The Application of Continuous Stationary Phase Gradients to High-Performance Liquid Chromatography and Its Potential to Improve Pharmacological Research

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Abstract

The separation of mixtures into different components is integral to experimentation and analysis in a multitude of fields. Chromatography is one of the most popular, well-developed, and well-studied methods used to examine the makeup of a mixture. Thus, the improvement of chromatographic procedures directly benefits research across many scientific disciplines. The application of a continuous stationary phase gradient to High-Performance Liquid Chromatography (HPLC) methods has been proposed to improve the separation of complex mixtures that are difficult to achieve with existing separation techniques. By incorporating a gradient stationary phase, analysts will create a more selective mode of separation to improve the efficiency and cost-effectiveness of their research. Particularly, this new protocol will enhance the function of the pharmaceutical industry, which relies heavily on chromatographic methods.

The Application of Continuous Stationary Phase Gradients to High-Performance Liquid Chromatography and Its Potential to Improve Pharmacological Research

The term “chromatography” refers to an analytical technique designed to separate the distinct chemical components of a mixture. Michael Tswett, a Russian-Italian botanist, developed this technique during the advent of the 20th century. He engineered his initial apparatus with the purpose of isolating chlorophyll from plant samples. The idea was inspired by his observation of the relative adsorption and extraction of chlorophyll by different organic solvents (Wixton, 2001). This first rudimentary model achieved separation through the application of an organic solvent to a vertical glass column filled with adsorbent, sand-like particles (Ettre, 2008). Although modern instrumentation varies significantly from this initial design, the basic principles remain the same. The chromatographic system consists of two phases: a mobile phase and a stationary phase. These phases and their unique interactions with each component in the mixture function concurrently to separate the compounds by their relative rates of motion or retention. The mixture that is analyzed for its contents, or the “analyte,” is adsorbed onto the stationary phase surface. Then, the mobile phase is allowed to move across the surface of the fixed stationary phase by diffusion. The components of the mixture move with the mobile phase and are deposited back onto the stationary phase at different points based on their relative affinity for each phase. Compounds with a greater affinity for the stationary phase will stay in the mobile phase for a shorter time, thus remaining near the point at which the mixture was added. Conversely, compounds with a greater affinity for the mobile phase will move within it for a greater time and deposit far from their initial position. By this mechanism, the analysts can separate individual compounds according to differences in their chemical characteristics.

High-Performance Liquid Chromatography

Over the past few decades, High-Performance Liquid Chromatography (HPLC) has become an indispensable laboratory technique. Scientists frequently use HPLC because of its advanced capability to separate chemical mixtures (Dong, 2006). HPLC consists of a stainless-steel column connected to a solvent pumping mechanism and a high-pressure sample injector (Wixton, 2001). The column contains an adsorbent packing material of small, coated beads, which function as the stationary phase. The mobile phase is composed of a solvent pumped through the column's interior, and the analytes are the various samples injected into the column for analysis (Blum, 2014).

The separation of analytes is quantified by pairing the HPLC system with a detector. Detectors frequently used to produce liquid chromatograms include UV-vis spectrophotometers, refractive index detectors, and fluorescence intensity detectors. The function of the detector is to visualize the elution time of each individual analyte by a corresponding peak on a graph (Akash & Rehman, 2020). This graph is referred to as a "liquid chromatogram," where the x-axis represents the time in the HPLC run, and the y-axis corresponds to the appropriate "response" unit (i.e., fluorescence or absorbance). Each detection method has unique capabilities. Since no single method can characterize all analytes, each run must be optimized by considering the chemical properties of the compound of interest and which system is best suited to the analysis of these characteristics. Furthermore, more than one detection method may be used in a single HPLC run, and multiple detectors working cooperatively can often characterize a broader range of analytes (Swartz, 2010; Scott, 1986).

The ability to separate analytes directly depends on the selected mobile and stationary phases (Felinger & Cavazzini, 2017). Optimization of an HPLC program must consider the interactions between each analyte and the two phases. These interactions are determined by the unique chemical characteristics of the compounds, such as their polarity, pH, temperature, and structure. (Robards & Ryan, 2022). Compound separation in HPLC can be optimized by selecting either normal-phase or reverse-phase conditions. Normal-phase and reversed-phase chromatography differ in the respective polarity of their phases. In normal-phase chromatography, the polar packing material of the column acts as the stationary phase, and a nonpolar solvent is used for the mobile phase. In reversed-phase chromatography, a nonpolar stationary phase is produced by the modification of the inner surface of the packing material with a nonpolar ligand, and a polar solvent is used for the mobile phase (Driskell, 2003; Moldoveanu & David, 2022; Robards & Ryan, 2022). If the compounds an analyst is working with are more hydrophobic and dissolve in organic solvents, they will typically opt to use a normal-phase method (Nesterenko & Palamareva, 2019). By comparison, analysts will utilize a reversed-phase method when separating samples that are highly hydrophilic and dissolved in polar solvents (Evans et al., 2009).

