickly later on. In addition, some guard columns are long enough that they can be used by themselves for fast separations. Testing the guard column separately will tell you if this is possible.

   Most columns come with a test chromatogram run by the manufacturer. Make sure to keep this chromatogram on file for later reference. Also, if you need to return a defective column, the manufacturer will want you to send a copy of this test chromatogram.

   Different manufacturers use different test mixes. For your reference mixture try to reproduce this test mix. Alternatively, or in addition, running a mix of methyl, ethyl, and propyl parabens is very diagnostic for reversed phase columns. C8 and C18 RP columns should be able to base line resolve the parabens in isocratic 70:30 methanol or acetonitrile. If the extra-column volume before the column is large, however, you may need to run a gradient from 10% to 80% organic to get a good separation. Make up an approximately $1x10^{-5}$M solution of methyl, ethyl, and propyl 4-hydroxybenzoate in 5% methanol for the "paraben" test mix. Remember to keep the injection volume small.

- **Cleaning by injection**

MS detection is very sensitive to contaminants. Contaminants can also decrease the efficiency of HPLC columns. Frequent rinsing of HPLC columns is vastly superior to full-scale, emergency cleaning when the resolution deteriorates. For reversed phase columns make sure to inject a good wash solvent every 5-10 injections or so. You will need to determine which solvent is best for your sample <u>and its impurities</u> before you start your first HPLC run. Isopropanol is probably the best overall choice. However, if isopropanol isn't a good solvent for your sample, try methanol, ethanol, cyclohexane, and methylene chloride. If you use cyclohexane or methylene chloride make sure to run at 90-95% organic as the eluant so the solvents will be miscible. Methylene chloride can also swell and weaken PEEK tubing and should be avoided if you are using PEEK tubing in your system. For proteins or oligonucleotides, methanol and 20% acetic acid can be used alternately. For stubborn samples, see the following table for solvent mixtures. Remember to use only <u>HPLC grade</u> solvents.

Solvent mixtures for column cleaning

25% cyclohexane, 25% acetonitrile, 50% isopropanol	good for phthalates (Agilent)
20% acetic acid, 30% water, 50% isopropanol	good for proteins
5% trifluoroethanol in 50:50 acetonitrile/H_2O	good for proteins (Vydac)
50% isopropanol, 50% methylene chloride	can attack some stainless steel parts

- **Multiple quick gradients**

Another commonly used and highly recommended technique for keeping your columns clean is to periodically run several quick gradients using your current eluants. For example, if your gradient usually takes 20 minutes, changing the gradient time to 5-10 minutes and running a good wash solvent as a sample can be very helpful. Use the maximum volume for your injector (8 µL for our system). You should periodically clean your columns, at least weekly or daily under conditions of constant use.

- **Overnight flushing**

Overnight flushing with a high organic eluant is also helpful for columns that are beginning to loose resolution. 70:30 methanol-water is good for this purpose, or for very contaminated columns change your eluant to 100% isopropanol. Use a flow rate that is 1/10 your normal flow rate. Because your column will be in contact with this solvent for a long time, it is best to not use any acidic or basic additives. You should also keep the column temperature at 40°C or lower to prevent hydrolysis of the stationary phase. Switch the pump from Micro Flow to Normal Flow to conserve on eluants. Remember to return the system to Micro Flow before doing any chromatography or before the next user starts.

- **Backflushing**

For strongly contaminated columns, backflushing is the best method. Running the eluant in the wrong direction used to be strongly discouraged. However, with currently used packing procedures, periodic backflushing is very helpful. For strongly contaminated columns, backflushing is certainly better than throwing the column away. The reason that backflushing works so well is that contaminants are often strongly adsorbed at the top of the column. Backflushing these contaminants prevents these materials from having to travel all the way through the column. Proteins are particularly susceptible to clogging the tops of reversed phase columns, and backflushing weekly is a good procedure. Backflushing is usually best done with several solvents applied one at a time. Alternating between solvents is very helpful. For example, 70:30 methanol, 20% acetic acid, methanol, isopropanol, methylene chloride, isopropanol, and finally 70:30 methanol is a good scheme. The mixed solvents listed above can also be used to great advantage.

The best way to implement backflushing with multiple solvents, is to have a separate pump set-up with 70:30 methanol. Then the other solvents can be flushed through the column using an injector set up with an injection loop with a volume corresponding to 3-5 column volumes. See the appendix for a table of column volumes. The column volume for a 0.5 mm x 150 mm column is 30 µL, so an injection loop of 150 µL will work well.

Instrument Setup

- **Tubing**

Yes we even need to worry about tubing and connections. The proper tubing and properly assembled connections can make the difference between good chromatography and very bad chromatography. Mishandling tubing connections can also cost a lot of money.

