Online Training Program

Methods of Analysis for Organic Chemical Contaminants in Food

Liquid Chromatography

Module 4

Janie Dubois, Ph.D.

Food Safety Specialist

parera
Food Risk Analysis and Regulatory Excellence Platform

INAF
INSTITUTE OF NUTRITION AND FUNCTIONAL FOODS

UNIVERSITÉ LAVAL
Faculty of Agriculture and Food Sciences

Table of Contents

Introduction

This module briefly reviews the fundamental principles of chromatography in general, the mode of action for different types of chromatography and goes deeper into the details of liquid chromatography (LC). The main parts of the LC system are presented, and a selection of detectors described. The focus of this chapter is on the application of these techniques in food safety for the detection and quantitation of organic chemical contaminants.

This module is designed as a part of a training program also including online and in-person components. As such, this text contains information that is complementary to the other portions of the training. It should not be used as a stand-alone reference.

Learning Objectives:

- Understand the principles of the analytical technique of liquid chromatography
- Understand the detection techniques used with liquid chromatography
- Understand the reasons why liquid chromatography is well-suited for food safety applications

Lesson 1: General chromatography

Chromatography is a technique that separates the components of a mixture. It can be used with solutions, suspensions and samples that are in vapor form. The technique was developed by Tswett in 1903[1] as a means of separating plant pigments. He devised a system that essentially used gravity and eventually a manual vacuum pump to flow a plant extract through a glass column filled with a medium, specifically powdered chalk or alumina. The differing levels of interaction between the packing material

[1]1. Ettre, L.S., *M.S. Tswett and the Invention of Chromatography*, LCGC North America (2003) Vol. 21, No 5., pp458-467 Available at: https://cdn.sanity.io/files/0vv8moc6/chroma/1d8bd9c34045ef61d93b9002b40e9335d19de2ed.pdf (Accessed 09/20/20)

and the components of the solution caused the separation of the pigments as the mixture progressed through the column and he was able to isolate them by changing the collection bottle as the color of the liquid dropping out of the column changed.

The initial experiments had a direct influence on the name chosen for the analytical technique. Chroma and graphein come from the Greek words that mean “color” and “to write”. Simply put, the technique could efficiently separate colors, or pigments. While the development of column technology and coupling with highly precise pumps has dramatically increased the number of chemical molecules that can be separated and the applicability of the technique, the principle has remained the same.

Chromatography is an essential analytical tool in food safety. Foods are very complicated matrices that are composed of proteins, water, lipids, carbohydrates, vitamins, minerals, even heavy metals at times, etc. The discipline of food safety is one that focuses its attention on either the nutritional value, which measures components present in relatively large quantities, or the contaminants that may also be present. The latter are typically found in very small quantities expressed in parts per million, per billion and even per trillion. Contaminants must therefore be isolated from the food matrix for detection and quantitation to reduce the challenge of the “needle in the hay stack”. Liquid chromatography performs this task very well and has gained popularity in contaminants analysis because it provides reliable results with relatively simple sample preparation. This can be thought of as finding the needle in the hay stack with only a few steps of pre-sorting. This concept will be discussed in more detail in Module 8 when we go through individual methods.

Throughout this and subsequent modules, we will use terminology related to the technique of chromatography. First and foremost, chromatography is the laboratory technique. A chromatograph is the instrument or the equipment we use, and it produces a chromatogram, which is the resulting chart.

Types of Chromatography

There are different types of chromatography, *i.e.* methods of separation based on the use of a medium where components move at different rates. Table 1 lists the types of chromatography that are suited for samples in different states.

Table 1: Types of chromatography appropriate for different sample states.

Sample state	Type of chromatography
Solution	Liquid chromatography Ion exchange chromatography Thin layer chromatography
Suspension	Size exclusion chromatography
Gas	Gas chromatography

The mechanism causing the separation within the medium is also a means of separating the different types of chromatography. Table 2 summarizes the mechanisms of separation and types of chromatography.

Table 2: Types of interactions that define different types of chromatography.

Interactions	Type of chromatography
Binding	Affinity chromatography
Charge	Ion exchange chromatography
Hydrophobicity	Hydrophobic interaction chromatography
Size	Size exclusion chromatography

Affinity chromatography uses specific interactions between molecules as the basis for separation. Interactions can be relatively weak and non-specific, such as attractions caused by polarity, or they can be extremely specific and quite strong, such as the interactions between antibodies and antigens. The main advantage of affinity chromatography is that it uses the structure, or the biological function, of the molecules and does not require a chemical modification. This means that the molecules are intact when they exit the column and can be measured quantitatively or used for biological processed or chemical reactions. Affinity chromatography is consequently a very useful technique for purification. We use it in food safety both as a sample preparation step, *i.e.* isolation of contaminants prior to their injection into an analytical instrument, and for identification and quantification of contaminants using an appropriate detector following the chromatographic separation. Many of the methods discussed in Module 8 use this principle of separation.

