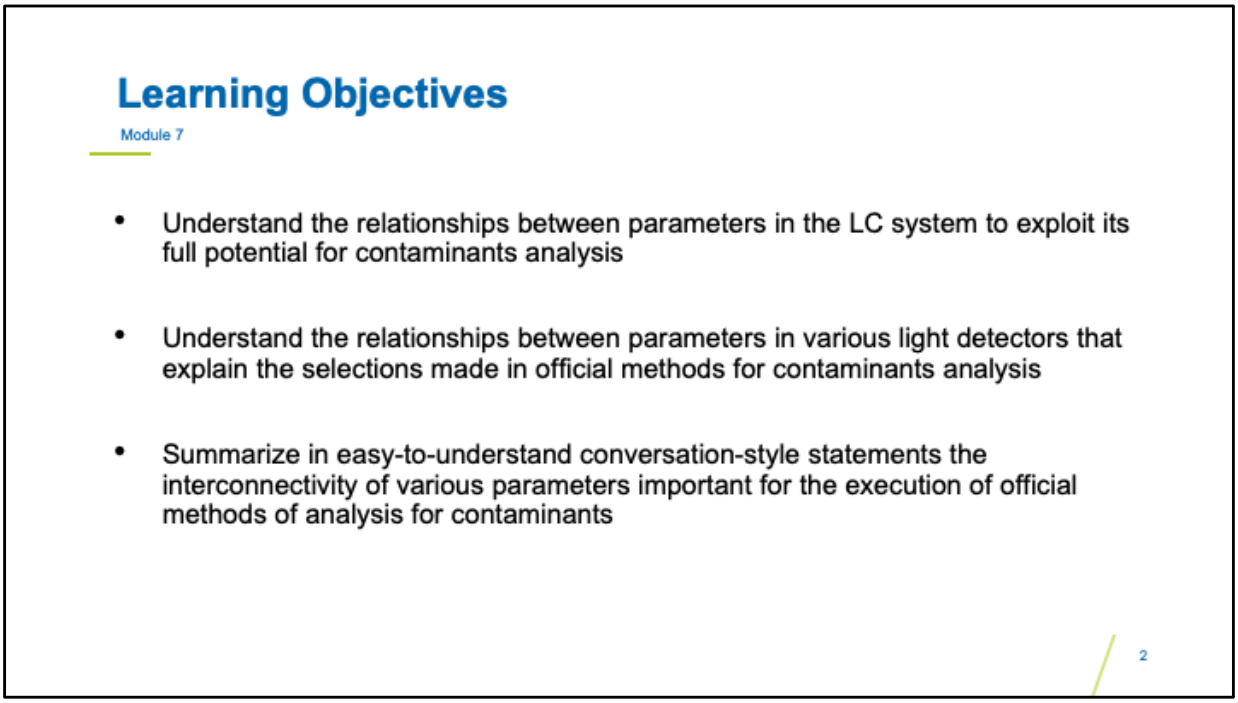
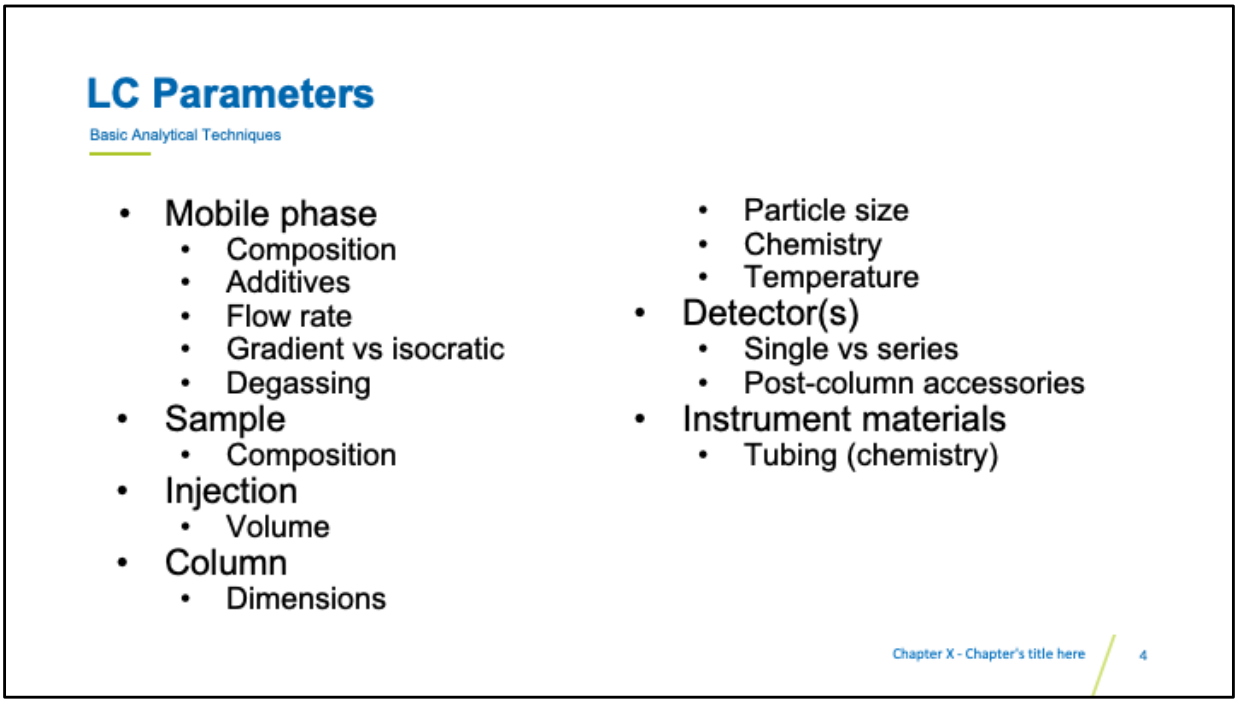
Previous modules 4, 5 and 6 have focused on the technology involved in the HPLC, GC, and mass spectrometer. Module is dedicated to understanding the parameters that must be set in these instruments in order to measure the components of interest in a reliable and accurate manner. In particular we are interested in the parameters that must be adjusted because of the combination of separation and detector types



The learning objectives of this module are to: Understand the relationships between parameters in the LC system to exploit its full potential for contaminants analysis; understand the relationships between parameters in various light detectors that explain the selections made in official methods for contaminants analysis; and finally summarize in easy-to-understand conversation-style statements the interconnectivity of various parameters important for the execution of official methods of analysis for contaminants

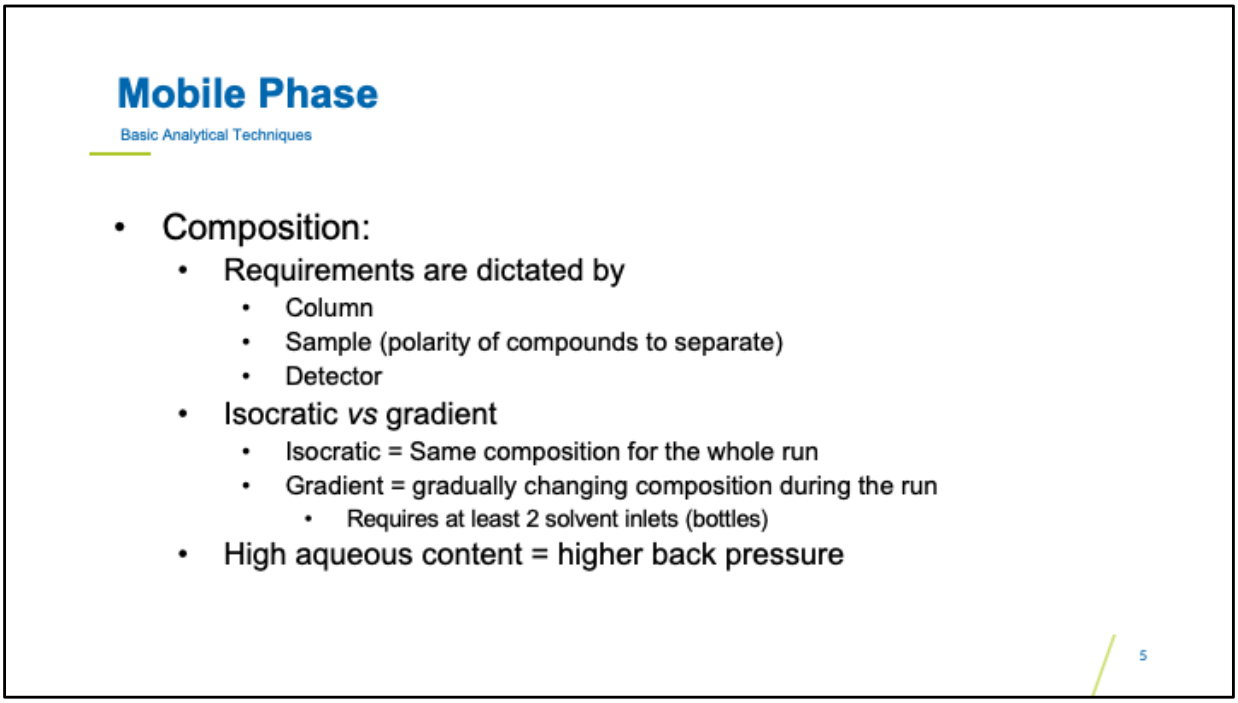
Section 1: Liquid chromatography parameters.



An HPLC method for the determination of food contaminants or other types of chemicals will contain a large number of parameters set to optimize both the separation and the detection of these analytes. The table here provides a list of parameters that must be considered in the optimization of the method and verified on one's own instrument.

We aim to take a look at all the different components of the method listed and how they affect the results, or even the applicability of a method.

This is not a list of ALL the parameters, but it is probably extensive enough to help analysts understand and troubleshoot their methods.