There may be three types of tubing in use on the system:
- o PEEK Coated Fused Silica Tubing: This is 1/32" OD PEEK tubing that has a core of fused silica capillary tubing. The ID's and the color codes are listed in the Tubing Internal Volume chart in the appendix. This is elegant tubing to use; it has a very small internal volume, excellent chemical resistance, and low protein adsorption. It is also easy to make pressure-tight connections. But this fused silica lined tubing can have some drawbacks. If you bend the tubing too sharply, you can break the inner fused silica, which will cause a leak and band broadening that will be very hard to detect. If you over tighten the fittings, you can also crush the fused silica. You can sometimes detect a break by running your fingers along the tubing to feel for a bump. This tubing must be purchased in fixed lengths. It cannot be cut in the laboratory.
- o PEEK tubing: This is 1/16" OD PEEK tubing throughout. This tubing comes in a wide range of ID's (see table). It can be easily cut, using the special tubing cutter. 1/16" PEEK tubing is the most robust tubing. However, it is less flexible than fused silica and PEEK coated fused silica.
- o PEEK capillary tubing: Capillary tubing is now available that is equivalent to fused silica in OD and ID. This tubing is very flexible and easy to use. Connections to capillary tubing are made using special fittings and sleeves that are specific to the OD of the tubing and the ID of the connector. So you need to be careful about selecting the correct sleeves. PEEK capillary tubing kinks easily, so be careful not to introduce flow restrictions from the kinks. PEEK fused silica is easy to cut with a razor blade, however, the cut may block the bore of the tubing causing high backpressures. The best method for cutting PEEK capillary tubing is to slip on a "silica seal" sleeve and then cut the sleeve and tubing using the 1/16" PEEK tubing cutter. Make sure to test your piece of tubing for blockages before you assemble the system.

Some solvents (like methylene chloride, THF, and DMSO) swell PEEK during long exposure; so if you use these solvents make sure the contact time is very short. More importantly, some classes of proteins may strongly absorb to PEEK. There are arguments as to whether fused silica or PEEK are worse for protein absorption.

Making connections to PEEK Coated Fused Silica Tubing is the trickiest, since the fused silica core may crack or break without you knowing it. To tighten PEEK coated fused silica, turn the nut until you just start to feel resistance. Then turn the nut until it feels snug; but don't turn it more than $1/16^{th}$ of a turn. Test your connection by pulling on the tubing. If the tubing lets go, loosen the nut, make sure the tubing is seated in the connector and then tighten again, this time turning the nut just a little more. Continue this careful tightening procedure until the tubing doesn't pull out of the connector anymore. This being said, you will find PEEK coated fused silica very easy to use and remarkably easy to make good high-pressure connections.

Testing for Leaks: Testing for leaks can be difficult in capillary HPLC since the flow rates are so low. It may take 15 minutes or more for leaks to show up. The easiest way to check for leaks is to insert the tip of a ChemWipe into the cavity of the connector, next to the tubing. Check the ChemWipe to see if it is wet. If you need to tighten, don't turn the nut more than $1/16^{th}$ of turn. Then check for leaks again. If you feel like you need to apply a lot of force, then something is wrong, and tightening any further will just make things worse. Just stop at this point, before frustration sets in and get help.

Pressure Conversion

Psi	Bar
500	35
1000	69
1500	103
2000	138
2500	172
3000	207
3500	241
4000	276
4500	310

Eluant pH

Ion Pairing or Buffering	Concentration	~pH
Trifluoroacetic acid, TFA	0.1%	2.0
Trifluoroacetic acid, TFA	0.02%	2.7
Acetic acid, CH_3COOH	0.1%	2.2
Acetic acid, CH_3COOH	0.05%	3.1
Formic acid, HCOOH	0.1%	2.7
Sodium dihydrogen phosphate, NaH_2PO_4	100 mM	4.4
Triethylammonium phosphate, TEAP		6
Ammonium Acetate		6-7

Column Characteristics

Column ID (mm)	Flow Range	Flow Range (μL/min)	Optimum Flow (μL/min)	Analyte Capacity (grams)	5 cm Bed Vol (μL)	15 cm Bed Vol (μL)	Injector Volume[+] (μL)	Peak Volume[#] (μL)
4.6	Standard	500-3000	1.25	10^{-4}-10^{-8}	830	2500	30	140
2.0	Microbore	100-1000	200	10^{-5}-10^{-9}	160	480	5	26.4
1.0	Microbore	20-200	50	10^{-6}-10^{-10}	40	120	1	6.6
0.5	Microbore	5-50	12	10^{-7}-10^{-11}	10	30	0.25	1.65
0.3	Capillary	2-20	5	10^{-8}-10^{-12}	4	11	0.128	0.64
0.2	Capillary	1-10	2	10^{-9}-10^{-13}	2	5	0.057	0.28
0.10	Nanoscale	0.25-2.5	0.5	10^{-10}-10^{-14}	0.4	1.2	0.014	0.07
0.05	Nanoscale	0.05-0.5	0.1	$<10^{-12}$	0.1	0.3	0.003	0.018

+   1/5 Peak Volume

The Agilent 1100 Diode Array detector cell volume = 0.5 μL. The detector volume should be <1/5 of the peak volume for 10% broadening

Reference: www.michrom.com/catalog/col_selection.html and
www.westernanalytical.com/western/microbore_props.htm