Ion chromatography is based on electrostatic interactions between opposite charges. Because it works with charged ions, this type of chromatography can be very useful to separate molecules that are very

similar by manipulating their charge. This is achieved by varying the pH of the solution. In the case of proteins for example, the isoelectric point of the protein is the pH at which the net charge of the protein is zero. We can cause the charge at the surface of the protein to become predominantly negative by raising the pH of the solution above the isoelectric point. We can do the opposite by lowering the pH. The protein would be more or less attracted to the stationary medium in the column depending on the resulting charge. Ion chromatography is used in food safety for the measurement of many acids, sugars, sulfates, phosphates, nitrates and chlorides, minerals in water and sometimes even for the measurement of some pesticides (polar ionic pesticides).

Hydrophobic interactions chromatography is based, as the name says, on the hydrophobicity of molecules. This is another parameter that can be manipulated, in this case using pH but also salt concentration, temperature and organic solvent concentration. This principle of separation is used in liquid chromatography for polar analytes. It is heavily used in the fields of proteomics, metabolomics and now foodomics. Foodomics is the study of chemical compounds in food aiming to define their nutritional properties using analytical tools and advanced mathematical processing specifically developed for chemistry (chemometrics). The main advantage of hydrophobic interactions chromatography is its ability to work with polar molecules.

Size exclusion chromatography separates particles in a suspension based on their size. It is important to keep in mind that some chemical molecules are large enough to form a “particle”; one example of this is proteins. The mode of action of this technique is based on the ability of small particles to enter the pores of the column medium and thereby be “slowed down” in their progression compared to particles that are too large to fit in the pores. There are no chemical interactions at play, it is solely about size in this case. The advantage of this technique for food safety is that it can be used as a sample preparation or purification technique. For example, we can use salt as a means to precipitate unwanted components in our sample and follow up with size exclusion chromatography to remove the salt.

In conclusion, chromatography, especially liquid and gas chromatography (Module 5), are used extensively in contaminants analysis for food safety. The main reasons for the popularity of liquid chromatography are the simplicity of sample preparation methods (and the availability of kits to further simplify them) and the flexibility of modern chromatography columns that enable simple methods to be applicable to a broad scope. This is achieved by exploiting the benefits of affinity, ion, hydrophobic

interactions and size exclusion chromatography applied to liquids and gases. This translates into the ability to test for large numbers of contaminants simultaneously and consequently significant time savings.

It is recommended to review the online materials of Module 4 Lesson 1 before moving to Lesson 2. It contains animation that clearly depicts the modes of separation, which must be understood as they constitute the basis of further information.

Lesson 2: Basic Principles of Liquid Chromatography

We defined the principles of chromatographic separation in the first lesson, and introduced the technologies adapted to the various states of samples. In this lesson, we focus on liquid chromatography, one of the cornerstone techniques of the food safety laboratory. This brief introduction illustrates the relationships between the various components of the modern liquid chromatography system. As a reminder, this text should be considered in conjunction with the video created for this lesson and will be complemented by the in-person laboratory training sessions.

First and foremost, it is important to understand the difference between LC, HPLC and UHPLC. These acronyms all represent liquid chromatography as an analytical technique, but they also inform about the performance expectations of the systems. LC refers to any type of liquid chromatography, which can be a vertical glass column filled with particles or salts, such as used by Tswett and in solid-phase extraction sample preparation steps (example in Module 8). HPLC stands for High Performance Liquid Chromatography or High Pressure Liquid Chromatography. The interchangeability of the terms performance and pressure is indicative of how this higher performance is achieved*, i.e.* with pressure. My point of view is that pressure is the engineering term while performance is the marketing one… UHPLC stands for ultra-high pressure (or performance) liquid chromatography.

Components of the HPLC System

Put simply, the HPLC system is a set of tubes where a solvent, mixture of solvents or gradient of solvents mixes with a sample and flows through a column packed with a separating medium and to a detector that can either simply determine the amount of signal generated at any given time during the experiment, or add some information useful for the identification of the sample and its components. The “magic” happens as a result of clever engineering and a deep understanding of the chemicals that need to be separated in various disciplines such as pharmaceutical, biopharmaceutical, food and petrochemical products to name only a few.

The solvent, mixture of solvents or sequence (gradient) of solvents are called the mobile phase. As the name indicates, it is what makes the sample mobile, *i.e.* move through the system. In LC, the mobile phase interacts with both the solute (the sample) and the stationary phase and, therefore, can have a powerful influence on the separation.

The stationary phase is the chromatographically retentive immobile phase involved in the chromatographic process. The stationary phase in LC can be a solid, a bonded, an immobilized or a coated phase on a solid support, or a wall-coated phase. We often speak of particles when describing the stationary phase of column liquid chromatography, while coating is more appropriately used for thin layer and gas chromatography. For example, a silica gel coated on a metal, glass of plastic plate is used in thin layer (*i.e.* adsorption) chromatography and octadecylsilane-bonded particles are used in reversed-phase chromatography. We will discuss the composition of the stationary phase both in this introductory module and in the instrumentation section of methods.

