STA

Methods of Analysis for Organic Contaminants

Chromatographic Instrument Operation

Module 7

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Overview

Introduction

Previous modules 4, 5 and 6 have focused on the technology involved in the HPLC, GC, and mass spectrometer. Module 7 is dedicated to understanding the parameters that must be set in these instruments 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.

Learning Objectives

- 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.
- Summarize in easy-to-understand conversation-style statements the interconnectivity of various parameters important for the execution of official methods of analysis for contaminants.

Lesson 1: LC Parameters

An HPLC method for the determination of food contaminants or other types of chemicals contains many parameters set to optimize both the separation and the detection of these analytes. Table 1 provides a list of parameters that must be considered in the optimization of the method, a part of method development, and verified on one's own instrument.

Table 1: Important LC Parameters

Component	Parameters
Mobile Phase	Composition Additives Flow rate Gradient vs isocratic Degassing
Sample	Composition (post sample preparation) Injection volume and method
Stationary Phase	Column dimensions Particle size Chemistry Temperature
Detectors	Single or series Post-column accessories

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 on reverse phase chromatography as it is the most popular type used in food safety for contaminants analysis. 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 of 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.

When deciding on a mixture of water and organic solvents for an 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 instruments, which would translate into a changing composition depending on how deep the instrument is picking from the bottle. Similarly, solvents used in sequence, or in gradients, must be miscible. 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 operating at around 40 degrees Celsius. There are numerous sources for tables of solvent miscibility; one table was provided in the video portion of this module. Most tables contain a very large number of solvents that are not used in food safety contaminants analysis, but the typical solvents applicable in our fields are present in these tables.

The incremental chromatographic 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 phase determines the retention time in the column and therefore the position of the peak in the chromatogram. In reverse phase chromatography, polar analytes elude the column first and this is independent of the use a gradient or an 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 phase 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.

There is an increasing interest in using highly aqueous mobile phases in laboratories around the world. Aqueous and weak organic mobile phases are safer for the laboratory analysts who are less exposed to harsh chemicals, and safer for the environment. The move to these health and environmentally friendly mobile phases was made possible through the engineering of columns that are well suited for aqueous solvents. The older traditional

alkyl chain media can be prone to phase collapse in less than 5% organic solvent. One other concerning aspect associated with the use of highly aqueous mobile phases is the possibility for microbial growth in the water. It is essential to prevent the growth of bacteria in our mobile phase bottles because it could result in the formation of films that can block the tubing or even the column of the instrument. We typically employ additives to prevent microbial growth, such as sodium azide, or mix a small amount of organic solvents in the water bottle (typically around 5%). Obviously, the addition of organic solvents in the water bottle means that the percentage of each bottle in the gradient design doesn't correspond to the percentage of organic solvent exactly.

While the selection of the solvents for the mobile phase is important, it is not the only parameter that will affect the efficiency of separation, and in some cases, of detection. Buffers are 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 risk 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 aqueous 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 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 salt that are best solubilized in water.

The mobile phase is pushed through the chromatography system using a system of pumps that delivers 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 right in order to

accelerate the analysis 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 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 above. 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 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.

Gas bubbles present in the mobile phase can cause noise spikes in the chromatograph. 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 the gassing 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 between 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. The inline degasser is different from the online version as it is typically a separate box.

As presented in the video portion of this training, the selection of an isocratic or gradient run is dependent on the number of components to separate by chromatography, the complexity of the sample itself, the column,

and the amount of time that we are prepared to spend on the chromatography. Even if one mode is preferred over the other, there typically is a number of combinations that will work. The rule of thumb is that simple mixtures of few components of interest perform very well with and 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 polarity in a shorter amount of time and consequently causes the illusion of analytes of interest to proceed faster.

Sample

The sample composition plays a critical role in chromatographic 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 tried to match the sample diluent with the starting composition of the mobile phase. If it is not possible to match the sample diluent with the mobile phase, then the sample may need to be injected in smaller volume to minimize the effect of the diluent. This can negatively impact the sensitivity of the method (LOQ and LOD).

The sample volume can further impact the quality of the chromatographic 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 in the linear range of the method. This is no longer true when the volume is so large that the sample spreads into the volume of the column and consequently elutes as a very broad band or causes the saturation of the detector. 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 elude at close retention times is too high, the peak width resulting from this high concentration may cause the peaks to merge and 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 maxima or centers of mass of the peaks.