Tubing Internal Volume

ID (inch)	ID (um)	Upchurch 1/16"	Upchurch 360um	Agilent 1/32"	Volume uL/in	Volume uL/cm	10cm Delay (sec)*
0.002	50		Natural	Green	0.050	0.020	1
0.0025	63.5	Natural			0.080	0.032	2
0.003	75			Blue	0.11	0.044	3
0.004	100	Black	Red	Black	0.20	0.079	5
0.005	127	Red			0.32	0.13	8
0.01	254	Blue			1.29	0.51	30
0.01	254	Natural			1.29	0.51	30
0.02	508	Orange			5.15	2.03	122
0.03	762	Green			11.58	4.56	274

*Delay in seconds for 10 cm of tubing at 10 uL/min flow rate

Filter Membrane Chemical Compatibility   Chart

C = Compatible   LC = Limited Compatibility (membrane swells and shrinks)   NC = Not Compatible   ND = No Data

Chemical	Nylon	PTFE	PVDF	Polysulfone	Cellulose Acetate	Polypropylene	Anopore	Regenerated Cellulose
Acids								
Acetic, Glacial	LC	C	C	C	NC	NC	NC	C
Acetic, 25%	C	C	C	C	C	C	C	C
Hydrochloric, 25%	NC	C	C	C	NC	LC	NC	C
Sulfuric, 25%	NC	C	C	C	NC	C	NC	C
Nitric, 25%	NC	C	C	C	NC	LC	NC	C
Phosphoric, 25%	NC	C	ND	ND	C	LC	C	C
Formic, 25%	NC	C	ND	ND	LC	C	LC	C
Trichloroacetic, 10%	NC	C	ND	ND	C	C	C	C
Bases								
Ammonium Hydroxide, 25%	C	C	LC	C	C	C	C	C
Sodium Hydroxide, 3 M	C	C	C	C	NC	NC	NC	C
Alcohols								
Methanol, 98%	C	C	C	C	C	LC	C	C
Ethanol, 98%	C	C	C	C	C	LC	C	C
Ethanol, 70%	LC	C	C	C	LC	LC	LC	C
Isopropanol	C	C	C	C	C	LC	C	C
n-Propanol	C	C	C	C	C	LC	C	C
Amyl Alcohol, Butanol	C	C	C	C	C	C	C	C
Benzyl Alcohol	C	C	C	ND	LC	LC	LC	C
Ethylene Glycol	C	C	C	C	C	LC	C	C
Propylene Glycol	C	C	C	C	LC	NC	LC	C
Glycerol	C	C	C	C	C	C	C	C
Hydrocarbons								
Hexane, Xylene	C	C	C	NC	C	C	C	NC
Toluene, Benzene	C	C	C	NC	C	C	C	NC
Tetralin, Decalin	ND	C	C	ND	C	C	C	ND
Halogenated Hydrocarbons								
Methylene Chloride	LC	C	C	NC	NC	C	NC	LC
Chloroform	C	C	C	NC	NC	C	NC	LC
Trichloroethylene	C	C	C	NC	C	C	C	C
Monochlorobenzene	C	C	C	LC	C	C	C	C
Carbon Tetrachloride	C	C	C	NC	LC	C	LC	LC
Ketones								
Acetone	C	C	NC	NC	NC	NC	NC	C
Cyclohexanone	C	C	NC	NC	NC	NC	NC	C
Methyl Ethyl Ketone	C	C	LC	NC	LC	LC	LC	LC
Isopropylacetone	C	C	NC	NC	C	LC	C	ND
Methyl Isobutyl Ketone	ND	C	LC	NC	ND	ND	ND	LC
Esters								
Ethyl & Methyl Acetate	C	C	C	NC	NC	NC	NC	LC
Amyl, Propyl, Butyl Acetate	C	C	ND	NC	LC	NC	LC	LC
Propylene Glycol Acetate	ND	C	ND	NC	NC	NC	NC	C
2-Ethoxyethyl Acetate	ND	C	ND	NC	LC	NC	LC	ND
Isopropyl Myristate	C	C	ND	NC	C	LC	C	ND
Oxides — Ethers								
Ethyl Ether	C	C	C	C	C	NC	C	LC
Dioxane	C	C	LC	NC	NC	NC	NC	C
Tetrahydrofuran	C	C	LC	NC	NC	NC	NC	C
Dimethylsulfoxide	C	C	NC	NC	NC	NC	NC	C
Isopropyl Ester	ND	C	C	C	C	ND	C	C
Solvents with Nitrogen								
Dimethyl Formamide	LC	C	NC	NC	NC	NC	NC	C
Diethylacetamide	C	C	ND	ND	NC	NC	NC	ND
Triethanolamine	C	C	ND	ND	C	C	C	ND
Aniline	ND	C	ND	ND	NC	LC	NC	ND
Pyridine	C	C	C	NC	NC	NC	NC	LC
Miscellaneous								
Phenol, Aqueous, 10%	ND	C	LC	NC	NC	NC	NC	C
Hydrogen Peroxide, 30%	C	C	ND	ND	C	C	C	ND

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