Most HPLC applications for contaminants detection in food safety use affinity mechanisms, more specifically polarity, to separate components of interest. The columns contains particles with either a polar or non-polar bonded chain and the solvents of the mobile phase have the opposite polarity. These topics will be discussed further in the section dedicated to columns.

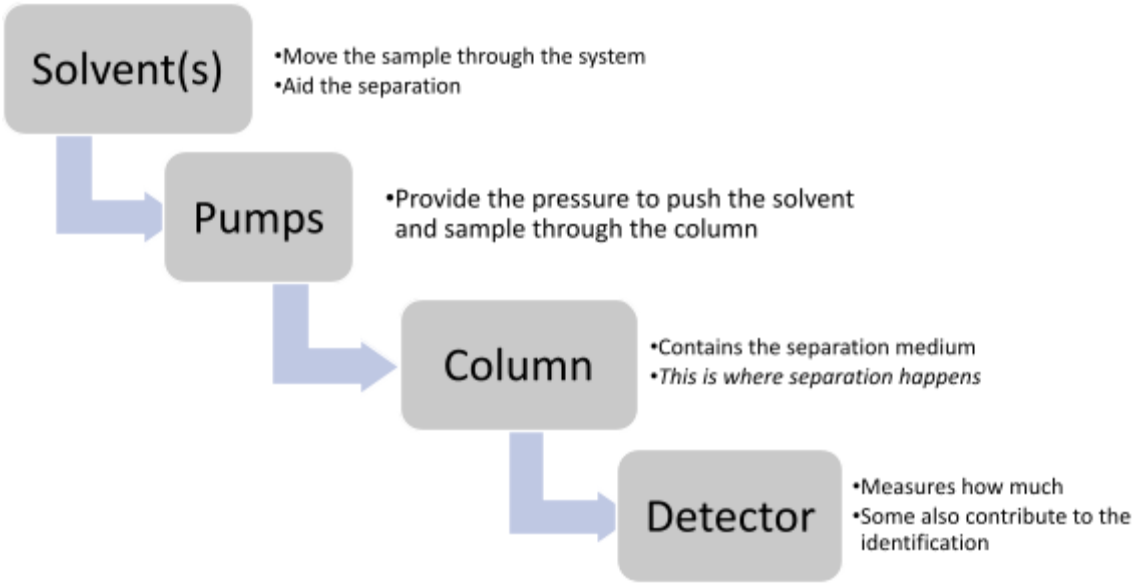


Figure 1:

Schematic representation of the main components of an HPLC system

Finally, a detector is used to convert the presence of solutes, other than the solvents coming out at the end of the column, into a measurable signal. The mobile phase sometimes also translates into a signal, but this is a source of what we will describe as interferences later on and definitely not an objective in the selection of a detector. The simplest form of detectors converts the presence of any chemical component other than the solvent(s) into a signal; these are non-specific detectors. Other detectors only measure certain chemicals and are therefore more specific. There are also different levels of specificity within this category where, for example, a fluorescence detector would measure only molecules that emit light at a specific wavelength range in response to an excitation wavelength, but also all molecules that emit in the emission range in response to the excitation wavelength. So while it would create a signal from select molecules of interest, there could be interferences from other compounds found in the sample that can reduce our ability to measure the compounds of interest, sometimes completely. An example of a more specific detector is a mass spectrometer that actually will only allow charged molecules of a certain mass-to-charge ratio to be measured, or the tandem mass spectrometer that will add another layer of specificity. These will be discussed in more details in the section on detectors and in Module 6.

The brain of any chromatograph is the column; it contains the medium causing the separation, but also is defined by a number of physical dimensions that impact separation efficiency. Moreover, it is the column that determines the range of workable pressure, which defines the limits of flow rate, as well as chemical compatibility, in turn defining the mobile phase options. The chromatograph was originally simply a vertical column, as illustrated in the video portion of this lesson. As the potential applications of the technique were discovered, a number of mechanisms were progressively added to improve extraction speed, efficiency and completeness. For example, longer columns showed the ability to separate more closely related compounds that eluted together in shorter columns. Of course, a longer column translated into a longer experiment as the flow was often only driven by gravity. When pumps were added, initially manual pumps, the length of the experiment was shortened. However, time was not the only benefit of pumps, and probably not the most valuable benefit. Indeed, pumps enabled the passage of liquid through more tightly packed columns that would be clogged, or at least very slow, if the flow was only aided by gravity. The addition of computer control to the pumps further increased the benefits by enabling the use of different solvents, in what we call a gradient run, and improving reproducibility of experiments through computerized control of the flow.

The result of a chromatography experiment is a chromatogram, or the graphical representation of the intensity of the signal from the detector as a function of retention time in the column. When compounds that generate a signal are present in sufficient quantity, they show in the chromatogram in the shape of a peak with a normal distribution. We identify the compound(s) giving rise to each peak by comparing the retention time (*i.e.* the time at which the compound eluted from the column) with that of standards ran in the exact same conditions on the same column.