Let's start with the mobile phase. The mobile phase used for HPLC separation is selected based on the requirements of the analysis, which include the type of sample, the column chemistry, and the detector. We will focus here on reverse phase chromatography as it is the most popular type used in food safety for contaminants.

When deciding on a mixture of water and solvents for the isocratic mobile phase, it is essential to consider their miscibility. Indeed, if solvents are not miscible, they will separate in the solvent bottles on top of the instrument, which would mean that the composition changes depending on how deep the instrument is picking from that bottle. Similarly, solvents used in sequence or in gradients must be miscible

The incremental chromatography separation of compounds is typically performed through utilizing the polarity characteristics of the analytes of interest in our sample, those of the particles in the stationary phase, and the solvents in the mobile phase. The competing attractions of the analytes between the stationary and mobile phases determines the retention time in the column and therefore the position of the peak in the chromatogram. In reverse phase chromatography polar analytes elute the column first and this is independent of they use a gradient or and isocratic mobile phase. It is strictly dependent

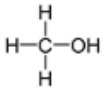
on the difference of polarity between the stationary phase and the mobile phase. While the gradient offers more opportunity to change the polarity of the mobile phase, and therefore the balance of attractions between stationary and mobile phase, it is possible to prepare a mixed mobile phase for isocratic run that will effectively separate the components of interest. As discussed previously, the choice of using an isocratic run may extend the duration of the run, but it should not prevent the separation of the components.

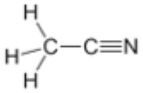
Finally, the increased interest in higher aqueous contents has brought about new challenges that need to be addressed in these methods.

Mobile Phase Composition

Basic Analytical Techniques

- In food safety, we typically don't need strong organic solvents in LC; mostly:
  - Methanol (MeOH)
  - Acetonitrile (ACN or MeCN)

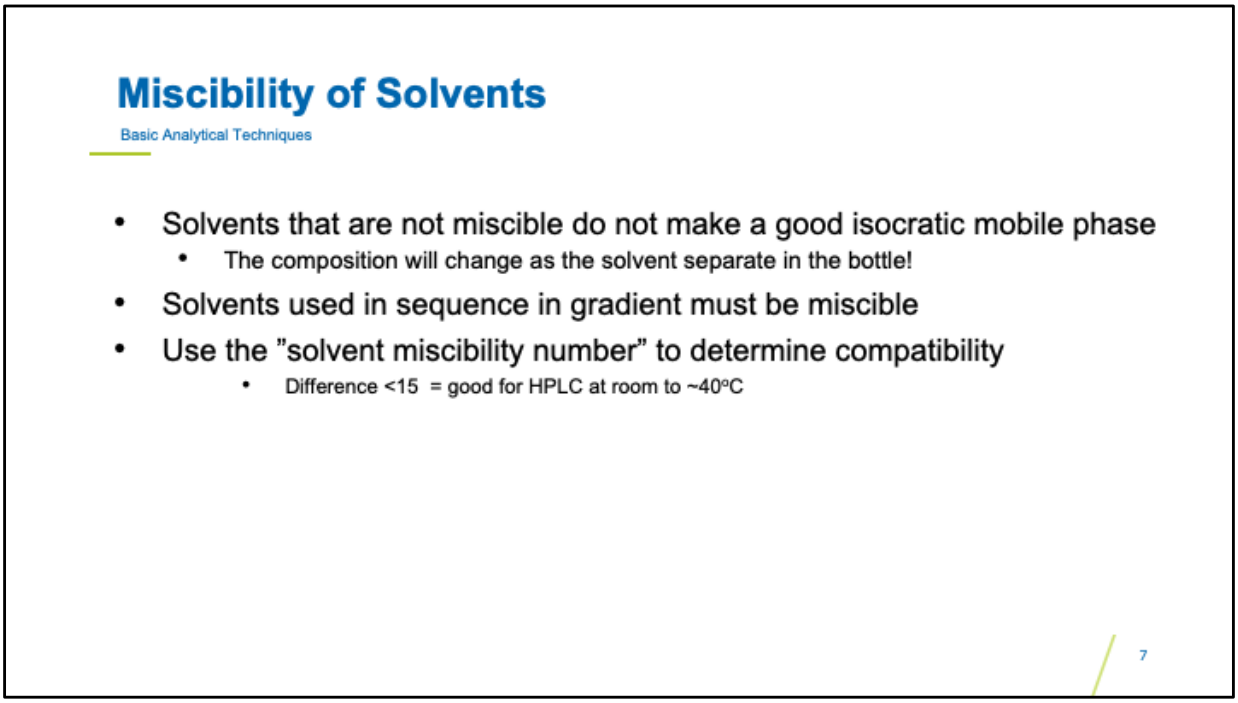




- Water
  - Distilled water for HPLC
  - Deionized water (18 ohm) for LC/MS
    - Ions present in water affect ionization in MS

6

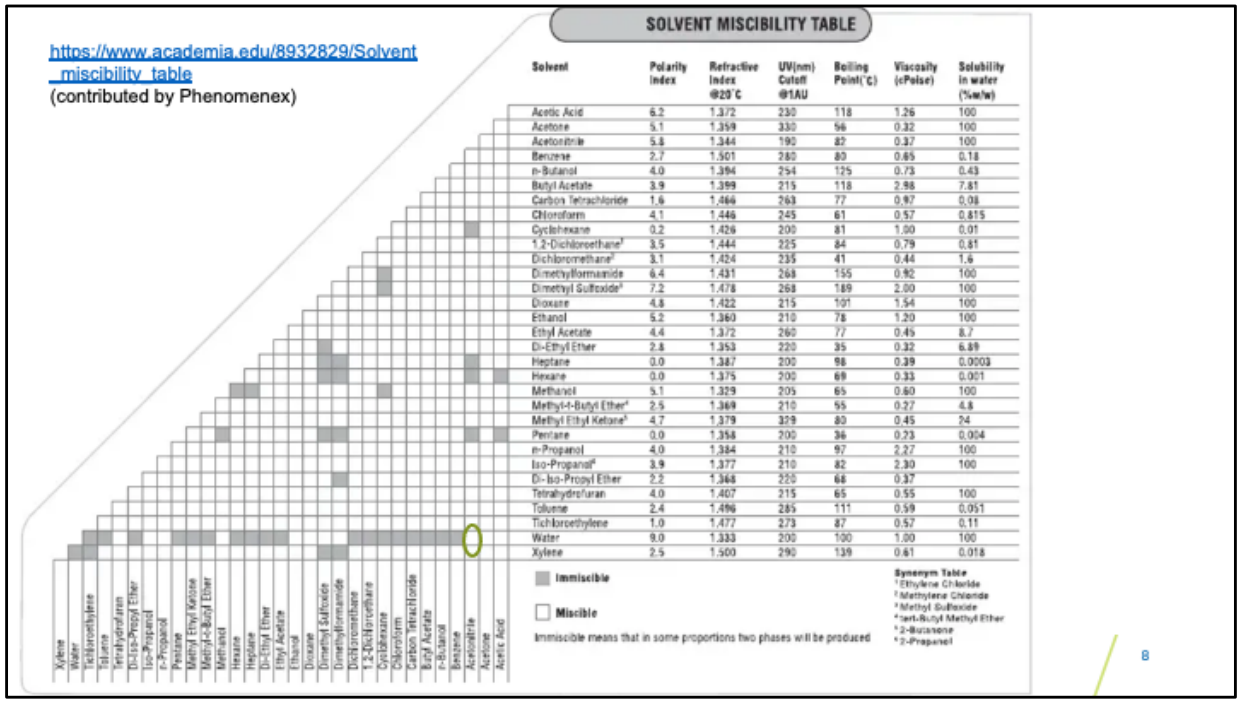
In food safety we typically do not need strong organic solvents in the LC. Methanol and acetonitrile are probably the most popular organic solvents. Of course, water is typically used in the mobile phase and it must be high purity to avoid effecting the components of interest. for HPLC distilled water is sufficiently pure, while mass spectrometry requires deionized water to prevent the ions in the water from affecting the ionization in the MS source.



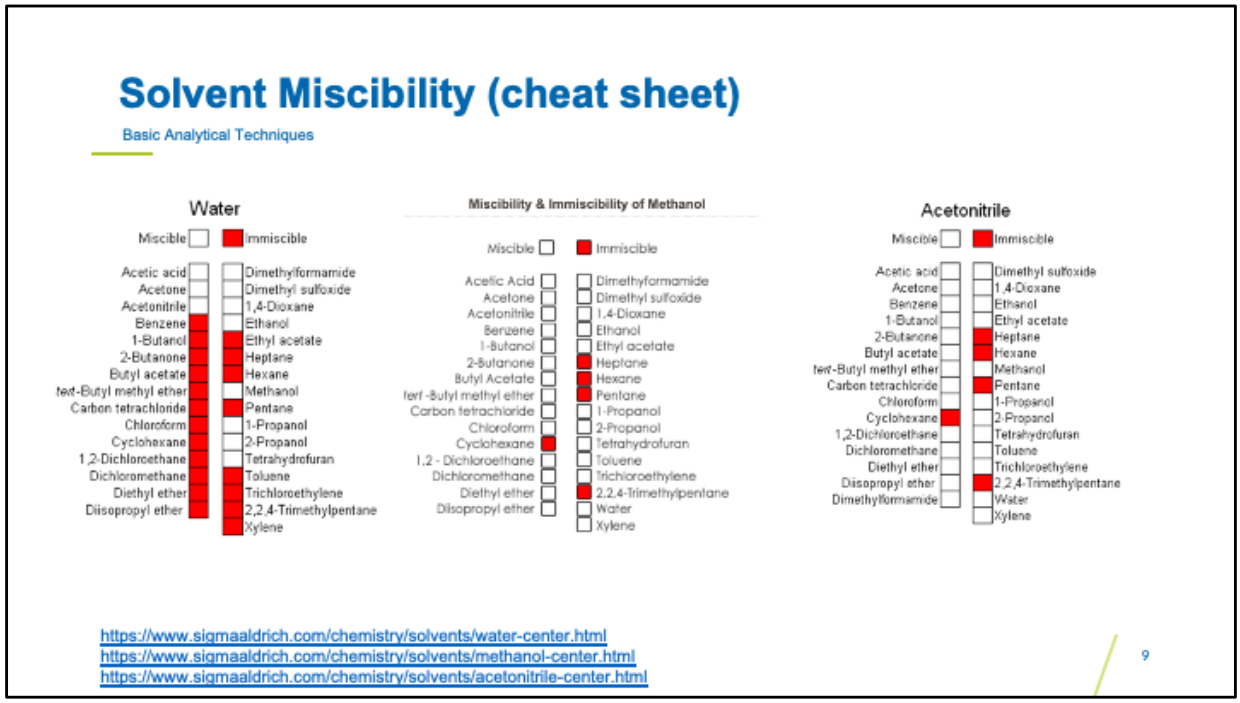
As we just mentioned, solvents that are not miscible do not make a good isocratic mobile phase because the actual composition will vary depending on how deep in the bottle we are taking from.