HPLC Parameters and the Qualification of a Method

The aptitude of an HPLC method is determined by different variables such as selectivity (α), efficiency (N), and resolution (R_s). The selectivity of a separation technique is defined as the relative retention of the analytes with respect to each other. This is quantified by the ratio of the "capacity factors," k_1 and k_2. The capacity factor measures the retention time of a particular analyte (t_R) when compared to the fastest possible elution time (t_0) (Moldoveanu & David, 2022).

$$\alpha = k_2/k_1 \quad (1)$$

$$k = (t_R - t_0)/t_0 \quad (2)$$

Efficiency describes the width of the peaks produced by an HPLC column. Peak widths can be measured either at the base (W_b) or at half of the maximum height ($W_{1/2}$). Some chromatograms will have low efficiency, where multiple peaks have a large margin of overlap, preventing the analytes from being clearly resolved. However, an ideal separation will produce peaks with elution times that differ enough and peaks that are narrow enough to distinguish between all the individual components of a mixture. The efficiency of a column is measured by the number of theoretical plates (N) it contains. The higher the number of plates, the more efficient the column. Each plate represents a hypothetical location where the stationary and mobile phases are in equilibrium (Jennings et al., 1997). The plate number can be expressed either in terms of the t_R and W_b or t_R and $W_{1/2}$.[17]

$$N = 16\ (t_R/W_b)^2 \quad (3)$$

$$N = 5.545\ (t_R/W_h)^2 \quad (4)$$

These relationships show that the peak width and theoretical plate number are inversely related. Therefore, a narrower peak means a higher number of plates and a more efficient column. Conversely, an inefficient column will produce wide peaks which become increasingly difficult to resolve. This phenomenon is referred to as “band broadening.” Band broadening is described by the van Deemter equation. The equation accounts for the three events that influence the efficiency of a column: multiple flow paths (A), longitudinal diffusion (B), and resistance to mass transfer (C) (Felinger & Cavazzini, 2017; Llovet, 2019).

$$H = A + B/\upsilon + C\upsilon \quad (5)$$

In the equation, υ represents the reduced velocity. This variable relates the diffusion of solute molecules throughout the mobile phase to the particle size of the packed column (Usher et al., 2008). The A, B, and C events are related to the height of each theoretical plate, H, by υ. The presence of multiple flow paths for solute molecules through a column (a.k.a. "eddy diffusion") is inherent and cannot be eliminated (Gritti & Guiochon, 2013). Thus, A is not a variable analysts try to optimize when developing a more efficient method for liquid chromatography. However, both B and C can be optimized by altering the flow rate of the mobile phase. The effect of longitudinal diffusion is minimized by increasing the flow rate through the column, while the effect of resistance to mass transfer is minimized by decreasing the flow rate. When A, B, and C are at their lowest possible value, the plate height decreases, the theoretical plate number is at a maximum, and the method has successfully been optimized to its peak efficiency.

The parameter of resolution is determined by both selectivity and efficiency. This mathematical relationship is called the Purnell equation (Purnell, 1960). It is the most useful way to measure the separation as a function of a set of HPLC conditions. Changing properties such as the column length, particle size, temperature, solvent, or ligand can improve the resolution of a technique.

$$R_s = \frac{1}{4} \cdot \sqrt{N} \cdot (\alpha - 1) \cdot \frac{k}{k+1} \quad (6)$$

Another important parameter that must be considered when optimizing an HPLC procedure is the overall speed of the method (Carr et al., 2011). If the method developed is extremely efficient according to the parameters of the van Deemter equation but takes an

extensive amount of time to carry out, then this decreases the usefulness of the method. A single HPLC protocol is often performed multiple times within a day in high-throughput industry labs. Therefore, a procedure that gives good results but takes up too much time is not adequate for pharmaceutical analysis.

Mobile Phase Gradient v. Stationary Phase Gradient

Incorporating a gradient mobile phase, also known as a "gradient elution" method, has become the most popular approach to increasing the selectivity of an HPLC technique since its invention in the 1950s (Howard & Martin, 1950; Robards & Ryan, 2022). There are many instances where an isocratic elution method, which uses a uniform composition of solvent, is not sufficient to separate a sample because of the large difference in retention times. Gradient elution improves upon the isocratic model by varying the composition of the solvent. This changes the strength of the phase interactions to reduce large gaps in retention times to minimize the effects of band broadening for samples with a wide range of polarities.