Normal and Reverse Phase Chromatography

As mentioned previously, most of the liquid chromatography done in food safety uses a column packed with particles and a separation process based on affinity, more specifically on polarity. This process can be used in two opposite manners named normal and reverse phase chromatography. In normal phase chromatography, the stationary phase is more polar than the mobile phase. This means that polar molecules will have more affinity with the stationary phase and therefore will take longer to elute than less polar molecules, as depicted in Figure 2 provided by Waters. In reverse phase chromatography, the stationary phase is less polar than the mobile phase.

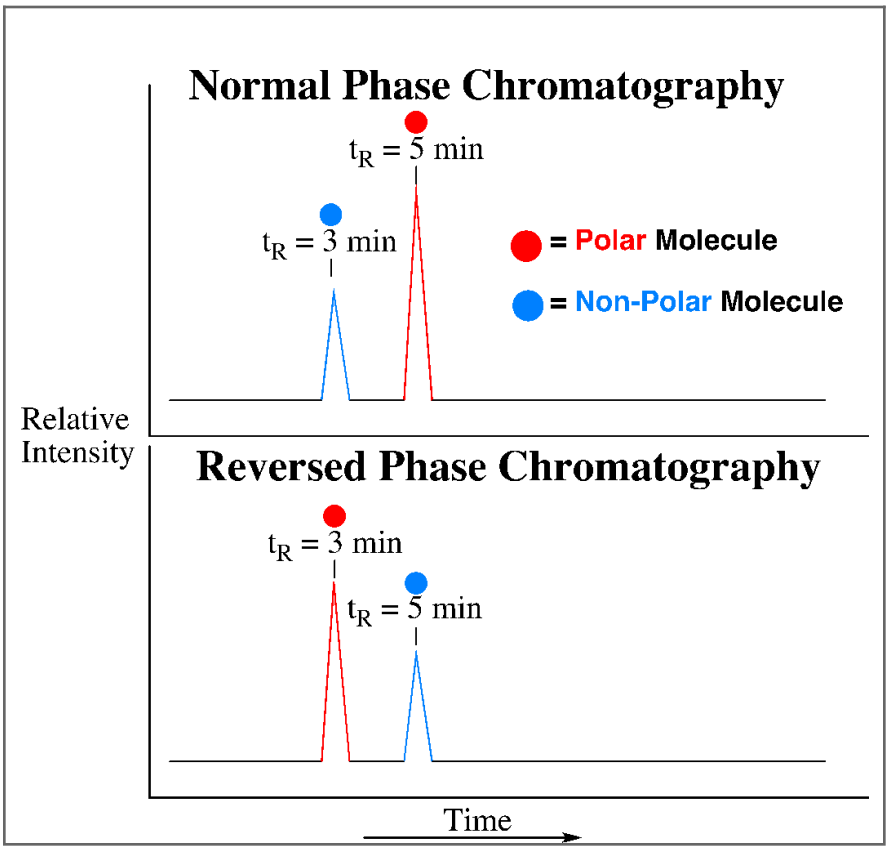


Figure 2: Typical chromatograms obtained in normal and reverse-phase chromatography (reproduced with permission from Waters, Inc.)

The selection of normal or reverse phase depends most importantly on the compounds to be separated, but can be influenced by availability of solvents and columns.

Mobile Phase

The mobile phase can be a single solvent, which can be a mixture such as 70% methanol or a 3:2 mix of methanol and acetonitrile, run through the column during the entire experiment. In this case, we call it an isocratic run. The polarity of the mobile phase is the same throughout the experiment in an isocratic run. This is widely used for the separation of relatively small numbers of compounds but it can lead to very long experiments when there is a large span of polarity. Indeed, the molecules more strongly attracted to the stationary phase may take a very long time to elute if the polarity difference with the isocratic mobile phase is large. Changing the composition of the mobile phase can be sufficient to solve this problem. When it does not create the separation needed, it is best to vary the polarity of

the mobile phase as the run progresses. This manipulation of the polarity of the mobile phase enables the scientist to influence the retention time of all compounds. Put simply, the scientist developing the method can accelerate the gradual change in polarity of the mobile phase to shorten the period of time allocated for a certain range of polarity where there may be a single peak of interest or no peak at all, while slowing down the polarity change to allow more closely related molecules to elute separately. These types of “designer runs” are called gradient runs and they can be more or less complicated depending on the task. In particular, they can use between two and four different solvents and comprise of as many segments of solvent proportion changes as needed. Examples of gradient runs abound in the determination of pesticide residues for example, where a single chromatographic run is designed to allow the identification and quantification of hundreds of compounds.

The gradient serves to improve the separation of compounds that elute too closely to each other in the isocratic run, thereby creating overlapping peaks, or to accelerate a run that would take too long to elute all compounds of interest. In the latter case, the acceleration also saves on the quantity of mobile phase, *i.e.* solvent, used.