Solvents used in sequence in gradient must also be miscible. If two solvents that are not miscible must be used, they must be separated by a transition into a solvent compatible with both.

The solvent miscibility number is used to determine compatibility. The rule of thumb is that a difference smaller or equal to 15 is good for HPLC operation around 40 degrees Celsius. There are numerous sources for tables of solvent miscibility; one table is provided the next slide. It contains a very large number of solvents that are not used in food safety contaminants analysis, but the typical solvents applicable in the field are present in these tables.



This is an example available from Phenomenex and it is found at the address on the top left of the slide. There are many such tables available with more or less solvents, but this one will cover everything you might need for GC or LC. Our need for completely different solvents in the food safety laboratory is quite limited, so we will use only a small portion of this table. For example, we can look at the miscibility of water and acetonitrile and see that they are miscible (highlighted with a green circle).



These are more examples of graphical representations of this miscibility and I like to just print these types of graphical representations and posting them either on the side of the instrument or close to the mobile phase preparation area in the wet lab to remind ourselves of what can be mixed and what requires a transition phase.

Solvent Polarity

Basic Analytical Techniques

- Drives the differential attraction between mobile and stationary phase.

https://www.academia.edu/8932829/Solvent_miscibility_table
(contributed by Phenomenex)

Solvent Polarity Chart

Relative Polarity	Compound Formula	Group	Representative Solvent Compounds
Nonpolar	R - H	Alkanes	Petroleum ethers, ligroin, hexanes
	Ar - H	Aromatics	Toluene, benzene
	R - O - R	Ethers	Diethyl ether
	R - X	Alkyl halides	Tetrachloromethane, chloroform
	R - COOR	Esters	Ethyl acetate
	R - CO - R	Aldehydes and ketones	Acetone, methyl ethyl ketone
	$R - NH_2$	Amines	Pyridine, triethylamine
	R - OH	Alcohols	Methanol, ethanol, isopropanol, butanol
	$R - COHN_2$	Amides	Dimethylformamide
	R - COOH	Carboxylic acids	Ethanoic acid
Polar	H - OH	Water	Water

Increasing Polarity

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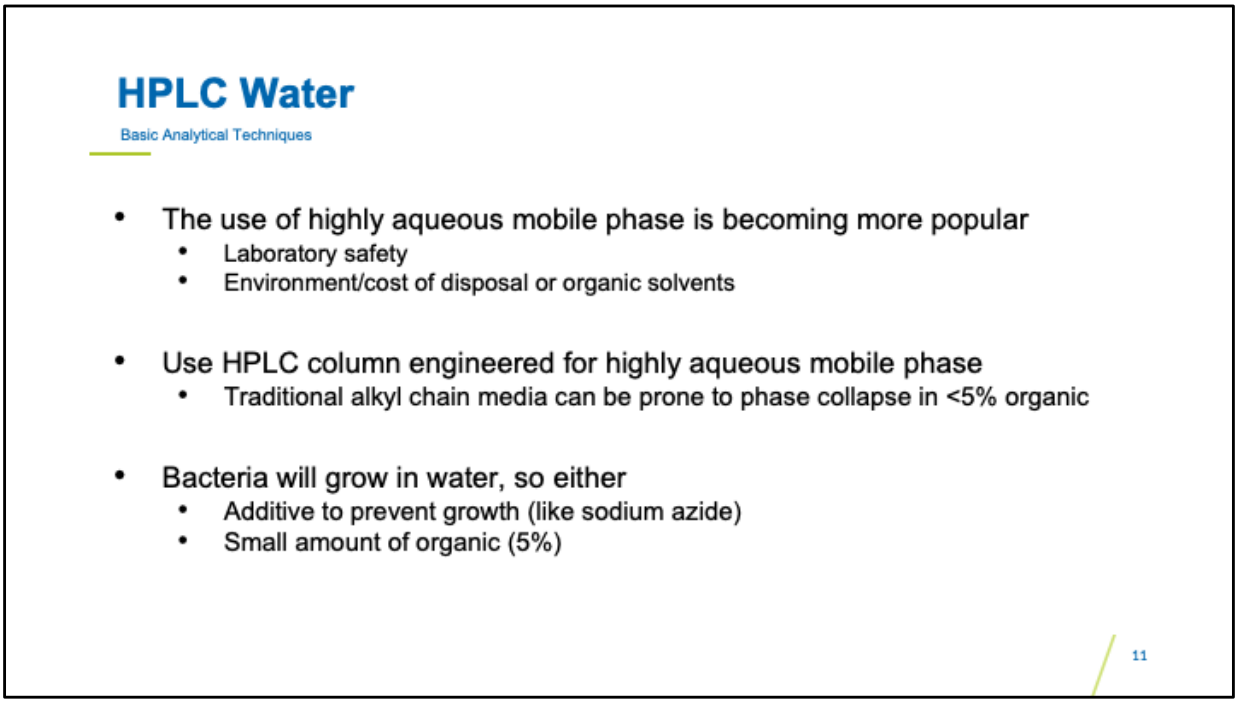
The next parameter is solvent polarity. It is the differentiating factor that either carries analytes through the column quickly or not. In other words, the difference in polarity determines whether the attraction is greater between the analytes and the stationary phase or between the analytes and the mobile phase.

This table, also from Phenomenex, gives examples of more or less polar solvents.

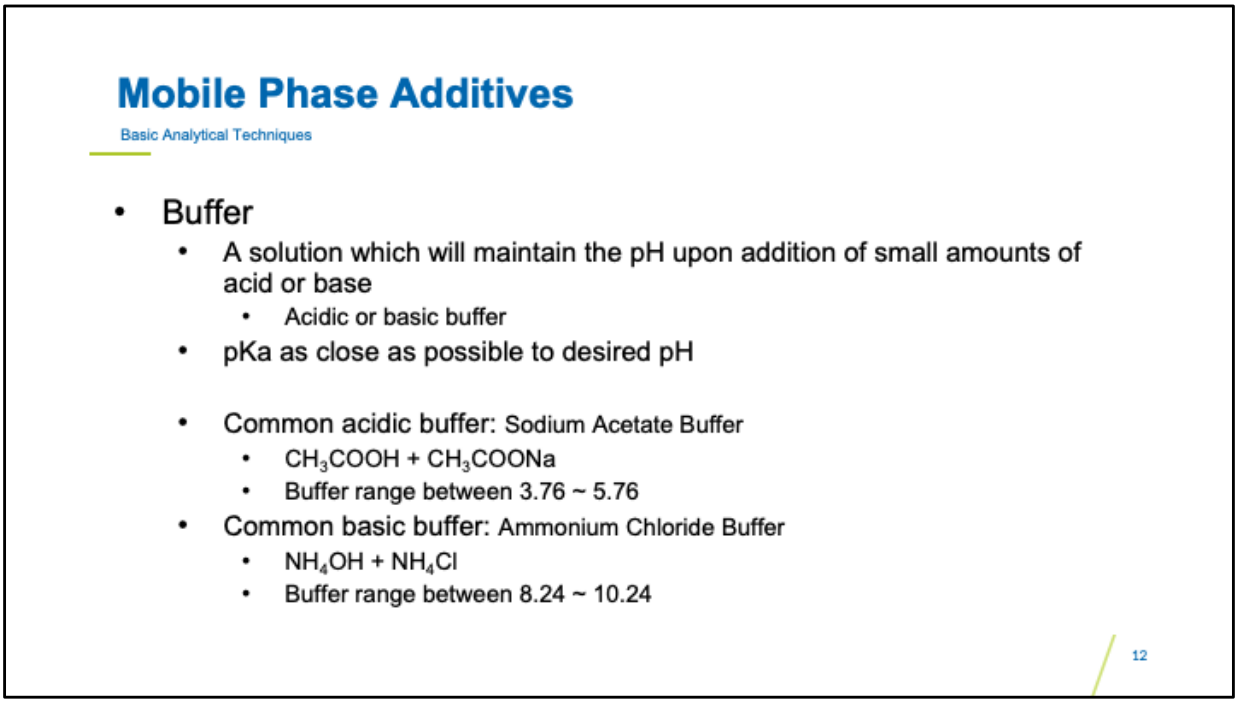


AS I mentioned a few slides ago, there is an increased interest in more aqueous mobile phases and this emphasized the importance of water quality. Water is safer for the analyst and less costly to dispose of because of the environmental impacts of organic solvents. However, highly aqueous mobile phases cause higher back pressure in the instrument because water is more viscous than organic solvents so the instrument may need to be run at higher temperature to reduce the viscosity. One has to carefully read the specifications of the column they are using as well because some columns are incompatible with high water content or 100% water. It would simply ruin the column.