While gradient elution in many ways outperforms basic chromatography methods, it also introduces more complications. For one, gradient elution is a lengthier process than isocratic elution. This creates a time-efficiency trade-off for the higher selectivity it produces (Schellinger & Carr, 2006). After a gradient elution is performed, the column must be "re-equilibrated" to return the column to the original condition before continuing to the next sample injection (VanMiddlesworth & Dorsey, 2011). First, the column must be "flushed out," emptying the column of all the remaining solvent in the system. Then, solvent conditions must be returned to the initial composition by pumping the prior solvent makeup into the column's interior. This can

take several hours and requires the use of large volumes of organic solvents, which are expensive.

Additionally, the programmed gradient and the gradient that is physically produced are never completely identical. There will always be some level of variability between the two because of gradient delay times. This gradient delay, also called the "dwell volume," represents the time between the initial injection of the mobile phase through the column and the point at which the prepared gradient reaches the column (Dong, 2006; Miller, 2009). Over this period, the column will exhibit an isocratic elution condition instead of the specific ratio of solvents that it is programmed to contain (Dolan, 2013). This will produce slightly different retention times for analytes and peak resolutions and will lengthen the time required for column equilibration and sample injection (Dong, 2005). Another source of gradient nonideality comes from the dispersion of the solvents over time. This slowly decreases the steepness of the gradient when the trend should be linear (Quarry et al., 1984; Snyder & Dolan, 2007). This variability makes it difficult to share protocols between instruments. A method established for one HPLC system may produce different results on a second system because each machine has a slightly different dwell-volume (Engelhardt & Elgass, 1975). This can prevent analysts from successfully imitating procedures from literature.

Analysts face another difficulty when trying to characterize a solvent gradient. The current literature describes only one method for measuring mobile phase gradients, and it is not very reliable (Dong, 2005). The procedure swaps out solvent A with 100% water and solvent B with a 0.1% acetone solution. UV absorbance is then used to measure the gradient. However, using water to represent the organic solvents creates irregularities because the measurement is

not made under conditions identical to the actual HPLC run. Additionally, the measurement must be made with an absorbance detector, meaning mobile phase gradients applied to LC systems with another detector cannot be analyzed with this technique (Mageea et al., 2014).

Considering the numerous issues associated with elution gradients, the development of a new technology for increasing the selectivity of HPLC would be ideal. One alternative that has been proposed is the application of a chemical gradient to the stationary phase. The advantages of using a stationary phase gradient in place of a mobile phase gradient include several nondestructive methods for characterization, the cost-effectiveness of the gradient preparation, the ability to have precise control over the shape of the gradient, and the flexibility of the method between instruments (Galea et al., 2015; Krupczyńska & Buszewski, 2004).

The idea of a "stationary phase gradient" goes back to the 1960s but still has not been incorporated into a standard analysis technique. This is mainly because the methods required to prepare the gradient columns were more complicated and time-consuming than performing a gradient elution (Urban et al., 2017). Early research on stationary phase gradients focused on creating a discontinuous gradient. Discontinuous gradients are made by piecing together columns synthesized with different polymeric materials. This creates a "step" gradient where the difference in ligand concentration across the column interior is not linear but segmented (Alvarez-Segura et al., 2016). This type of gradient was shown to increase the selectivity and theoretical plate count by adding the properties of each column type (Berna et al., 2004; Nyiredy et al., 2006; Ortiz-Bolsico et al., 2013); however, because the gradient was not linear, there were void volumes of solvent between each column section, leading to band broadening (El Rassi & Horváth, 1986).

In recent years, separation scientists have shifted their focus within research on stationary phase gradients toward the development of a continuous, linear gradient. Continuous gradients have an advantage over the previous discontinuous prototypes because they can optimize separations without unnecessarily complicating chromatography procedures. If the stationary support of a system could be modified to have a linear trend in functionalities across its surface, the efficiency of the separation should increase, and no band broadening should occur (Gritti & Guiochon, 2014).

Methods for Creating Stationary Phase Gradients

The production of chemical gradients falls within the field of nanotechnology. Nanotechnology investigates the control of matter at the atomic and molecular levels for industrial purposes. Researchers have ceaselessly explored the realm of possibilities for the synthesis of surface gradients. The range of applications of surface gradients extends from protein separations to electrical engineering to environmental science. The copious amount of existing literature on chemical surface gradients is what informs and inspires separation scientists in their endeavors to apply a gradient of functionalities to the surface of stationary phases for high-resolution sample separations.