Column

We reviewed important aspects of column selection in module 4 and discussed that generally, 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. We discuss these topics and their inter-related impacts in this section.

Column Dimensions

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 (*i.e.* selectivity or separation capability). 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 very good 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 a larger number of analytes, a longer column with a higher flow rate provide 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 short column with a slower 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 the chromatographic method. The concentration range can be quite narrow, such as expected in the determination of pesticide residues in an environment where good agricultural practices are applied, while a very broad range of concentrations that can reach three or more orders of magnitude may exis, 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.

The second dimension of the column is its diameter. A smaller column diameter is typically considered better because it can produce narrower peaks due to the smaller amount of lateral dispersion of the sample in the column. However, for the same packing material, narrower column will cause an increased back pressure and will consequently require a stronger pump to circulate the mobile phase through the column. To optimally benefit from the narrower column with higher peaks, the analyst must pay attention to detector saturation when evaluating the suitability 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.

The rule of thumb[1] about column dimension is that the injection volume must be smaller than 10% of the column volume.

Particle Size

The column packing material is described as the column chemistry and particle size. Both are also an important factor in the selection of the column. Larger 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 accommodates 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 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 flow the sample and mobile phase. As the particles get smaller, the interstitial space in which the mobile phase and sample must flow also 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.

[1] A rule of thumb is a guide based on experience

The particle size limit that characterized HPLC systems prompted changes in the engineering of UHPLC system to allow the use of sub 2-micron particles. It is beyond the scope of this training to discuss the materials, connections and pump differences between the two types of systems and the reader is referred to Waters' UPLC Primer[2] for more details.

Chemistry

Column chemistry must be selected as a function of the analytes being separate, the compatibility of pH tolerances and requirements for both the column and sample analytes, compatibility with the mobile phase and the targeted 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.

Table 2: Sample characteristics to consider for the selection of the column chemistry

Characteristic	Consideration
Analytes structure	Polarity Column lifespan
Analytes hydrophobicity	Partitioning between aqueous and organic phase (LogP value > 0 or <0)
Number of analytes	Resolution, peak width, theoretical plates (mostly affects column dimensions and particles)
Analytes pKa	pH range

The column chemistry is almost exclusively selected based on characteristics of the sample analytes. Matrix components remaining in the injection sample should also be considered, but there usually is no compromise for them and any problems they cause are solved by cleaning up the sample better (*i.e*.

[2] Available at: https://www.waters.com/waters/en_US/Primers/nav.htm?cid=10048920&locale=en_US

modifying sample preparation). As mentioned above, the concentration range and number of analytes impact the selection of the column dimensions. In this section, we review some of the most important factors considered in the selection of the column chemistry.

Analytes structure is the most important consideration because the column chemistry must affect the travel speed of the analytes differently. Analytes hydrophobicity is determined by the LogP value. If LogP > 0, meaning that the analyte is hydrophobic, reverse phase chromatography is preferred and the column chemistry is therefore more non-polar. If the analytes are charged, *i.e.* LogP value below 0, hydrophilic liquid chromatography (HILIC) columns are better suited. Food safety contaminants analysis overwhelmingly uses reverse-phase mode. The most common ligands for reverse phase mode were reviewed in the online portion of this module and will not be discussed here.

We focus on C18 columns, which are the most common in the food safety laboratory because they generally offer an appropriate pH range for operation, have a good durability in the relevant conditions, and offer broad range selectivity and retention. Moreover, the core-shell C18 are physically homogeneous, which results in reproducible retention times. As discussed in the online document, free silanol groups characteristic of these columns can be a good or a bad thing for the retention behavior of the column. Silanol groups can cause peak tailing for basic compounds, but they can also improve the retention and resolution of other analytes. End-capped C18 columns have a secondary bonding that limits the free silanol groups and may also involve additional polar groups to change the separation characteristics of the column. There is no single column associated with any group of chemical contaminants and the choice is really driven by method performance parameters such as the resolution needed (*i.e.* the selectivity), the time we are prepared to spend on the chromatography, and the commercial availability and cost of the columns.

Selectivity is calculated as the ratio between the retention factors of two analytes in a given column (all other conditions must be the same). The retention factor is calculated according to the following equation:

$$k = \frac{t_R - t_0}{t_0}$$

Where k is the retention factor, t_R is the retention time of the analyte and t_0 is the column void time.

