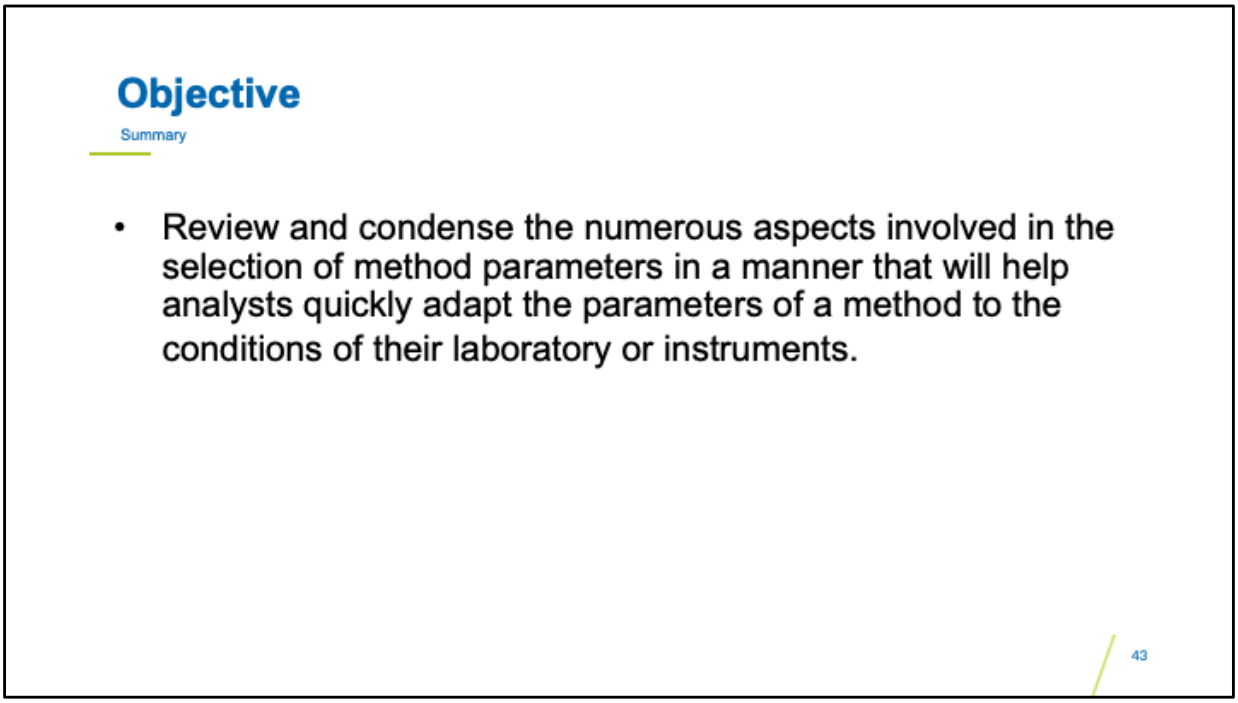
Welcome to Module 7 on instrument operation.

SECTION 3
Parameters Summary



This section aims to review and condense the numerous aspects involved in the selection of method parameters in a manner that will help analysts quickly adapt the parameters of a method to the conditions of their laboratory or instruments. This is accomplished by focusing on the relationships between parameters with the largest impact on the results.

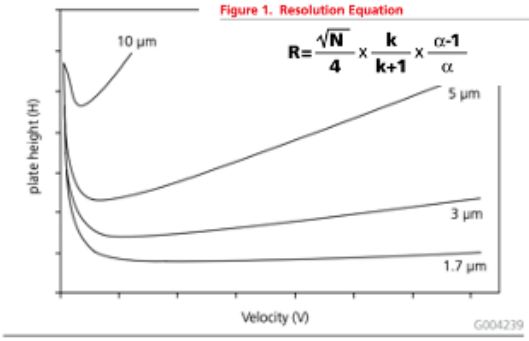
The level of adaptation or modification that is acceptable for an official method will be discussed in module 9, but the focus is placed here on those parameters that can be adjusted in a method without requiring full revalidation. Of course, any changes applied to an official method should be documented to demonstrate that the method is still fit for purpose.