As most stationary phase supports (i.e., the small beads packed in the HPLC column) are constructed from silica, their modification is attained by the addition of compounds which can bind to silica through a simple reaction. To achieve this, researchers commonly use a class of compounds called organosilanes. Organosilanes are silicone-based monomers resembling hydrocarbons that contain at least one silicon-carbon bond (Dawood, 2014). Silanes react with the silanol groups on packed silica columns via a condensation reaction, where an ester bond is

formed between the two compounds through the displacement of a water molecule (Figure 1) (Schoell, 2012).

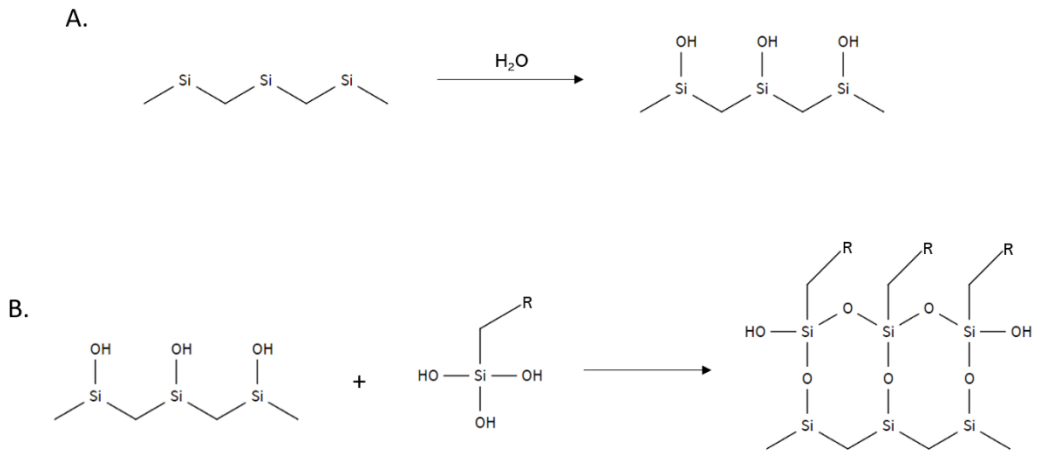

Figure 1

This schematic shows the steps of the condensation reaction that takes place to modify a silica surface with an organosilane (Schoell, 2012).

The neighboring silanol groups from the organosilane molecules will also cross-link to form a network, creating what is called a "self-assembled monolayer" (SAM) (Escorihuela & Zuilhof, 2017). The "silanization" of a column can occur by either a wet method or through vapor deposition. Under the wet method, the stationary support is soaked in hydrofluoric acid prior to its immersion in the silane solution so that hydroxyl groups form at the surface for reaction with the silane (Maruška et al., 2007). Alternatively, vapor deposition does not require the use of a solution to hydroxylate the surface. Instead, the low vapor pressure of the silane is used to achieve modification through the movement of the compound across the surface (Klages et al., 2019).

One-dimensional, continuous gradients can have either a positive or negative orientation. Positive gradients begin with a low ligand concentration, and the ligand density increases across the surface. Negative gradients begin with a high concentration of the ligand, decreasing over the support length. Each gradient type increases the resolution of the separation by a different mechanism. Positive gradients increase the analyte resolution because of "chromatographic zone focusing." Negative gradients increase resolution by the "pre-concentration effect" (Maruška et al., 2007). Stationary phase gradients can also be either linear or exponential. A linear gradient will increase ligand concentration incrementally, while an exponential gradient possesses an exponential relationship between ligand density and location.

So far, three methods have proven successful in creating a continuous gradient on a stationary phase support and separating analytes with high resolution. The first of these methods is called "photo-initiated grafting." The grafting of polymers on a surface using a photo-initiator has existed as a method of chemical surface modification for over three decades. It is based on the phenomenon of "photopolymerization," where a specific class of compound called a photo-initiator reacts with monomers in the presence of light to form a polymer (Dessauer, 2006; Rohr et al., 2003). This reaction can easily be manipulated to create a gradient because the amount of linking or "grafting" of localized monomers has a direct relationship to the amount of light exposure. Therefore, if the amount of light exposure is varied, the regions of the substrate that have had a longer time in direct contact with the light source will have a higher density of the polymer.

The second method has been used to develop what is called a "continuous bed" or "monolithic" stationary phase. The main difference between a monolithic column and a standard

chromatography column is that monoliths are “non-particulate” in nature. The traditional particulate column is packed using tiny beads of an inert material, such as silica, while a non-particulate monolithic column is synthesized by *in situ* polymerization (Dessauer, 2006). Columns constructed via *in situ* polymerization possess a greater number of channels and a higher porosity (González-González et al., 2017; González-González et al., 2020). The porosity of the continuous bed column increases the number of theoretical plates, producing higher resolution and efficiency in separations (Ghose & Cramer, 2001). To apply a gradient of modification to the continuous bed column, researchers used a vacuum to draw different monomer solutions up the interior of the column. As these solutions were taken into the column, they polymerized to form the stationary phase at the interior of the support (Klages et al., 2019). Alternating which solution the column is immersed in creates a change in the polymeric properties of the inner surface.