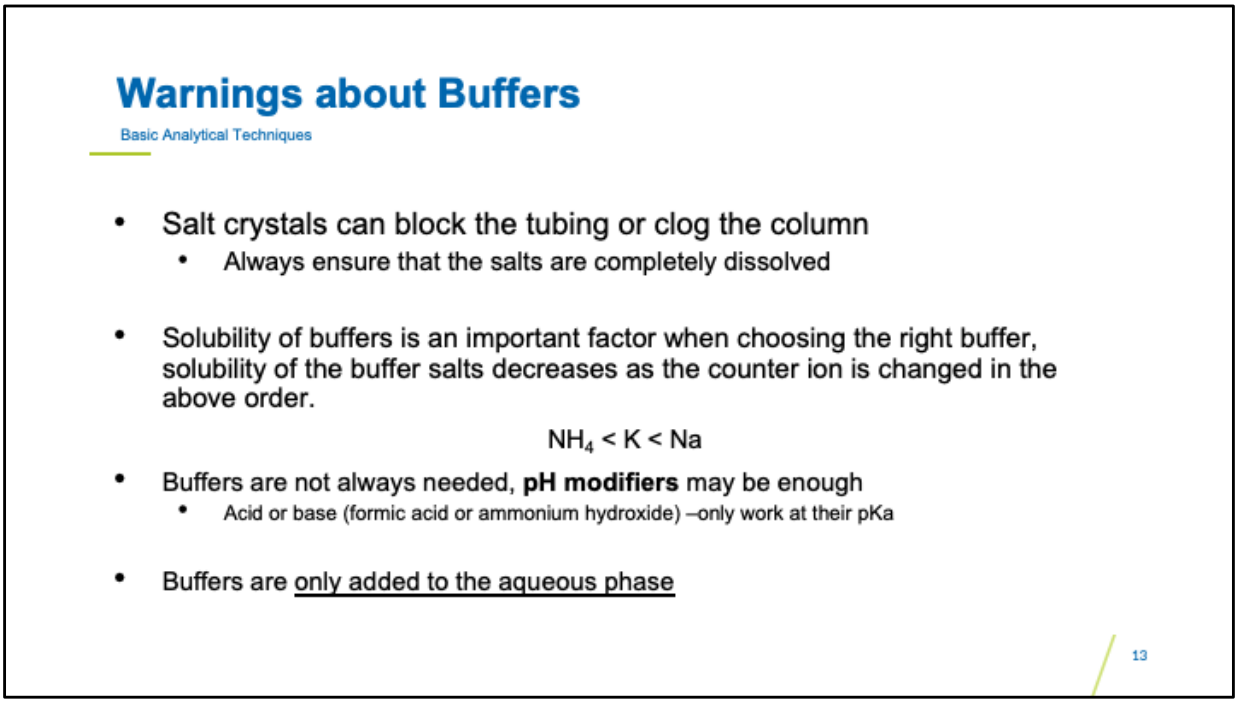
Water is also at risk of spoilage with bacteria and must therefore be supplemented with additives that prevent bacterial growth. Sodium azide is a popular additive for this purpose, but another option is also to add a little bit of organic solvent in the bottle of water to prevent bacterial growth.

Finally, the purity of the water is critical because the presence of any contaminants, even ions, in the water, can change the separation and the back pressure generated. In the case of using mass spectrometry as the

detector, free ions in the water can compromise the ionization in the source for our compounds of interest.



Buffers are also typically used in the mobile phase. They are solutions which maintain the pH of the mobile face upon addition of small amounts of acid or base. It is important to choose a buffer based on a pKa that is as close as possible to the desired pH of the solution. A common acidic buffers is sodium acetate which maintains the pH between approximately 3.76 and 5.76, while a common basic buffer is ammonium chloride which maintains the pH range between 8.24 and 10.24



It is important to recognize the risks associated with using salts to prepare buffers. Crystals can block the tubing or the columns of the instrument. The analyst must always ensure that the salts are completely dissolved in the mobile phase bottle. Solubility of buffers is an important factor when choosing the right one. Buffers are actually not always needed and a pH modifier such as an acid or a base for example formic acid or ammonium hydroxide can maintain the pH close to the right pKa when the sample is not expected to contribute significantly to the pH. Finally, buffers are only added to the aqueous phase because they are generally salts that are best solubilized in water.

Flow Rate

Basic Analytical Techniques

- Flow rates too low or too high = Band broadening
- Too high also increases back pressure

- HPLC and UPLC use different flow rates –at least to start
  - Best practice is to start with same linear velocity
    - Conversion factor is square of ratio of column diameters = $(D_{UHPLC}/D_{HPLC})^2$
  - Then increase flow rate to accelerate the method gradually
    - Watch back pressure remains within system specifications

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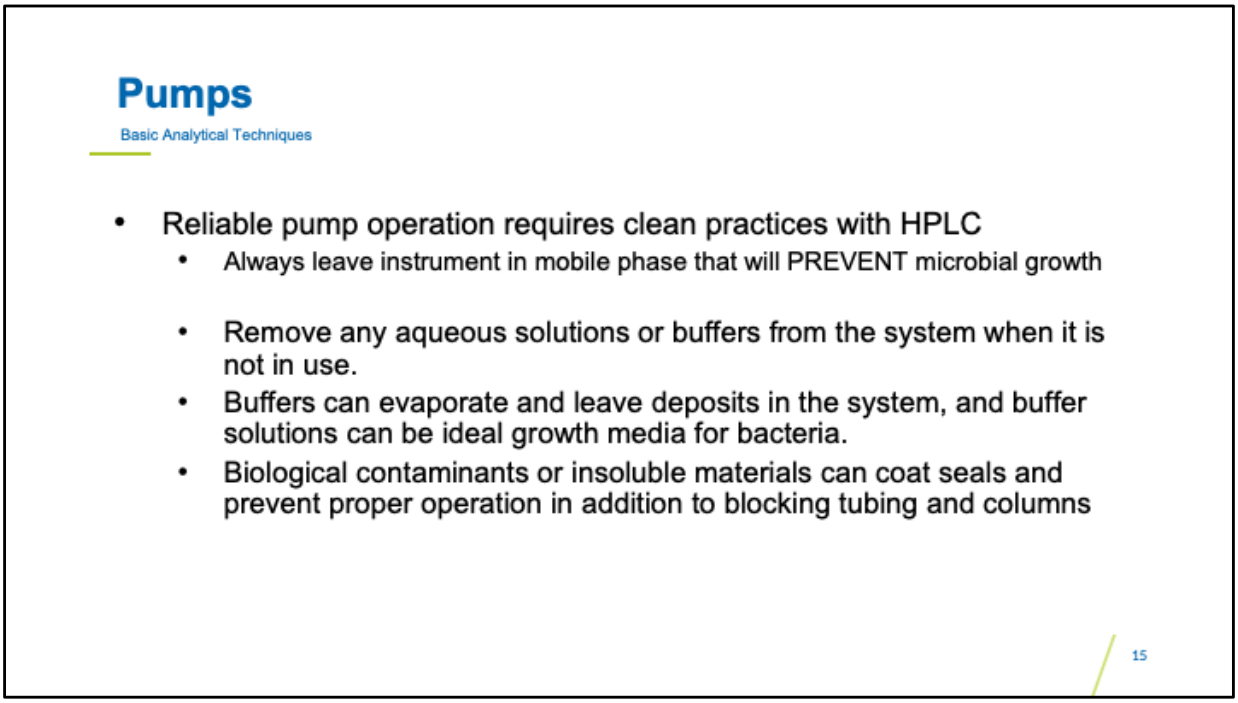
Now let's talk about flow rate. The mobile phase is pushed through the chromatography system using pumps that deliver an even flow throughout the run. The flow rate has an impact on band broadening and the optimal rate will create the most symmetrical and narrow band possible. Flow rates that are too high or too low will both cause band broadening, while an exceedingly high flow rate will increase the backpressure in the instrument and could damage the column or connectors. HPLC and UHPLC use very different flow rates; the rule of thumb when converting a method from HPLC to UHPLC is to maintain the same linear velocity, which is converted through a consideration of the column diameters. The analyst could then proceed to increase the flow in order to accelerate the analysis , but gradually; the critical factor to monitor in this process is the backpressure to ensure that it remains within the system specifications.