Resolution, Flow Rate and Particle Size

Summary

- Big particles (10µm) – resolution gets bad as you increase velocity
- Medium particles (5µm), some degradation with increase
- <2 µm particle, almost no difference, so can increase flow rate
- We will discuss the resolution equation and van Deemter curve in class

Figure 2. Graph illustrating reduced plate height as a function of mobile phase velocity for several particle sizes



https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Datasheet/t308183.pdf

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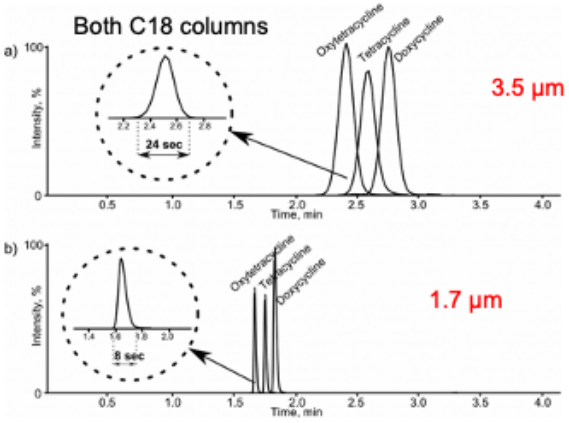
We have seen in module 4 that particle size effects the resolution in liquid chromatography. Generally speaking, smaller particles deliver higher resolution through a greater number of theoretical plates for the same column length. We also saw that increasing the flow rate may reduce this resolution obtained with particles of the same size. The van Deemter curve explains this phenomenon. As the velocity increases, we see from the top curve that the plate height initially decreases, which increases resolution, but quickly starts going up. The following curves for 5 and 3 micron particles, show much slower increase as the velocity increases.

The phenomenon however does not apply to particles smaller than two microns, those used in UHPLC, we can see that the curve stays flat, so one can increase the flow rate of a method without losing resolution.

Particle Size, Resolution and Run Time

Summary

- Reducing particle size increases resolution and shortens run time



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Reducing particle size increases the resolution and shortens the runtime for an analysis. This is the main advantage of UHPLC over HPLC, and the reason for the development of smaller particle size columns for HPLC, going from 10 to 8 to 5 and to 3.5 microns.

The resolution and time gains provided by UHPLC technology are significant advantages for laboratories that can afford both the technology itself and the increased maintenance cost associated with it. However, it may not be an appropriate solution for a laboratory that does not benefit from a stable electrical supply, tightly controlled environmental conditions, such as temperature and humidity level, and sufficient budget. In these cases, it is preferable to look for HPLC columns with smaller particles to benefit from the increased ruggedness of the instrumentation and lower cost of the instrumentation and columns.

Increasing the flow rate has also been shown to reduce the runtime for methods utilizing the same size of particles. This can result in reduced resolution, which may not be concern if the sample contains few analytes, as in the example on the slide. or if the detection technique also acts as a separator such as is the case with mass spectrometry.

Mobile Phase, Temperature and Pressure

Summary

- Temperature decreases viscosity of liquids = reduce pressure
- Pressure is directly proportional to flow rate

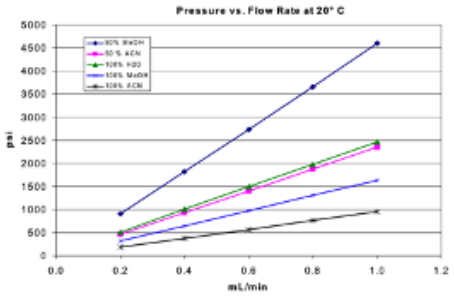

Figure 2. Pressure vs. Flow Rate

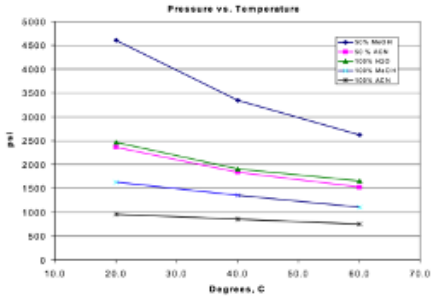

Figure 3. Pressure vs. Temperature

https://www.waters.com/webassets/cms/library/docs/watersamd30.pdf

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Back pressure increases when increasing the flow rate, as shown in the graph on the left, but it may be possible to shorten the runtime of an experiment by increasing the flow rate and limiting the impact on the backpressure by increasing the temperature of the column. Increasing the temperature of the column decreases the viscosity of the fluids and consequently reduces the backpressure in the system, as seen in the graph on the right.

The effect of temperature on the sample analytes and the chemistry of the column should be considered carefully before a large increase in temperature is applied. Columns are rated for a specific temperature range and should not be used outside of these specifications.

The chemical composition of the mobile phase is selected for optimal separation of analytes, but its composition also affects the backpressure of the instrument as increased organic content produces a lower viscosity which reduces back pressure. The HPLC or UHPLC instruments come with a specified working pressure range that should not be exceeded.