The third method that has been explored for gradient creation is called “controlled-rate infusion” (CRI). CRI functions by using a syringe pump to inject the desired silane solution for modification into a vessel containing the stationary phase. The controlled flow rate at which the solvent is pumped into the vessel determines the steepness of the change in modification along the stationary support surface (Forzano et al., 2019; Kannan et al., 2011a; Kannan et al., 2011b).

Methods for Gradient Characterization

The type of chemical modification in these gradient creation methods does not produce any alteration which is visible to the naked eye. Therefore, researchers must develop detection methods that allow them to analyze the surface functional groups to verify there is a change in the ligand concentration. Historically, most gradient detection methods have been invasive to the

structure of the stationary phase. The analysis of specific chemical composition required the destruction of the column to retrieve data (Petter et al., 2008). Developing a non-invasive method of detection is an integral part of making the use of stationary phase gradients a practical option for laboratory research. An LC column must be able to be tested for its composition and remain functional for subsequent runs.

Several different methods have been developed within recent years for non-invasive gradient characterization, a select few of which have been specifically used to analyze stationary phase supports. One method which was successfully applied to stationary phase characterization is called scanning capacitively coupled contactless detection (sC^4D) (Gillespie et al., 2006). The sC^4D method visualizes the distribution of charged functional groups on the inner surface of the column by scanning the length of the column with two electrodes. An optimized electron current is passed through the detectors, and the strength of the signal received is proportional to the conductivity of the charged functional groups at that point on the longitudinal gradient (Connolly et al., 2007).

Another method used Water Contact Angle (WCA) measurements to characterize the stationary phase gradient by its "wettability." In this technique, a drop of deionized water is mechanically administered to the surface of the support, and the angle the droplet makes with the surface is measured according to a computer program. The surface tension of the water is directly linked to the chemical composition of the support at that location, thereby producing different WCA measurements at different points in the gradient (Uyama et al., 1991).

Other less common methods include X-ray Photoelectron Spectroscopy (XPS), Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (AT-FTIR), and Scanning

Electron Microscopy (SEM) or Electron Probe Microanalysis (EPMA). XPS uses a radiation source to generate photoelectrons from the surface compounds, determining the chemical composition according to their kinetic energy (Axnanda et al., 2015). AT-FTIR is a form of mid-infrared (MIR) spectroscopy which allows for efficient and nondestructive production of spectral data on the surface functionalities (Buffeteau et al., 1996). SEM and EPMA use electron beams to produce high-resolution images with resolving power of up to 1-20 nm (Llovet, 2019; Smith & Oatley, 1955). This is achieved by the small wavelength of energy associated with electrons. The decrease in wavelength between the electron beam and visible light is so significant that the magnification powers of these techniques are about 1000x more powerful than those of a normal optical light microscope. These methods appeared less frequently in the literature on stationary phase gradients as they are more costly and require highly specialized equipment.

The simplest and most recurring technique in all the experiments on stationary phase gradient characterization was UV-light visualization. This method can reveal the varying ligand concentration to the human eye by applying a UV light source to the area of interest. UV visualization is much cheaper and more easily accessible for most laboratories than any of the other characterization methods; therefore, it was used by most of the studies. However, this method does require the stationary phase materials must be UV-sensitive, or else a nondestructive dye must be used before analysis.

Experimentation and Results

In one successful application of a stationary phase gradient to chromatography, researchers used the photopolymerization method to produce a gradient of sulfonic acid functionalities on the surface of a monolithic column used for capillary electrochromatography

(CEC) (Pucci, 2004). This was the first time a gradient of functionalities was applied to a CEC column. Teflon-coated silica capillary columns were modified using a hydrophobic poly (butyl methacrylate-co-ethylene dimethacrylate) ligand. Two distinct UV illumination systems were used to graft the polymer poly (2-acrylamido-2-methyl-1-propanesulfonic acid) (AMPS) onto the column surface. The first method utilized a moving shutter to control UV light exposure. The second method used a neutral density filter. EPMA was then used in conjunction with SEM to confirm the presence of the polymeric gradient on each CEC column. The data from the analysis revealed that a linear gradient of AMPS was produced when the moving shutter was used, while an exponential gradient was produced from the use of a neutral density filter. A third column was prepared as a positive control. This column was given a uniform distribution of the AMPS from equal exposure to UV across the length of the column.