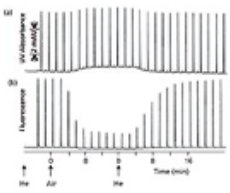
The pumps in the HPLC system act like the heart in the human body. Pump health is maintained through the utilization of best practices for the selection of solvents, buffers, and cleaning routines. The risk of microbial growth in the aqueous mobile phase was discussed before. Microbial growth can also happen in the column if it is left filled with water. Similarly, the buffers that could crystallize in the solvent bottle could deposit in various locations in the instrument, especially if there is an opportunity for the solvent to evaporate. We must work hard to prevent bacterial growth in the flow path of the instrument because insoluble materials and films, the biofilms, can coat seals and prevent proper operation in addition to blocking tubing and columns. Each laboratory should have an SOP developed and implemented to ensure the prevention of microbial growth in the HPLC instrument. This SOP will contain procedures for solvent exchange in the column at the end of the day and possibly different procedures for solvent exchange when the instrument is to be left idle for an extended period of time.

https://www.chromatographyonline.com/view/how-does-it-work-part-1-pumps

Mobile Phase Degassing

Basic Analytical Techniques

- Outgassing was a common problem in older HPLC systems
  - Modern systems have online degassers
  - BUT: Watch for signs that you have gas issues
    - Pump stops (air bubbles)
    - Retention times not stable (air bubbles)
    - Noise spikes in UV or fluorescence chromatograms
- If needed, degassing should remove at least 50% of gas
  - Vacuum (with a clean stir bar) - 50-70% of gas (5 minutes)
  - Sonicating – 20-25% (not enough)
- In-line degassers are common now
  - Use a porous membrane to allow air out of mobile phase



Noise spikes in FLD

https://www.chromatographyonline.com/view/how-does-it-work-part-ii-mixing-and-degassing

16

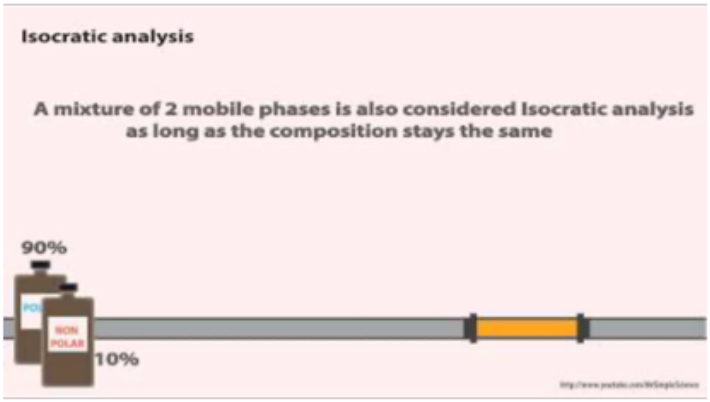
Gas bubbles presence in the mobile phase can cause noise spikes in the chromatogram. Outgassing solvents was a common problem in older HPLC systems; modern systems commonly have an online degasser. However, it is still important to watch for signs that there might be a release of gas from the mobile phase. Symptoms include a pump that stops because of an air bubble, retention times that are not stable, or noise spikes in UV or fluorescence chromatograms. If degassing is needed, either because the instrument does not have an online degasser or it is not sufficient to remove the bubbles, a separate step of degassing should be applied to remove at least 50% of the gas. Stirring the solvent under vacuum for five minutes can remove 50 to 70% of gas. Sonicating is also a popular method, but it only removes 20 and 25% of the gas, which may be sufficient to supplement the online degasser. Inline degassers are also quite common now and they consist in a porous membrane that allows air out of the mobile phase before it reaches the pumps.

https://www.chromatographyonline.com/view/how-does-it-work-part-ii-mixing-and-degassing

Isocratic or Gradient?

Basic Analytical Techniques

- No one-size-fits all…



https://youtu.be/tAcfJPveWwM

17

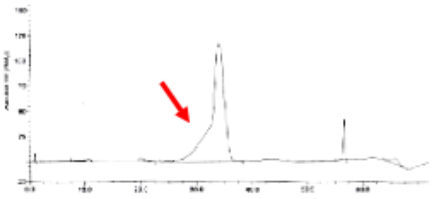
This cute video from Simple Science shows us the effect of the use of a gradient in a chromatographic run.

The selection of an isocratic or gradient run is dependent on the number of components to separate, the complexity of the sample itself, the column, and the amount of time that we are prepared to spent on the chromatography. Even if one mode is preferred over the other, there typically are different combinations that will work. The rule of thumb is that simple mixtures of few components of interest perform very well with an isocratic method, while solutions containing a large number of components of interest perform better in gradient methods. The gradient method goes through a broader range of polarities in a shorter amount of time and consequently causes the elution of analytes of interest to proceed faster.

Sample

Basic Analytical Techniques

- Composition
  - Peak fronting or splitting can happen when sample diluent is more eluting than mobile phase (higher organic content)
  - Best to approach composition of the mobile phase at start of the run
  - If not, reduce the injection volume



https://phenomenex.blog/2015/12/10/technical-tip-fronting-peaks/

18

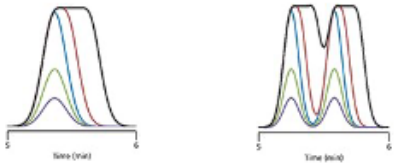
The sample composition plays a critical role in the separation despite the very small volume of sample that is actually injected. For example, a sample presented in a highly organic solvent may cause peak fronting or splitting as it effectively tries to move faster than the mobile phase. This is the main reason why we try to match the sample diluent with the starting composition of the mobile phase. If it is not possible to match the sample diluent with mobile phase, then the sample may need to be injected in a smaller volume to minimize the effect of the diluent. This can negatively impact the sensitivity of the method.

https://www.crawfordscientific.com/technical/chromatography-blog/hplc-chromatography-tips/hplc-practice/sample-diluent-effects-in-hplc

Sample Injection

Basic Analytical Techniques

- Volume:
  - Peak area is proportional to quantity, until
    - So much that we can see spread from volume in the column
    - Saturation of the detector
  - Higher peak = higher S/N = lower LOQ
  - Too high → close peak can no longer be resolved



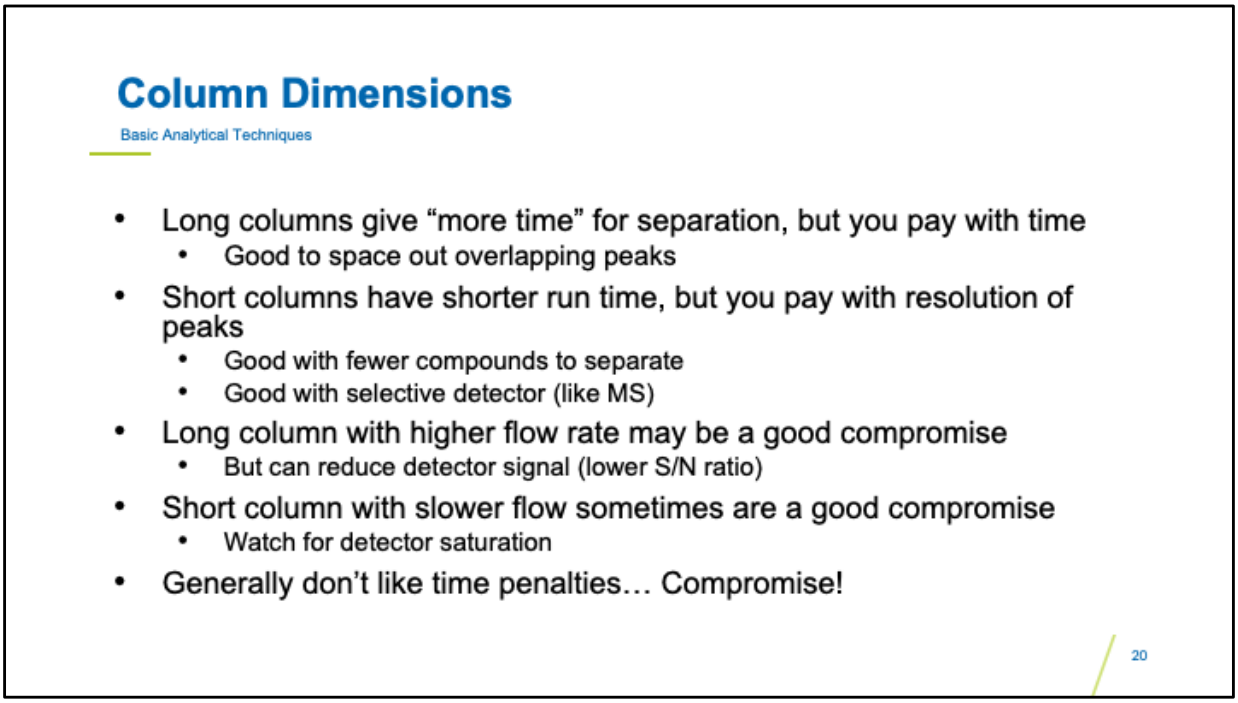
https://www.chromatographyonline.com/view/how-much-can-i-inject-part-i-injecting-mobile-phase

19

The sample volume can further impact the quality of the peak and consequently the ability to identify and quantify analytes. The peak area in the chromatogram is proportional to the quantity of analyte injected in the column. This is no longer true when the volume is so large that the sample spreads into the volume of the column and elutes as a very broad band or causes the saturation of the detector. While we attempt to optimize parameters to obtain the highest peak possible because it translates into a higher signal to noise ratio, which in turn means a lower limit of quantitation for the method. However, if the peak is too high, the signal may saturate the detector. This is characterized by a flat top on the peak. If the concentration of two analytes that elute at close retention times is too high, the peak width resulting from this high concentration may cause the peaks to no longer be resolved. This would make the quantitation of either analytes impossible and their identification would be less reliable due to the influence of the neighboring peaks on the position of the top of the peaks or center of mass of the peaks. In this case we would need to either inject the analytes at lower concentration or to change our gradient run to separate them.

https://www.chromatographyonline.com/view/the-lcgc-blog-inexpensive-quick-and-selective-seeking-the-holy-grail-of-sample-extraction

For advanced, calculation here:
https://www.chromatographyonline.com/view/how-much-can-i-inject-part-i-injecting-mobile-phase



We have already discussed some of the important aspects of column selection in module 4 and also some “cause and effect” relationships such as how reducing column volume increases sensitivity, but that selectivity, back pressure and durability must be considered. We also reviewed the most popular column chemistries and their applicability and will do more of that in the in-person portion of this training.

The dimensions of the column affect the chromatogram by impacting the amount of time given to the analytes to elute the column at a given flow rate. A longer column gives us more time, while a shorter column translates into a faster analysis. The best compromise between increasing the flow rate in a longer column and slowing down the flow rate in a shorter column can be calculated but needs to be verified empirically. The longer columns, with their greater amount of available time, are our best options to space out overlapping peaks. It can be a problem to attempt to excessively accelerate a method by simply switching to a shorter column. This remedy works well for samples containing fewer analytes or when the detection technique coupled to the chromatograph performs an additional filtering or selection step such as in the mass spectrometer.