The mobile phase typically also contains modifiers that further improve the separation. The additives may be simple acids or bases that modify the pH to favor charged ions in ion chromatography or affect the polarity in affinity chromatography. They may also be buffers to maintain the pH at a desired level. Competing bases can be used to negate the effects of silanols in some columns, while chelating agents may serve to block heavy metal sites. Another type of additive could be a light or UV absorbing compound for indirect photometric detection. One of the greatest challenges faced during the preparation of the mobile phase is to ensure that the additives, which are often salts, are fully dissolved and that no crystal remains, which could block the tubing or damage the column. As discussed in the online lesson, the pKa of any acid or base and its buffering range define when they are used.

Stationary Phase

A column containing a more polar stationary phase is used for normal phase chromatography, and one packed with a non-polar stationary phase is used for reverse phase chromatography. In normal phase, non-polar compounds in the sample will elute first, while they would elute last in reverse phase. These

basic principles remain the same for all columns of one type or the other. However, the retention time, or any retention at all in some cases, is affected by many other parameters describing the column.

The dimensions of the column have many interrelated implications on the chromatography. For example, the sample loading capacity, or the amount of sample that can be injected in the column, is larger for columns with a larger diameter or a longer column. In a wide column, the sample is present as a relatively narrow band at the beginning of the column, which will of course widen as the mobile phase flows and causes separation. In a very narrow column, the sample volume is disproportionately large and spreads over a long section of the column, which effectively only leaves a shorter section for the separation to occur. Sample loading volume must therefore be appropriate for the diameter of column.

The diameter also influences the flow of the mobile phase, but mobile phase flow rate is even more dependent on the particle size of the stationary phase. When a column is packed with very small particles, the mobile phase must flow through very small interstitial spaces, which generates a high backpressure. Consequently, there is only room for a small volume of mobile phase in the column because it has a small diameter and most of the space is occupied by particles, and the flow rate of the mobile phase must be adjusted accordingly. A larger column packed with larger particles displays larger interstitial space and therefore can accommodate a larger volume of mobile phase. The flow will also create less backpressure, which means that weaker pumps can be used to push the mobile phase. This last sentence effectively describes HPLC and the parameters that differentiate it from UHPLC.

The diameter of the column and the particle size impact the peak shape in the chromatogram. The peak will be lower and broader in a column containing larger particles and the opposite combination of a narrow column filled with very small particles will create narrow and tall peaks. Once again, this represents the difference between HPLC and UHPLC. However, there are different combinations of column diameter and particle size available in both HPLC and UHPLC to accommodate the method's needs. For example, one might use a column with larger particles for a faster experiment when few compounds need to be separated.

The peak height and width have an impact on the separation efficiency of a column, as discussed above. Larger column, filled with larger particles, are characterized by a broader dispersion of the sample as it progresses through the column. The physics of the effect of the distance from the walls is

beyond the scope of this module, but it is important to understand the impact of this dispersion. Dispersion is effectively a dissolution of the compound of interest into a larger volume of mobile phase, which causes a broader and lower peak.

Finally, all these effects combine to yield a certain peak height and width for a particular concentration of the compound of interest in the sample. Higher and narrower peaks are favored because they can still be seen clearly even when the concentration is small. A broad and relatively flat peak disappears quickly into the background noise. The noise is the random signal generated by the detector, which is not correlated with the concentration of analytes. It is sometimes called random noise or background noise. It is essentially due to the electronics and cannot be removed. Consequently, we strive to develop methods producing peaks that are high enough to be distinguished from this background noise even for very low concentrations. The comparison of the height of the peak to the height of the noise is called the signal to noise ratio (S/N). A high S/N allows the detection and quantification of low concentrations, while low S/N will limit the method's suitability to detect and quantify similarly small concentrations. These parameters are called the limit of detection and limit of quantitation. They will be discussed further in later modules.

The column length effectively determines how much time is available for the separation when the column diameter, particle size and flow rate of the mobile phase are the same. A short column only allows interactions between the stationary phase and sample analytes for a short duration before they elute, which may not be sufficient for a complete separation into clearly separated peaks. A longer column run under the same conditions may offer the necessary amount of interaction to cause a clean separation.

The particle size and the surface chemistry of the stationary phase are critical parameters, as described in the previous examples. In the early days of high-pressure liquid chromatography, particles were around 0.1 mm in diameter. Advances in manufacturing technology and the understanding of the effect of particle size have resulted in the use of increasingly smaller particles. Nowadays, HPLC particles are around 3.5 to 5 micrometers in diameter, while UHPLC particles are smaller than 2 micrometers. Particle size affects the loading capacity of the column, the back-pressure created by the flow of the mobile phase, and consequently the power of the pumps needed to drive it forward, peak height and width, S/N and column separation efficiency. The separation efficiency of a column is characterized by the number of theoretical plates. As seen in the examples used in the online portion of this lesson, a

15-cm column packed with 5-micron particles has 12,000 theoretical plates, while the same column packed with sub-2-micron particles has >30,000 theoretical plates.