Following this, Pucci et al. ran samples of acetylsalicylic acid (ASA) and salicylic acid (SA) on each of the three experimental columns to model the effect of the photopolymerized gradients on analyte separation. A comparison of the chromatograms showed that both gradient columns improved the separation of ASA and SA when compared with the positive control. However, the exponential gradient synthesized from the neutral density filter yielded a much larger difference in the retention factors of the analytes than the gradient from the moving shutter because there was a longer stretch of un-grafted, hydrophobic functionalities to interact with the more nonpolar analytes. The greater separation between the analytes proved that the exponential gradient provided a better peak capacity and selectivity than a linear gradient of the same composition. Although the use of a neutral density filter improved separation abilities, this improvement came at the expense of the analysis time, as the neutral density filter diminished the UV intensity requiring a longer exposure time to overcome the grafting energy.

Another study developed gradient stationary phases for reversed-phase CEC and capillary liquid chromatography (cLC). The gradients were created by the control of capillary forces. The columns were immersed sequentially in monomer solutions of increasing hydrophobicity, switching to a new solution when the meniscus reached a certain height (Maruška et al., 2007). A control column of uniform surface modification by a 70% hydrophobic monomer solution was also produced for comparison. These columns were then used under isocratic conditions to separate four different benzoates. The chromatograms showed that the gradient column had a better resolution. It was able to resolve all four benzoates into distinct signals, while the uniform CEC column only displayed three peaks.

Researchers in another study utilized photoinitiated grafting to create a gradient of sulfopropyl methacrylate monomers for cation-exchange chromatography (Currivan et al., 2010). They formed the gradient on two different polymethacrylate monoliths, poly(butyl methacrylate-co-ethylene glycol dimethacrylate) and poly(lauryl methacrylate-co-ethylene glycol dimethacrylate). The synthesis of a gradient via photopolymerization was achieved by using a photo mask. The photo mask is a patterned opaque material used to cover certain regions of the column, blocking its exposure to the UV light source. This mask was used in conjunction with the photo-initiator compound benzophenone to achieve a varied polymerization. The gradient types, positive and negative, were created by this method. The positive gradient was exposed to increasing amounts of UV light along the length of the column, while the negative gradient experienced a decrease in UV exposure. This photopolymerization method proved more efficient than previous ones because of the ease with which the gradient could be characterized. The researchers in this study used plotted the distribution of functional groups by measuring the high

conductivity response of the cationic sulfopropyl methacrylate monomers via scanning capacitively coupled contactless detection (sC^4D).

The conductivity responses along the length of the column showed that the density of the functional groups increased proportionally to UV intensity. The columns were then used to separate a mixture of two divalent metal cations, Mg^{2+} and Ba^{2+}. The efficiency of the column increased upon the use of the stationary phase gradient, with the peak width at half height decreasing by over 30% for both analytes compared to the uniform column chromatogram. The negative gradient proved to improve resolution more than the positive gradient.

The same was true when capillary action was used to create the gradients. The homogeneous capillary column was not able to separate analytes with the same resolution that the gradient columns could, showing overlapping and indistinguishable peaks. The chromatogram of the gradient stationary phase had significantly narrower peaks, showing four distinct analytes, while only three could be distinguished on the chromatogram of the homogenous column. The efficiency, or ability to resolve the components of the mixture, was much higher for the gradient column (Maruška et al., 2007).

Stationary phase gradients created from the CRI method also demonstrated an improved separation of analytes. Researchers created a multicomponent gradient on a silica TLC support by placing the plate inside a graduated cylinder and injecting 3-aminopropyltriethoxysilane (APTEOS) and phenyltrimethoxysilane (PTMOS) solutions through a programmable syringe pump (Forzano et al., 2019). As the volume of APTEOS increased, the plate was more densely modified with the amine. When the volume of PTMOS increased, the density of phenyl modification increased. Positive and negative control plates were also prepared, with uniform APTEOS modification on the positive control and no modification on the negative control. The

presence of a gradient was confirmed by covering the plates in ninhydrin, which reacts with amines to form a purple product. The intensity of the colored product across the plate revealed the distribution of these ligands. The positive control showed a constant intensity of the dye across the plate, and the negative control showed no formation of a purple product.

A mixture of four weak acids and bases were separated on the positive control, negative control, gradient A (Ga), and gradient B (Gb) plates, using a mobile phase of methylene chloride: methanol: acetic acid (90:10:0.5). On gradient A, the analytes were spotted at the end with the highest amine concentration. On gradient B, the analytes were spotted at the end with the lowest amine concentration. After the separation of the analytes, the plates were visualized under UV light, and fluorescence detection chromatograms were created using the TLC Analyzer Software. It was found that the mixture exhibited the best separation on gradient A, with all four analytes clearly resolved into distinct peaks. The first two analytes (BA and ABA) did not resolve on gradient B because the acids interacted strongly with the high concentration of amine at the start of the gradient. Since gradient A started with a lower amine concentration, the acids were able to travel farther up the plate and separate. The same setup was repeated, this time using a basic mobile phase of methylene chloride: methanol: triethylamine (90:10:0.5). The chromatograms received from these plates showed that the use of mobile phases with varying pH affected the separation on the unmodified TLC plate much more than it affected separations on the gradient plates.