Beyond its effect on fluids viscosity, temperature also impacts the retention of analytes on the column by affecting the chemical interaction between the ligands and the analytes. This in turn can affect peak resolution in otherwise

identical method conditions. Official methods have usually gone through significant steps verifying the impact of these parameters and optimizing the selection of the mobile phase composition. So, it is usual for any method adaptation to NOT deviate very significantly from the mobile phase composition defined in the method. For example, the mobile phase composition would generally not be modified to compensate for excessive back pressure in the instrument. Other factors affecting back pressure such as sample cleanup, column age, column cleanliness, and any maintenance issues such as a blockage at any point in the instrument would be investigated instead.

Temperature and Retention Time

Summary

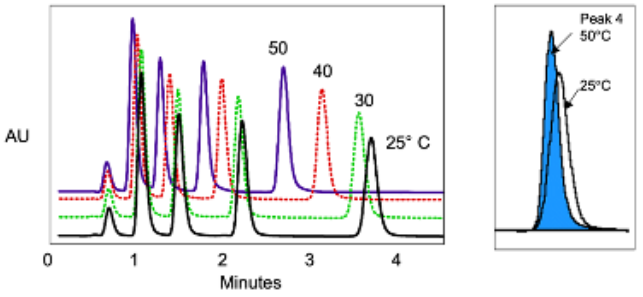

Figure 4. Effect of temperature on retention time and peak shape
Isocratic separation of acetone a C2-C4 alkylphenones in 60:40 methanol-water on a Symmetry® C_{18} 2.1x30mm column

- Increasing temperature accelerates the run
- Also increases sensitivity of ~15% at 50°C compared to 25°C

https://www.waters.com/webassets/cms/library/docs/watersamd30.pdf

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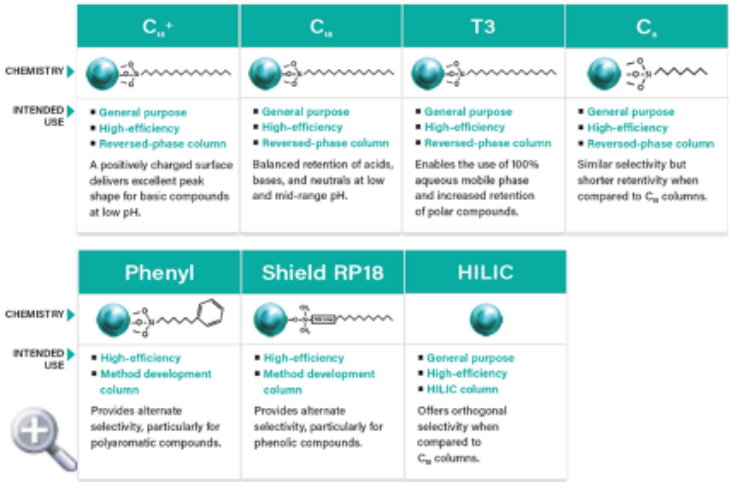
We discussed in the previous lesson how temperature can also affect retention time and how this is one of the reasons we must always run standards to confirm the retention time in our specific conditions. Some of the factors one must keep an eye on when increasing the temperature are the retention time of the first peak following the void retention time of the column to ensure that this first peak is resolved at the baseline, that all peaks resolved at lower temperature still resolved are at the new higher temperature and verify that the narrowing of the peak caused by temperature does not result in saturation of the detector. In other words, we want to ensure that the advantages associated with a more intense and narrow peak, such as increased sensitivity and separation, are not defeated by these side effects.

Column Chemistry and Mobile Phase

Summary

- C18 is most common in food safety



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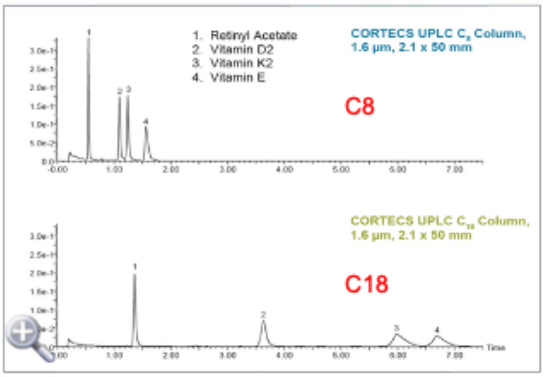
The impact of the column chemistry and the reasons for selecting one over another are critical. Luckily, many of the food contaminants of interest with relations to regulatory limits can be analyzed in relatively similar conditions by liquid chromatography. The C18 column is by far the most common chemistry used because of its wide range of applications and high efficiency in reverse phase chromatography. The C18 column is however not friendly to the use of 100% aqueous mobile phase; in such a case the T3 column would offer the benefits of allowing the use of 100% aqueous mobile phase and increased retention of polar compounds.