In many cases dealing with samples with many analytes of interest, a longer

column used with a higher flow rate provides a better compromise, but the high flow rate could result in reduced signal to noise ratio, which may in turn affect the limit of quantitation of the method. For simple samples, a shorter column with a s lower flow rate can provide a good compromise by producing high peaks, but it is important to pay attention to detector saturation. Understanding the concentration range expected from the samples in a given application is a critical factor in the development of the method.

The concentration range can be quite narrow, such as expected in the determination of pesticide residues in environments where good agricultural practices are applied, while a very broad range of concentrations that can reach three or more orders of magnitude such as may be found in highly heterogeneous samples submitted for mycotoxins analysis. It is not always possible or wise to develop the method with the intention to cover the entire concentration range. It is often best to restrict the concentration range of the method to ensure that the signal remains within the linear range of the detector, and dilute samples that fall outside this range. We generally don't like to extend the time of analysis if we don't have to because it limits the number of samples we can analyze in a day.

Column Dimensions (cont.)

Basic Analytical Techniques

- Smaller column diameter produces narrower peaks
  - Watch for increased back pressure
  - Watch for detector saturation
  - More sensitive to buffer crystals and biofilms

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I wanted to illustrate in a very simple schematic what happens when you move from a wide to a narrow column. First, if you try to inject the same volume of sample, it will occupy a much larger proportion of the length of the narrow column. This means that you only have what is left on the right hands side to separate the analytes contained in that sample. Consequently, we normally reduce the volume of sample when we move to a narrower column.

Of course, this is possibly an oversimplified schematic as it doesn't talk about other phenomena that happen such as the sample dissipation in the larger column, but I hope it helps understand the impact of sample volume.

The dimension of the column that we are talking about here is the diameter. A smaller column diameter is typically considered because because it can produce narrower peaks due to the smaller amount of lateral dispersion of the sample in the column. Another benefit of the narrower column is to produce higher peaks; the analyst must pay attention to detector saturation when evaluating the suitability of a narrower column and adapt either the flow rate or the sample volume to ensure that the peak intensity falls within the linear range of the detector. If the sample volume is too large, the sample

front in the column may occupy an exceedingly long portion of the column that will leave very little length for actual separation. Once again, many parameters are interrelated and must be optimized simultaneously to obtain the most appropriate separation for the application at hand. As a reminder, we do not always need to obtain the best possible separation, especially when using mass spectrometry for the detection following chromatography.

Rule of thumb about column dimensions: Injection volume must be smaller than 10% of the column volume.

A rule of thumb, for those who are less familiar with the term , is a guide based on experience

Column Particle Size

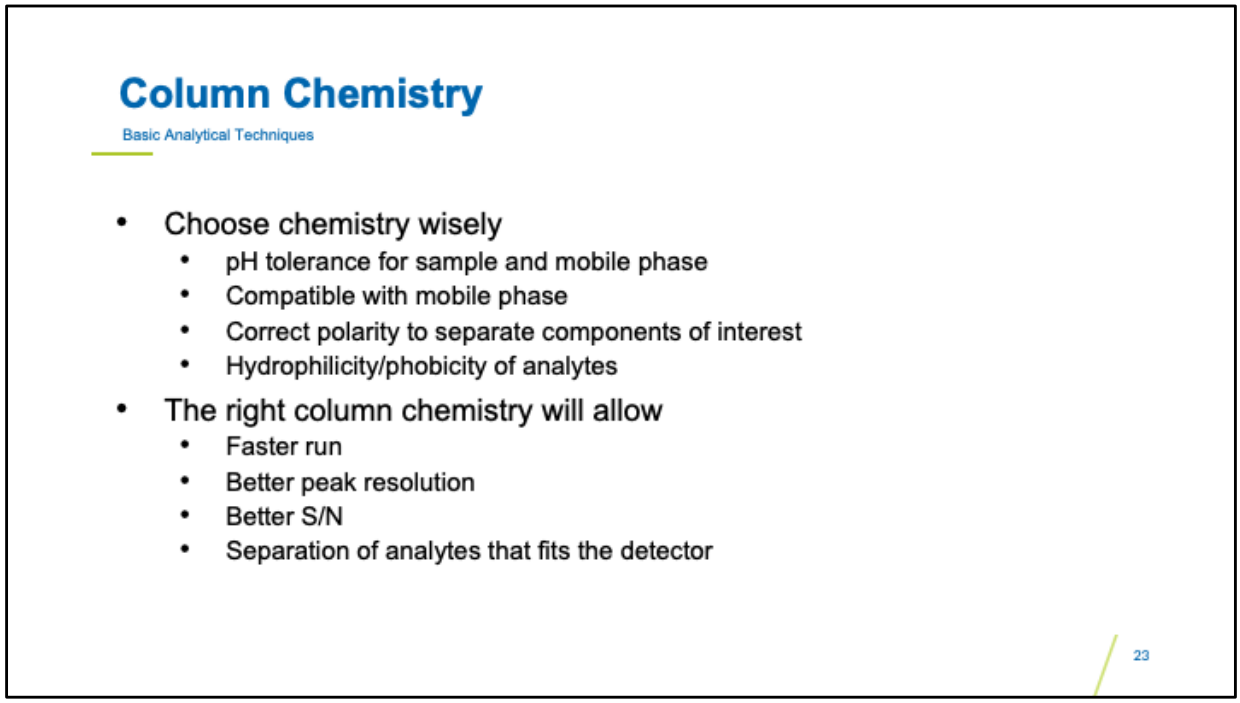
Basic Analytical Techniques

- Larger particles require less pressure from pumps
  - But fewer particles in column means less separation capacity
- Smaller particles lead to better separation (sharper resolved peaks)
  - Require special instrument <2 um -> UHPLC
  - Columns clog more easily, no reverse flow possible to clean the column

22

Let's move on to particle size. Large particles require less pressure from the pumps to move the sample and the mobile phase through the column. The downside of using larger particles is that the column contains fewer of them which means there is less opportunity for interaction with the stationary phase, and consequently less separation capacity. Smaller particles lead to better separation and it shows in the form of sharper well resolved peaks. However, there is a physical limit to how small the particles can be and still allow the mobile phase to flow through. As the particles get smaller, the interstitial space –as seen here as the triangular hole between the green circles, becomes smaller, which creates an increased amount of back pressure in the system.

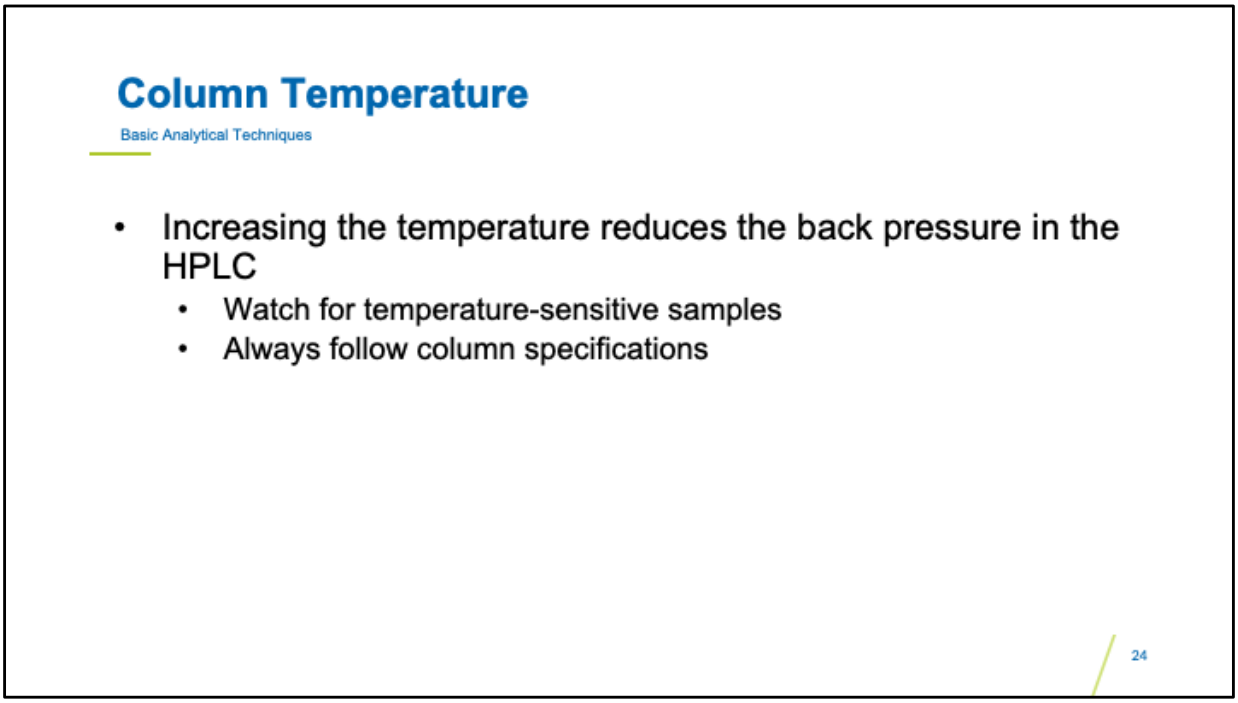
The back pressure can be reduced to an extent by increasing the temperature of the column, but the temperature cannot be set so high that it would cause heat-damage to the analytes of interest, the silica particles in the column, their shell or ligands. The particle size limit that characterized HPLC systems prompted changes in the engineering of the UHPLC system to allow the use of sub 2-micron particles.