Additional single-component gradient plates were prepared to investigate the effect of the flow rate of the silane during plate preparation on the separation of analytes. Three gradient plates were prepared with the APTEOS flow rates of 400 mL/hr, 700 mL/hr, and 100 mL/hr. The gradients were then characterized by applying a ninhydrin dye, showing that a slower flow rate

produced a steep gradient, and a fast flow rate produced a shallow gradient. A mixture of three commonly used over-the-counter drugs, doxylamine succinate (Dx), diphenhydramine hydrochloride (Dp), and acetaminophen (Ac), were then separated on the three gradient plates. A mixture of these drugs was also separated on a positive and negative control plate. Chromatograms were produced from UV visualization of the TLC plates and capturing the image on a TLC Analyzer Software. The results from this analysis showed that the highest resolution separation was achieved from the 1000 mL/hr APTEOS flow rate. The researchers concluded that there was an inverse relationship between gradient steepness and peak resolution. When there was a slight change in ligand density over the length of the support, a better resolution separation was produced than when a dramatic increase in density over a short length of the stationary support.

When a gradient TLC plate was used for separations, as compared with a plate of uniform modification or no modification, the resolution of analytes increased (Kannan et al., 201b). Additionally, using the method of CRI, researchers were able to show that the creation of a two-dimensional gradient, where two different gradients of functionalities are deposited in opposite directions, further improved the overall resolution of the analyte separation.

Application to Pharmaceutical Research

While there are countless potential applications for the use of stationary phase gradients in liquid chromatography, this paper seeks to address how it will impact the field of pharmaceutical research. Drugs produced by pharmaceutical companies are vital to the health of communities, providing life-saving solutions to a myriad of diseases. However, both time and money can be a roadblock to connecting individuals with the treatments they need. For the

development of every new drug, approximately one billion dollars and one decade of time is spent to get that drug to market (Pandey et al., 2010). Therefore, any discovery that can increase the time or cost-efficiency of drug development is invaluable not only within the industry but also to the health of communities worldwide.

Liquid chromatography is a central part of the drug development pipeline, with both manufacturing and research heavily depending on the technique (Castro-Perez, 2007; Hassan, 2012; Nikolin et al., 2004; Siddiqui et al., 2017). Specifically, HPLC is utilized for the identification of structures, formulation, detection of contaminants, analysis of drug interactions, determination of shelf-life and degradation products, and analysis of pharmacokinetic data (Chewa et al., 2021; D'Atri et al., 2019; Hsieh & Korfmacher, 2006; John et al., 2004; Petruczynik et al., 2020). Therefore, optimizing the efficiency of the chromatography techniques employed by pharmaceutical researchers will greatly increase the throughput of pharmaceutical labs (Howard et al., 2021).

As the use of stationary phase gradients in previous studies has proven to increase the resolving power of various chromatography techniques, there is significant indication that their application to the drug development pipeline could have a similar effect. An improvement in the chromatographic separation resolution would mean an increase in reliability in drug screenings, producing higher-quality data from LC analysis. Stationary phase gradients can increase the speed of investigative and formulation steps as well as the resolving power of the chromatographic technique. They may even minimize costs if the amount of expensive organic solvents required for the LC system can be reduced by the amplification of the column by stationary phase modification alone.

Future Work

The stationary phase gradient creation methods described thus far have exhibited success in their application to capillary electrochromatography (CEC), capillary liquid chromatography (cLC), and Thin Layer Chromatography (TLC). They have even provided significant evidence that the incorporation of a stationary phase gradient improves the resolution of each of these separation techniques. However, despite the extensive amount of research published on the topic of stationary phase gradients, scientists have yet to apply a stationary phase of varying functionalities to High-Performance Liquid Chromatography (HPLC), the most frequently used chromatography technique. Therefore, it is suggested that in order to explore this new area of application, the experimentation already performed with other techniques could be used to inform the creation of an HPLC mode. By modifying existing protocols to fit an LC system, a study could be designed to reliably test the effects of a stationary phase gradient on analysis by HPLC.