The column void time is the time “spent” on the column void volume, which is the volume of empty space not occupied by particles. It is dependent on the flow rate and calculated as V/F, where V is the column void volume and F is the flow rate. Column manufacturers will generally provide either the value of V, or the pore volume and internal dimensions of the column needed to calculate the column void time. Note that they cannot provide the void time itself because it depends on a user-selected parameter, *i.e.* the flow rate. Obviously, the column void time should not contain any peaks; in general it is agreed that the first peak of a chromatogram should be at a retention time of at least twice the column void time.

As indicated above the selectivity is the ratio of the retention factors between two peaks.

$$\propto = \frac{k_2}{k_1}$$

Where alpha is the selectivity and k are the retention factor of the two analytes of interest.
It is important to use two analytes that we expect to find in our samples and that have a similar structure in this calculation, *i.e.* that elute close to each other or could even elute at the same retention time in sub-optimal conditions. What we aim to express with the selectivity is the separability of two closely related analytes.

As discussed previously in the section on the mobile phase, the interaction between the stationary phase the mobile phase and the analytes all affect the separability. A greater difference between the interactions of the analyte with the mobile and with the stationary phase will result in a greater separation in the chromatograms. Consequently, the selectivity is not only influenced by the column chemistry, but also by the selection of the mobile phase and the flow rate. Nevertheless, column chemistry impacts selectivity. Among the variety of C18 columns available, the decision is based on the ligand used and ligand density, which must be matched with the analytes of interest, for example pesticides or persistent organic pollutants, and provide the most appropriate selectivity needed for the purpose and depending on the detection technique. These topics will be discussed extensively during the in-person segment of this training.

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 analytes. The rule of thumb in this case is that an increase of temperature of 1 degree Celsius decreases the retention time by about 1 or 2%. Increasing the temperature can therefore be a way to accelerate an analysis. However, 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.

Detector

While the detector is a completely separate component in the chromatography system, the selection of the column must consider which detector will follow. For example, at detector with no mechanism for selectivity, such as a detector that counts photons or measures current, means that the chromatography must produce peaks that represent the elution of a single analyte. Indeed, if the peak resulted from more than one analyte, then the peak area would not be indicative of either the presence or the concentration of any single one of the analytes characterized by this retention time. In addition, these non-selective detectors impose an absolute necessity for baseline-resolved peaks, in other words these methods cannot tolerate overlapping peaks. Selective detectors such as the mass spectrometer, or in certain circumstances fluorescence detectors, can measure a specific analyte of interest in isolation by either not producing any signal for other analytes that don't fluoresce at the methods wavelength, or by preventing analytes from progressing through the system as is the case in mass spectrometry.

Another important consideration is whether it is possible or useful to combine detectors to obtain different types of information from the same eluent. The normal practice is to use a single detector because it limits the length of tubing post column, which in turn effects the dispersion of the chromatographic peak. 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 uncommon to see more than one detector used for regulatory analysis, and this type of setup is more appropriate in the research laboratory.

It is important to note here that a mass spectrometer, while described in this training as a detector, may be used as a filter, and in such case, precede the actual detector. We discussed the example of the QTOF system in module 6, where the quadrupole acts as a filter for the time of flight measurement and nonspecific detection.

Post-Column Accessories

Some analyses require the use of accessories added 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 and B2. 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 therefore reduce the sensitivity of the method. As we have discussed before, peak broadening translates into lower signal to noise ratio, higher limit of quantitation, higher limit of detection and the possibility of overlapping peaks.

Lesson 2: Parameters in Light Detectors

Light, or photometric, detectors are very popular in the food safety laboratory because of their relatively low cost and broad applicability. Fluorescence, UV and visible detectors are some of the default detectors associated with liquid chromatography instruments. Method development with these

detectors is straightforward; the analyst determines the best wavelength to use by running a wavelength scan and identifying the wavelength or wavelengths at which maximum absorbance or fluorescence occurs. A routine method would then use that single wavelength for the detection and quantitation of the target analyte. For most food safety laboratories, this development work has been done elsewhere and the official method states the wavelength to use. In this section, we very briefly review the principle of these techniques in order to understand the parameters that affect analytical results.