Column chemistry must be selected as a function of the analytes being separate, pH tolerance or requirements for both the column and sample analytes, compatibility with the mobile phase and the target performance parameters.

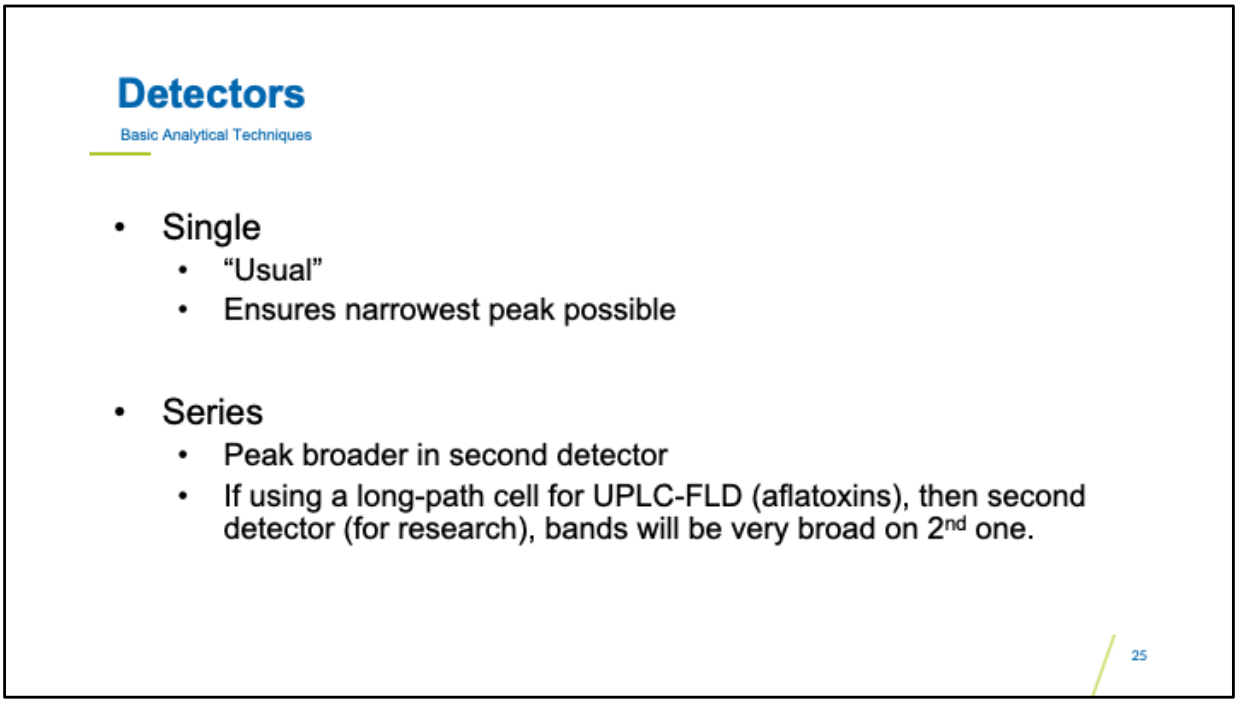
A suboptimal column selection may provide a level of separation that is sufficient for peaks to be present in the chromatogram, but their shape may be asymmetrical or flattened, which would limit their usability for quantitation, or even qualitative identification of an analyte. The right polarity, pH tolerance, and separation capacity should allow a faster run, providing better peak resolution, and high S/N. A poorly chosen column could also block, risking to damage the pumps, connections and tubing of the instrument.

The last element in this list specifies that the separation only needs to be as good as your application requires and this really is a segway into a discussion of mass spectrometry. We saw in Module 6 that the mass spectrometer in the tandem quadrupole, Q-ION TRAP or QTOF formats use the first quadrupole to filter the ions of interest and discards the others. This means that we can live with overlapping peaks from the chromatography if we have a tool to further separate the analytes ahead of detection.



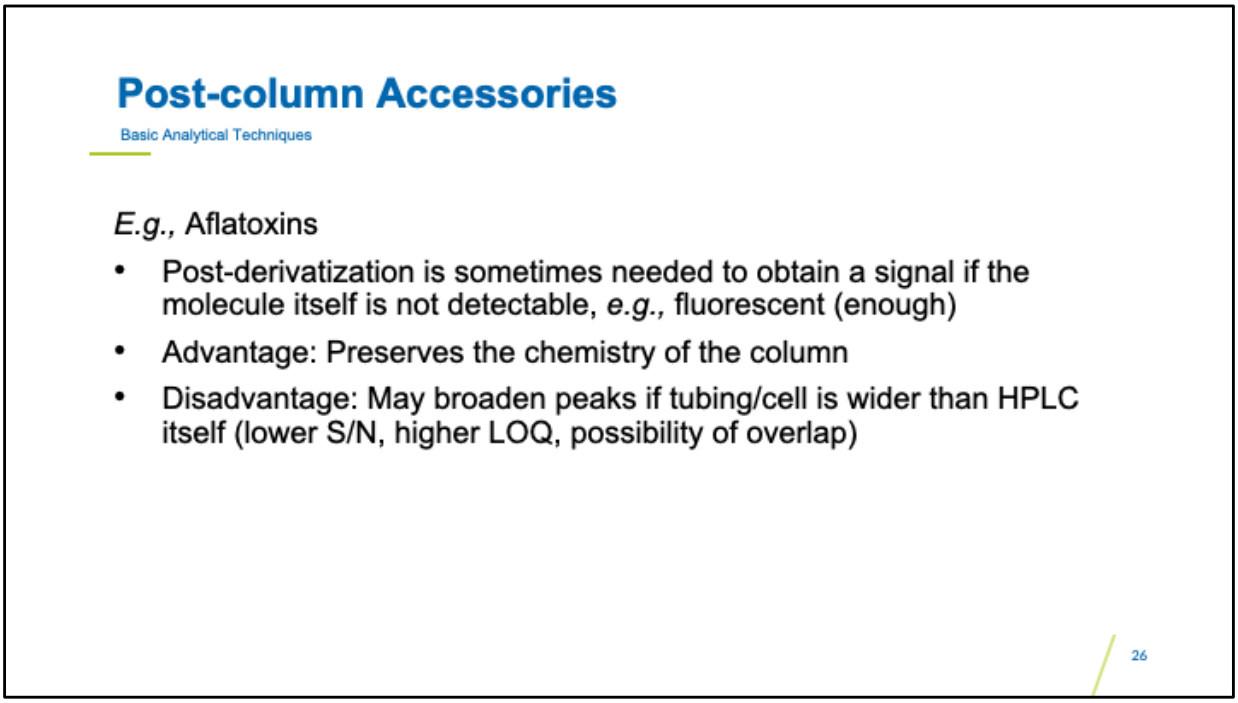
Column temperature. In most modern HPLC and UHPLC systems, the column is housed in an enclosed chamber that enables temperature control. The temperature of the column affects the flowability of the mobile phase and consequently the backpressure resulting from the selection of a particular flow rate for a column of set dimensions and particle size. By changing how the mobile phase flows through the column, the column temperature also effects the retention time of or analytes.

Care must be taken to keep the temperature low enough to avoid any degradation of the analytes of interest or the column itself. Commercial columns always come with specifications relating to their operating temperature range.

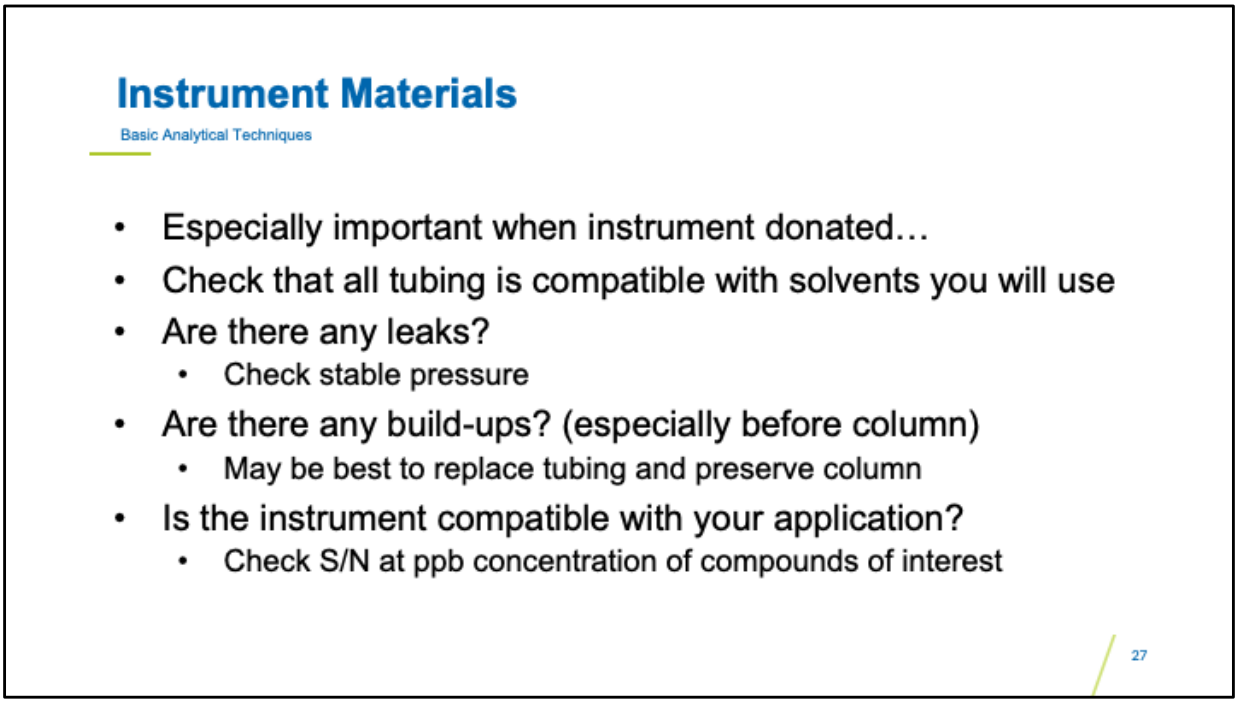


While the detector is a completely separate component of the chromatography system, the selection of the column must consider which detector will follow. We just mentioned how a mass spectrometer can dramatically changes the requirements from the chromatography as an example.