The column packing chemistry also plays a crucial role in the separation of compounds and affects the chromatography mode (normal *vs* reverse phase), pH range of applicability, the mobile phase options and sample preparation requirements. The most popular packing materials in HPLC and UHPLC methods for food safety are C18, C8 and various high strength silica materials. C18 is also known as ODS, or octadecyl group-bonded silica gel. C8 is an octyl group-bonded silica gel.

Standards

Finally, we introduced the fact that identification is performed by comparing the retention times of the peaks in a chromatogram with those of standards run in the exact same conditions and using the same column. Standards are samples containing a formally identified compound, in isolation or in a mixture, in a known concentration. There are different levels of quality of standards. For example, there are internal, external, calibration, certified and other types of standards. They will be discussed in Module 9 dedicated to quality assurance, but it is essential to note that any identification of a contaminant performed for regulatory purposes requires that its retention time matches that of a standard ran on the same instrument, using the same experimental parameter (including the column) and on the same day (ideally in the same sequence of samples).

The position of the center of the peak or the top of the peak provides information about the identity of the compound. This is considered qualitative information. The peak height or the area under the peak is used to determine the concentration; this is quantitative information.

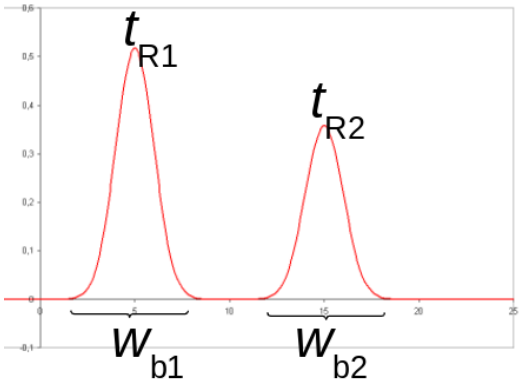
The basic principles of liquid chromatography reviewed up to this point constitutes the level of information that is typically sufficient for a laboratory technician or associate who will run methods but will not be involved in method development or validation. These latter roles require a deeper understanding of the science behind the instrument to ensure that appropriate adaptations and substitutions are made when necessary. The next sections present some of the information needed for these functions.

HPLC Parameters: Causes and Effects

Resolution

Resolution is a term used the qualify and quantify the ability of a method, column or instrument to separate chromatographic peaks. The measurement resolution is the ability of an instrument operated according to a method to separate chromatographic peaks. It is calculated using the following equation:

$$R_s \equiv \frac{2\,(t_{R2} - t_{R1})}{w_{b1} + w_{b2}}$$



where:R_s = Resolution

t_R = Retention time of each of the two peaks

W_b = Baseline width

The measurement resolution is very important for the identification and quantification of compounds in a mixture. A minimum resolution of 1 is typically required for separation to be considered sufficient for quantitation, while a lower resolution may be enough for presumptive identification (0.6 for example), but higher resolution is needed for a method to be considered rugged (typically a minimum of 1.7).

The resolution of a column is calculated using the Purnell equation as follows:

$$\mathbf{Rs} = \frac{\sqrt{\mathbf{N}}}{\mathbf{4}} \cdot \frac{\boldsymbol{\alpha}\text{-}1}{\boldsymbol{\alpha}} \cdot \frac{\mathbf{k}}{\mathbf{k}+1}$$

Where: α = Selectivity factor

N = Number of theoretical plates in the column

K = Capacity factor

The parameters contained in the green portion of the equation represent the theoretical plates. As discussed previously and more extensively in the online module, the number of theoretical plates relates to the physical properties of the column, such as the particle size, dispersion and capacity to handle higher pressure. The orange portion of the equation relates to the chemistry of the columns, the pH of operation and, generally, the ability of the column to retain compounds.

More specifically, one can calculate the number of theoretical plates (N) using one of the two formulas:

$$N = 16(t_R/w_b)^2$$

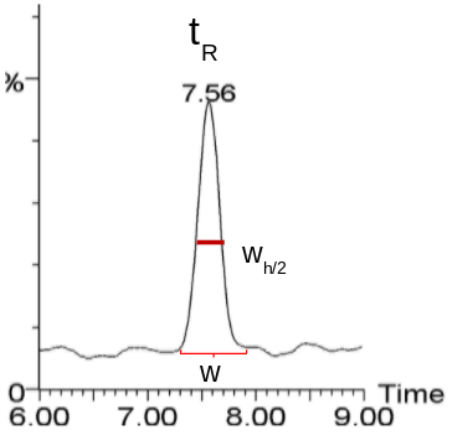
Or

$$N = 5.54\ (t_R/w_{h/2})^2$$

Where: w_b = Width at the peak base

t_R = Retention time

$w_{h/2}$ = width at half height



Students are encouraged to review the glossary of terms published by Chromatography Online[2].

The science (or art) of method development is to use the interactions between different factors to increase the ability to obtain a narrow and high peak at the low concentrations of interest for the method. In the case of compounds with similar polarity, and therefore close retention times, increasing the measurement resolution may be the key to separate them. Based on the equation for the measurement resolution, it means that increasing N, controlling k and increasing α all contribute to a higher resolution.