UV-Visible Detectors

The absorption of ultraviolet light occurs when the energy applied to a molecule causes some of its electrons to jump from their ground state to an orbital at higher energy. This is called the excited state. The detector therefore measures the loss of UV light from the source as it went through a sample and was absorbed, and presents it inverted as a peak. UV detectors are very common and can be in the form of a diode array detector called DAD or PDA, which stands for photodiode array. The advantage of the arrays is to offer a wavelength range instead of a single or small selection of few wavelengths for data acquisition. While only a single or a few wavelengths are required for any given method, the array broadens the range of applicability of the instrument to more methods that aim to measure different analytes with different UV absorption characteristics.

Being a very simple detector, there are only a few parameters that affect the signal reported in the chromatogram. The selection of an appropriate wavelength is critical to obtain any signal at all, but the intuitive selection of the wavelength of maximum absorption, while usually optimal, may in certain cases not be. This would happen if two analytes absorbing UV light at nearby wavelengths are targeted by the analysis. It would be best for selectivity to use wavelengths at which there is no overlap in signal. The sensitivity of the method is lower when not using a peak maximum, but the benefit of using a simple and inexpensive detector can outweigh this loss for analystes present at higher concentrations. Figure 1 illustrates this example.

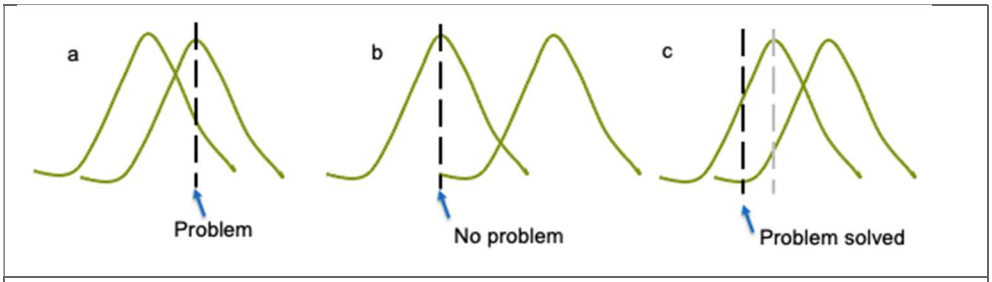

Figure 1: The wavelength measures a) contributions from two components; b) contribution from a single component without interference; and c) contribution from a single component by moving away from the common absorption wavelength range.

A method developed to measure a larger number of analytes has an increased risk of seeing overlapping bands. Overlapping bands can be separated by changing the column chemistry or the mobile phase composition, or both. This essentially corresponds to going from the chromatogram depicted in Figure 1a to the one depicted in 1b. If it is not possible to modify them, a measurement could be made away from the top of the absorption peak, where the second component does not absorb. As mentioned above, the disadvantage of this approach is that it reduces the sensitivity of the method and consequently increases the limit of detection and limit of quantitation. Flow rate and column length also impact overlap; a column that is too short may not allow sufficient interaction with the stationary phase to cause the separation of the two peaks. Similarly, a mobile phase moving at an excessively high flow rate will prevent these interactions or at least limit them. Slowing down the flow of the mobile phase may result in the separation of the peaks and using a longer column could lead to the same outcome (*i.e.* going from a to b in Figure 1). When it is not possible to change the column chemistry or the column length, the chemistry of the mobile phase and the flow rate should be considered first before settling on using a wavelength of lower sensitivity (Figure 1c). If using a wavelength of lower sensitivity is the only solution, it may be possible to increase the pathlength of the cell, which results in an increased signal intensity and lower limits of detection and quantitation. However, this is best done when it is possible to increase the pathlength without widening the diameter of the cell because this would cause peak broadening and possibly increased overlap of the bands. The regulatory requirements for the confirmation of identity of a contaminant will be discussed further in module 9, but briefly, for UV-visible detectors, a confirmation of identity would require the use of either two different chromatography columns or two chemically-independent wavelengths of absorption to deliver the level of certainty required for regulatory action.

Fluorescence Detectors

Fluorescence is emitted when a higher energy short wavelength of light is absorbed and causes a lower energy longer wavelength burst to be emitted. Few compounds fluoresce naturally so derivatization is common in methods using fluorescence detectors. Generally, pre column derivatization accessories are easier to install but can be damaging for the column. Post column derivatization can be a little more complicated to install but it is often preferred because it ensures a longer column lifespan. This is an important consideration because of the high cost of chromatography columns.