Every once in a while I get asked if it is possible or useful to combine detectors to obtain different types of information from the same eluent. Usually, we work with a single detector because it limits the length of tubing post column, which in turn effects the dispersion of the peak before the concentration is measured. If two or more detectors are set up in series, the second detector will display broader peaks because of the dispersion happening in the first detector. The dispersion can be quite large if the first detector uses a cell whose dimensions depart from the diameter of the transfer tubing. It is quite uncommon to see more than one detector used for regulatory analysis, and this type of setup is more appropriate in a research laboratory.



Some analyses require the use of accessories to the chromatography system in order to create a detectable signal. The best example in food safety is the derivatization process used to increase the fluorescence signal of aflatoxins B1. While these analytes are naturally fluorescent this fluorescence is quenched and results in very low intensity peaks, which in turn restrict the sensitivity of the measurement. In order to obtain the limits of quantitation and limits of detection desired for the measurement of aflatoxins by HPLC and fluorescence, it is necessary to derivatize the analytes. This can be performed before the column, but the harsh chemicals used for the derivatization can negatively impact the durability of the column. Consequently, post column derivatization is often preferred to preserve the chemistry of the column. However, the derivatization set up must not cause excessive dispersion of the sample as this would result in a broader peak and there are four reduce the sensitivity of the method. As we have discussed before peak broadening translates into lowered signal to noise ratio, higher limit of quantitation, higher limit of detection and the possibility of overlapping peaks.



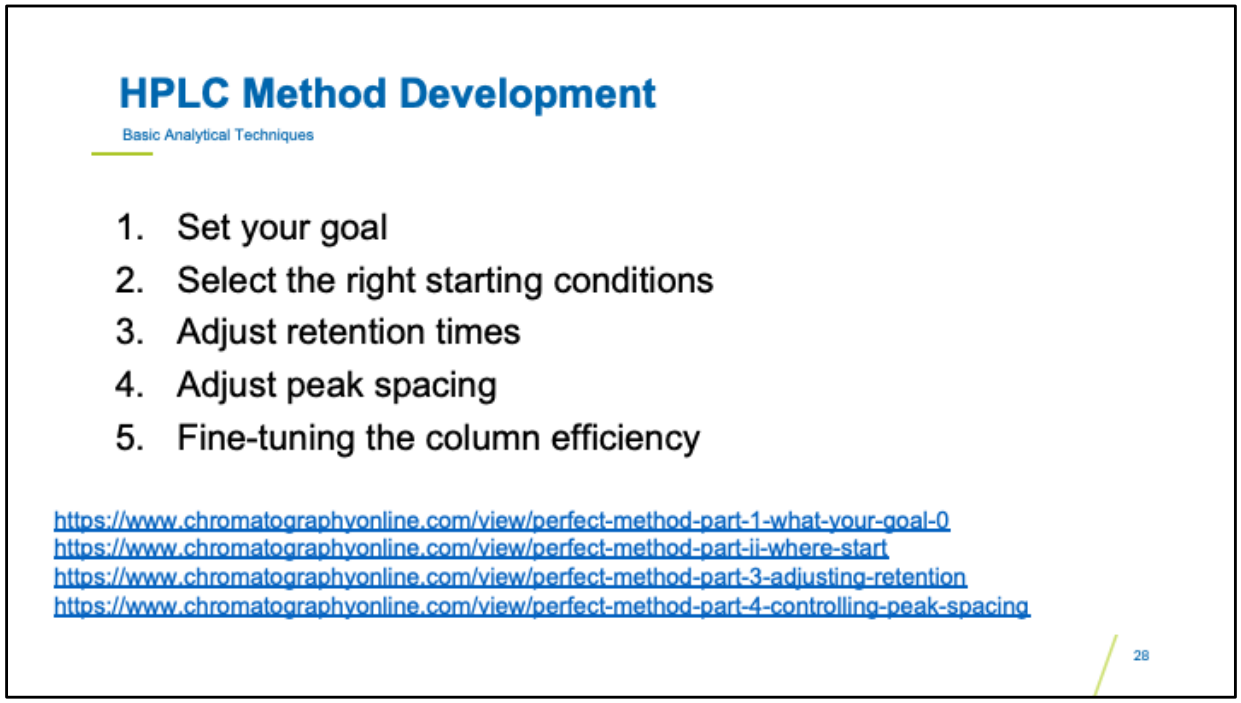
The materials used for the construction of an instrument matter. In food safety, we have the advantage of mostly working with weak organic solvents, so we don't need to worry too much about what the tubing is made of for example. I still want to bring the topic to our attention here because this can be challenging in laboratories that rely on instrument donations. Some materials are more rugged and while you may not need it because you use mild conditions, it could make a difference in how worn the instrument is depending on what the previous owner did with it.

Some of the critical materials to look at are those used to make the numerous seals in the instrument. If harsh chemicals were used, the seals may be damaged, which would cause an unstable pressure in the instrument. It is not possible to do reproducible chromatography with an instrument that does not maintain a stable pressure. There is no compromise possible here.

Incompatible materials, or simply poor maintenance, may have caused an accumulation of residues around connections or even in the tubing. It is a good idea to replace tubing and connectors that appear dirty or damaged before build up detaches and lodges itself in the column. Back-flushing the

column may work to fix the problem, but it also may not. Back flushing is also not an option in UHPLC.

Last but not least, it is critical to check if the instrument provides the S/N that you need at the concentration of food contaminants. No amount of time spent playing with the user-accessible parameters such as flow rate to try to increase the signal will make up for the low sensitivity of a detector that was designed for a completely different application…



In summary, the key to good method development and application is to set your goal and select the right starting conditions. You may then adjust retention times using the parameters we just discussed to adjust the total run time, but also peak spacing. Fine tuning to obtain the best column efficiency will provide the levels of peak intensity and S/N ratio needed for contaminants analysis at trace levels.

The following links can be accessed for additional information.

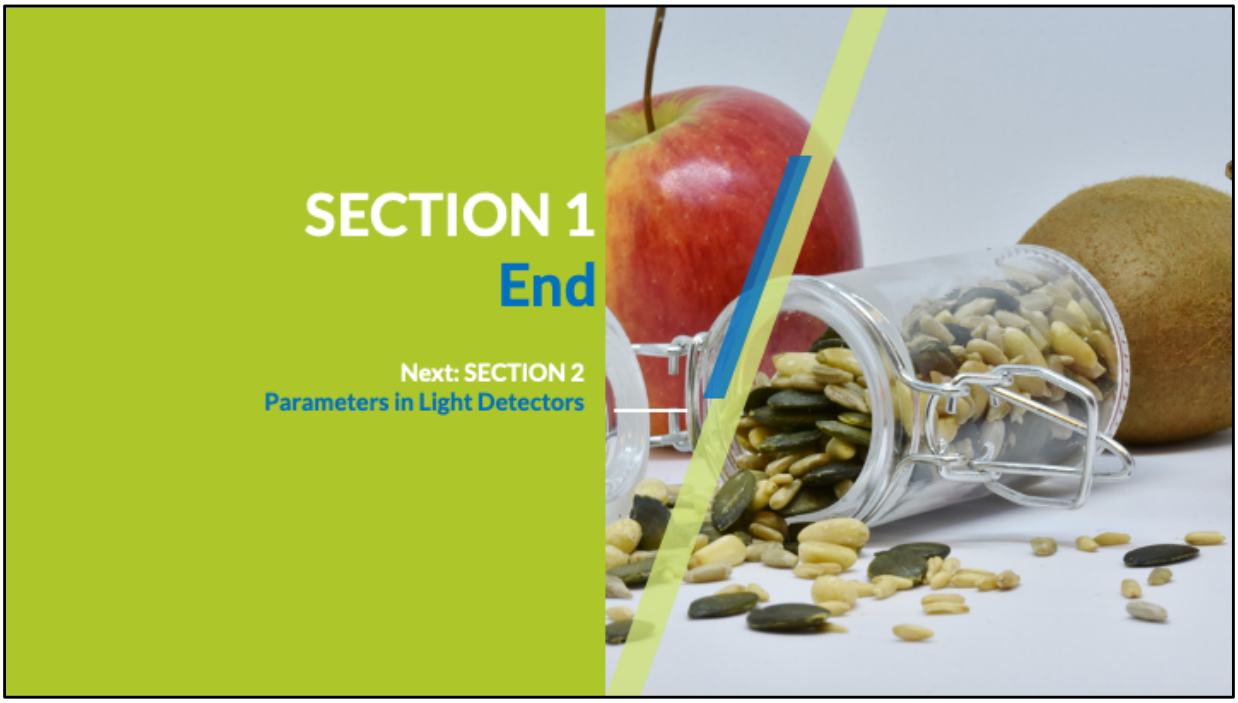
https://www.chromatographyonline.com/view/perfect-method-part-1-what-your-goal-0

https://www.chromatographyonline.com/view/perfect-method-part-ii-where-start

https://www.chromatographyonline.com/view/perfect-method-part-3-adjusting-retention

https://www.chromatographyonline.com/view/perfect-method-part-4-controlling-peak-spacing

https://www.chromatographyonline.com/view/perfect-method-part-6-make-it-faster



You have reached the end of section 1. Section 2 discussed the parameters in light detectors.