Increasing N may be achieved by:

[2] https://www.chromatographyonline.com/view/glossary-hplclc-separation-terms (accessed 11/30/20)

- Using a longer column
- Reducing the particle size of the stationary phase

Controlling K can be achieved by changing the composition of the mobile phase, and increasing α can be achieved by:

- Changing the composition of the mobile phase
- Changing the column temperature
- Changing the chemistry of the stationary phase (including additives)

Particle Chemistry

The particle chemistry and physical composition is one of the factors affecting resolution and the ability of a method to measure components of interest. Modern particles are either fully porous or porous only on the surface with a solid core. The latter are called core-shell and present a number of advantages that justify their use despite some down sides like higher cost and lower resilience. Core-shell particles can be manufactured with a very narrow particle size distribution, *i.e*. particles are uniform in size, they allow for the use of ligands that would not be attached easily to a porous bead and are tolerant of a broader pH range of applications. The impact of the uniformity of the particle size is a more direct and reproducible path for the sample through the column than is achieved with more heterogenous packing. The ligands and the shallower porosity also contribute to the movement of the mobile phase in more of a straight line through the column. As introduced previously, a more direct and less dispersed path through a column leads to narrower bands with a higher intensity, and therefore greater S/N, and the solid core particles also show these outcomes at higher mobile phase velocity.

Mobile Phase and Additives

The mobile phase composition, including additives, and the option between gradient and isocratic runs also impact the measurement resolution, peak height, width, shape and S/N. Typical solvents used as mobile phase alone or in mixtures in food safety applications of LC include, in order of polarity, water, alcohols and acetonitrile.

Pure water is rarely used as a mobile phase alone or in a gradient because microbial growth in the solvent bottle could cause the production of biofilms that may block the tubing or the column. To prevent microbial growth, the “water” bottle usually contains at least 5% organic solvent. Methanol, isopropanol and acetonitrile are the most popular organic solvents in LC for food contaminants. Acetonitrile is often the best option, but its cost and a shortage that happened about a decade ago prompted its replacement with methanol in many routine methods used at large scale.

The additives, or modifiers, must be selected carefully to achieve the best outcome. There is often a need to achieve a balance between using additives that yield the best LC separation and those that provide the best detection. This is especially true when using mass spectrometry as a detection method because ions must be formed from the solution eluting from the LC. This subject will be discussed in greater detail in Module 6 on mass spectrometry.

Many additives serve to set and maintain a desired pH in the mobile phase. While the pH selected is dependent on the compounds to be separated in the column, the applicable pH range of the column is a critical factor as operating outside of this range could damage the column. The pH also impacts the shape of the baseline of a chromatogram. The ideal baseline is flat, and a mismatch between the optimal operating range of a column and the pH of the mobile phase can result in a sloping baseline.

The additives are typically present in the aqueous solvent bottle (the water with possibly a small amount of organic solvent to prevent biofilms) because many of the salts used as additives don’t dissolve well in organic solvents. Since the presence of any crystals could damage the system by blocking tubing or making its way into the column, it is essential to ensure a complete dissolution of the additives.

Lesson 3: Detection Techniques for Contaminants separated by HPLC

Different principles of measurement can be applied to the eluent of the LC. Generally, the food safety laboratory includes detectors that use the interaction of light with molecules to measure absorbance of light at various wavelengths in the visible and UV spectrum, fluorescence, light scattering and others measure completely different properties such as mass to charge ratio. Other detectors measuring circular dichroism, radiochemistry, chemiluminescence, laser induced fluorescence, conductivity of nuclear magnetic resonance to name a few. The reasons for selecting a detector over all other options often are cost, ruggedness, ease of use and throughput requirements in addition to the appropriate selectivity and sensitivity for the problem at hand.

In this lesson, we review the principle of measurement for the main detectors used in the food safety laboratory. As a starting point, it is important to understand that detectors can be more or less specific. Specificity is the ability to measure a compound without interference from others. UV and visible absorption detectors for example are not very specific because many different compounds may absorb the same wavelength of light. Fluorescence detectors are more specific because fluorescence, or the absorption of energy at one wavelength and release at a different one, is characteristic of fewer compounds.

Photometric detectors

Absorbance detectors, known as UV-Visible (or UV-VIS) use a source made of a tungsten or a simple filament visible lamp to emit a beam of light that is diffracted through a prism or a grating, which essentially produces a rainbow of color bands. A slit is aligned at the appropriate angle to pass the selected wavelength onto a cell containing the sample and a light receiver measures the amount of light coming through the sample. Please refer to the video in the online module for an animated description of the system (also available here[3]). In this system, the entire or at least a large fraction of the spectrum of light is emitted by the source and later a selected portion of the wavelength range is blocked out and a non-selective detecting element captures all the light that makes it through the sample.

Photodiode array (PDA) detectors use the same principle of absorption of light a specific wavelength, but the entire spectrum of light is pointed at the sample cell and a grating placed after the sample cell diffracts the light onto a diode array. Each diode on the array measures its own wavelength range, allowing for multiple parallel measurements at once. A video prepared by Arden Scientific is suggested in the online lesson [4]. In both cases, the amount of light absorbed is directly related to the concentration of light absorbing molecules in the sample.