Fluorescence is very common for contaminant analysis because it is a very sensitive technique. It is characterized by a high signal to noise ratio which enables the use of faster flow rates and delivers suitably low limits of quantitation. Fluorescence is considered very selective because few components are naturally fluorescent. However, interferences from matrix components that may result in diminished fluorescence or fluoresce themselves impose a requirement for sample preparation steps that create a very clean sample. The complicated matrix characteristic of food safety applications often requires that we use sample preparation methods that are either time consuming or expensive or both. In the case of aflatoxins for example, the extract from corn must be purified through an immunoaffinity column or at least a highly selective chemical clean-up column to remove all matrix components that are naturally fluorescent. In general, fruits and vegetables must go through extensive cleanup before they can be measured using fluorescence detection because they contain chlorophyll which displays a really strong fluorescence that would overwhelm most peaks of interest.

The analysis of aflatoxins using fluorescence provides an example of the impact of the type of chromatography used ahead of the detector. When HPLC is used, it produces relatively broad peaks that do not allow for appropriate limits of detection and quantitation to be achieved without the need to derivatives aflatoxins B1 and B2. However, using UHPLC can remove the need for derivatization because it produces very tall narrow peaks, *i.e.* more concentrated elution. The UHPLC narrow band is not by itself sufficient to produce the signal intensity needed to reach the LOQ and LOD necessary for aflatoxins testing, but combining the UHPLC with a long path fluorescence cell solves the problem.

In fluorescence measurements, a confirmation of identity for regulatory purposes would require the use of either two different columns or two different fluorophores to deliver the level of certainty required for regulatory action.

Conclusions

Once again, there is no “one-size-fits-all” solution That can satisfy the breath of requirements of food safety contaminants analysis. A detector must be chosen on the basis of its selectivity, sensitivity, the cost of the detector itself and any costs associated with special sample preparation needed, and the amount of time available for the analysis. The detector and HPLC parameters must be adjusted to obtain the best signal to noise ratio peak resolution and peak isolation. It is important to also monitor the deterioration of the signal as detectors age as they may require adjustments in the method parameters to compensate for peak distortion or reduced intensity. Similarly, method parameters are likely to need adjustment when a new detector is installed.

Lesson 3: Parameters Summary

This lesson aims to recapitulate 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 own laboratory or instruments. We will not reproduce the graphics presented in the online portion of this training, but rather focus on delivering a compact set of considerations that will enable efficient and effective method adaptation. This is accomplished by focusing on the relationships between parameters with the largest impacts 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 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

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 the resolution obtained with particles of the same size. This is true for the particles found in HPLC systems, *i.e.* approximately 5 micron particles. This phenomenon does not apply to particles smaller than two microns, those used in UHPLC, so one can increase the flow rate of a method without losing resolution. The van Deemter curve that describes this phenomenon is shown in online module 7 lesson 3 and will be discussed in the in-person portion of this training.

Resolution, Particle Size and Run Time

Reducing particle size increases the resolution, allows the use of a shorter column and consequently 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 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 and level of technical specialization 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 or 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 both the instrument 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 or if the detection technique also acts as a separator such as is the case with mass spectrometry.

Mobile Phase, Temperature and Pressure

Increasing the temperature of the column decreases the viscosity of the fluids and consequently reduces the backpressure in the system. Back pressure increases when increasing the flow rate so 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. The effect of temperature on the sample analytes and the chemistry of the column should be considered 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 mobile phase composition also affects the backpressure of the instrument as increased organic content produces a lower viscosity, which reduces back pressure. The HPLC or UHPLC instrument comes with a specified working pressure range that should not be exceeded. The chemical composition of the mobile phase also needs to be

considered in the selection of the operating temperature in addition to its impacts on back pressure (and obviously most importantly on the separation on analytes) because it affects the miscibility of solvents.

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 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 pressure such as sample cleanup, column age, column cleanliness, and any maintenance issue such as a blockage at any point in the instrument would be investigated instead.

Temperature and Retention Time

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, ensuring that all peaks resolved at lower temperature still 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, the advantages associated with a more intense and narrow peak, such as increased sensitivity and resolution, should not be defeated by the above mentioned side effects.

Column Chemistry and Mobile Phase

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 HPLC. 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

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 an 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.

Conclusions

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, which appears only greater when combined with mass spectrometry. We hope that the 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. These will be discussed extensively in the in-person portion of this training.