Column Chemistry and Run Time

Summary



- The right column could accelerate your analysis significantly
- Don't just use what you have, get what you **NEED**

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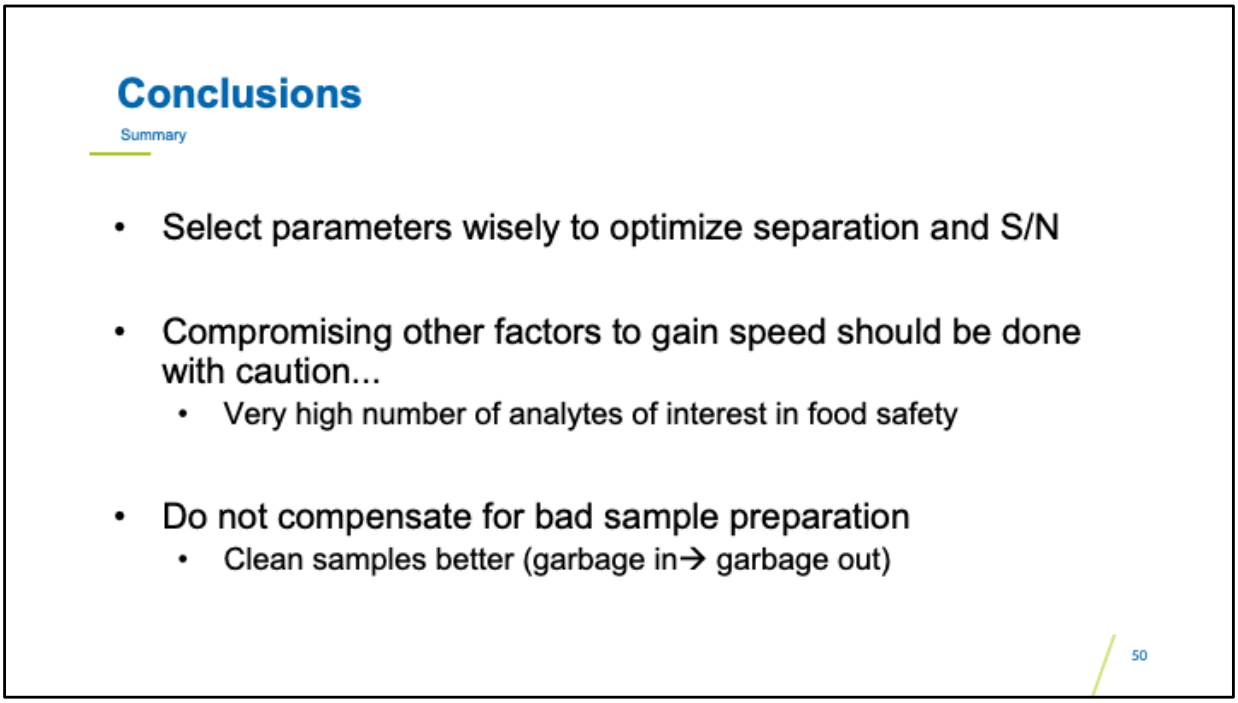
The column chemistry not only influences the separation of components but also the runtime of the experiment. For example, the longer chain of the C18 column promotes increased retention in the column which means a longer runtime then the shorter chain of the C8 column. Consequently, a column chemistry could be preferred for its ability to accelerate the run time when two options provide similarly suitable resolution. It is important to plan the budgets to purchase the columns that we need for a method rather than try to adapt a method to a suboptimal column.



In conclusion, it is important to select parameters wisely to optimize separation and S/N within the restrictions of your laboratory. We usually cannot get instruments with better performance in the timeframe that we need, so you have to optimize with what you have.

Compromising other factors to gain speed should be done with great caution... We deal with a very large number of analytes of interest in food safety, so methods grow in scope all the time. If we compromise on resolution or peak intensity to go faster, we may need to revisit these choices when more analytes are added to the method.

Finally, it is important to not go through all sorts of method optimization experiments to compensate for bad sample preparation... Just clean your samples better. We have a saying: garbage in→ garbage out. If you put dirty samples in your instrument, you will damage your column more quickly and obtain sub-optimal chromatography that will eventually limit the applicability of your method. We will practice sample preparation extensively during the in-person segment of this training.

The inter-connectivity of different parameters is sometimes perceived as an important barrier for new analysts coming into the field of food safety using liquid or gas chromatography. And the hurdle appears even greater when combined with mass spectrometry. We hope that this quick review of a number of the most critical parameters for the implementation of a method in one's laboratory has brought some clarity and will help understand the reasons for the selection of parameters in specific methods.

The next module discusses a few methods used for regulatory purposes.