[3] https://youtu.be/sfxEj_MxBcs (accessed 12/12/20)

[4] https://youtu.be/6K7EYMkBbFk (accessed 12/12/20)

Fluorescence Detectors

The fluorescence detector uses one wavelength of light to excite molecules to a higher energy level, and measures the light emitted at a different wavelength by the molecules as they return to a stable state of lower energy. Consequently, the two critical parameters are the excitation wavelength and the emission wavelength. There is also a consideration for the width of the bands produced by the compounds of interest; this width determines how many bands can be differentiated in a single experiment. The width of the band depends on the column, the flow rate of the stationary phase and the dissipation that may happen in any accessory used to induce fluorescence. For example, fluorescence detection is used for the determination of aflatoxins, which are molecules that do not naturally fluoresce sufficiently for a direct measurement with HPLC. While the aflatoxins G1 and G2 fluoresced enough for measurements following their separation by HPLC, after toxins B1 and B2 quench and therefore result in a very low signal. A derivatization step is typically used to form the aflatoxin B1a and B2a from after toxins B1 and B2 because these derivatives show a better fluorescence response. The addition of a reaction cell for the derivatization causes the equivalent of a small dilution of the aflatoxin's which results in broader chromatographic bands.

In UHPLC, it is possible to use a fluorescence detector with a long path cell to measure the fluorescence of aflatoxins B1, B2 and G1, G2 without the need for derivatization. This is possible because UHPLC produces much narrower bands of greater intensity for the same concentration of compound. Consequently, UHPLC methods do not need derivatization cells. This can be an advantage in a laboratory because it reduces the use of harsh chemicals such as iodine.

Other Detectors

The next type of detector is the refractive index detector. It measures the change of the refraction index caused by the presence of a compound or multiple compounds in solution. It is typically used for substances that do not fluoresce or absorb UV light such as sugars. The refractive index detector is not specific and therefore the sample stream coming out of the HPLC must be highly purified.

Light scattering detectors are very popular especially for the measurement of particle size. They operate by shining a laser onto a suspension. As a result, light is scattered and the quantity of scattering depends on concentration, while the pattern of scattering depends on particle size. These detectors are very useful for compounds that do not absorb UV-visible light or fluoresce. They are also quite unique in their ability to measure particle size.

A number of other detectors exist but they are not used as much in the food safety laboratory. For example the conductivity detector measures electronic resistance caused by ions present in solution. This is very useful for ionic solutions in ion chromatography. Chemiluminescence is very similar to fluorescence, but it uses a chemical reaction instead of wavelengths of light in the excitation source. Finally, optical rotation can detect the difference between optical isomers and is very useful in the pharmaceutical industry but not used widely in food safety.

Mass Spectrometry

The mass spectrometer is increasingly popular in food safety. It uses the mass to charge ratio (m/z) to select molecules that will pass through an electromagnetic field. The mass spectrometer is a selective detector because everything else is rejected and therefore not detected. This adds a level of selectivity after the HPLC separation. Mass spectrometry is typically abbreviated as MS, while MS/MS is a combination of two quadrupoles into a single instrument. In MS/MS the mass to charge ratio that is allowed through is selected twice while a high energy collision is induced between the two quadrupoles, which causes the conversion of the original molecule, known as the precursor ion, into a number of smaller molecules known as the product ions. MS/MS is highly selective.

In mass spectrometry, the mass of a molecule originates from its protons and neutrons as well as minimally from its electrons. Its charge comes from an excess of either protons which cause a positive charge or electrons that create a negative charge. It is important to remember that mass spectrometry only detects charged species. The sample eluting from the HPLC does not typically contain charged molecules. Therefore we must add a proton or remove a proton within the mass spectrometer itself. It is also possible to have multiple charges, especially in large molecules or in small molecules with well separated functional groups. The charge is necessary because the movement and selection of ions is performed using a magnetic field and an electric field. Consequently only charged species (ions) are affected by these fields. Neutral molecules are unaffected and will therefore not move through the instrument.

The mass used to characterize a particular compound can be expressed in one of a number of ways. Monoisotopic mass is calculated using precisely known mass values for the most abundant isotope for each element present; this is also known as the nominal mass. For example, the most abundant isotope for carbon has a mass of 12.0000. The most abundant isotope of hydrogen has a mass of 1.0078. The exact mass is calculated using a precisely known value for a specific isotope for each element, while the average mass, as the name says, is calculated using the average mass for that atom considering all possible isotopes. This information will be particularly important when discussing the new technology of high resolution mass spectrometry in module 6.

Conclusion

In summary, there are many detectors available for the measurements of the element coming out of an HPLC. The selection of the detector has important implications as it affects the limit of quantitation, the limit of detection and specificity of our methods. The most common detectors in the food safety laboratory for the detection and quantitation of contaminants are UV visible, fluorescence, mass spectrometry, and MS/MS.