To validate an HPLC method with a gradient column, the experimental design should mimic those designs used for the validation of the gradient stationary phase in other chromatographic systems. Following the previous prototypes described in this report, both a negative and positive control should be prepared to qualify the capabilities of the gradient column. The positive control should be a uniformly modified LC column, and the negative control should be an unmodified LC column. Borrowing from the modification techniques established by previous publications, the gradient LC columns could be synthesized according to either the sequential immersion (Maruška et al., 2007), controlled rate infusion (Forzano et al., 2019; Kannan et al., 2011a; Kannan et al., 2011b), or photopolymerization (Dessauer, 2006;

Rohr et al., 2003) methods. A survey of these modification techniques indicates that photopolymerization would be the best method to apply to HPLC.

Photopolymerization is superior to sequential immersion because it provides greater control over the gradient shape. When the techniques were compared, the gradients produced by photopolymerization were much more linear than those produced by sequential immersion in monomer solutions. Photopolymerization also has advantages over the method of CRI because it has been applied to CEC, a chromatographic method that uses a column stationary phase like HPLC. This similarity between the CEC and HPLC formats makes it easier to adapt previous photopolymerization protocols to LC systems. Meanwhile, CRI has only been demonstrated on TLC, which uses a two-dimensional plate stationary phase and differs greatly from the format of HPLC.

As the intention behind the experimentation is to apply the technique to pharmaceutical production, the compounds chosen for analysis should reflect the composition of commonly used pharmaceutical products. Following the example of the study conducted by Kannan et al. (2011) which used acetaminophen, diphenhydramine hydrochloride, and doxylamine succinate, a similar set of easily acquired over-the-counter drugs could be chosen for analysis (Forzano et al., 2019).

A successful study would show an increase in resolution between peaks produced by the appropriate detection method when the gradient column is used. HPLC is a highly flexible technique that can be paired with numerous detection methods. Therefore, the experimental design could incorporate many different methods for the analysis of separations. However, the most commonly used detection methods in the pharmaceutical industry are fluorescence

intensity, mass spectrometry, and UV-vis spectroscopy, so the best representative model would incorporate one of these detectors.

Although the first step is to build off what is already tested and known from the literature and apply it to HPLC, future work will also include branch points into novel areas of gradient technology. For example, there are many other methods that exist within the field of nanotechnology for creating chemical gradients which have not been applied to the modification of stationary phases. Among these methods, the use of vapor phase deposition looks promising for the modification of an LC column (Bautista-Gomez et al., 2018; Kobayashi et al., 2006).

Another possibility yet to be explored is the application of a multidimensional stationary phase gradient. The theory of multidimensional HPLC says that the use of multiple separation schemes in a single system amplifies its separation capabilities because the overall resolution is a product of the respective resolutions of the multiple systems involved (Aly et al., 2020). This is shown in the equation, where $n_{\text{2D-HPLC}}$ is the peak capacity (i.e., the number of peaks that can be separated distinctly within the retention time) of the entire 2D system, $n_{\text{1st-D}}$ is the peak capacity of the first dimension, and $n_{\text{2nd-D}}$ is the peak capacity of the second dimension.

$$n_{2D-HPLC} = n_{1st-D} \times n_{2nd-D} \tag{7}$$

The validity of this theory has been demonstrated by repeated studies that utilized two different gradient elution schemes within one HPLC method and elevated the peak capacity of the individual elution mechanisms (Acevedo et al., 2020; Erni & Frei, 1978; Hirsh & Tsonev, 2012; Liang et al., 2012). A similar approach could amplify the selectivity of single dimension stationary phases. Instead of one linear gradient formed across the surface of the stationary support, a multidimensional gradient would provide additional points of differentiation by

utilizing two or more independent separation mechanisms simultaneously. This technique would mimic the coupling of columns with different elution gradients, this time coupling stationary phase gradients of different modifications to leverage the different analyte interactions they would promote.

Yet another extension of this method involves the coupling of both a stationary phase gradient and a mobile phase gradient for maximum selectivity. The effects that this type of procedure would have on chromatography separations is largely unknown and little has been done by way of experimentation. One study by Cain et al. used both experimental and mathematical models to analyze the outcome of utilizing both solvent and column gradients (Cain et al., 2019). However, the scope of this study was limited to one gradient type and a single mixture of compounds. Much more remains to be accomplished regarding the impact of different gradient shapes and ligand types used in conjunction with solvent gradients on separation efficiency.

Conclusion

Chromatography is an invaluable method for analysis across many fields of scientific study. The optimization of chromatographic separations will directly improve the research capabilities of scientists across the world, decreasing the time required for experimentation and as well as the overall cost of the procedure. Several methods have demonstrated the effectiveness of continuous stationary phase gradients in improving chromatography procedures. However, these methods have yet to be applied to HPLC. If stationary phase gradients can be incorporated into HPLC, this will specifically impact pharmaceutical research, where HPLC is crucial to creating and developing drugs. If separation scientists can introduce a practical way to

incorporate gradient stationary phases, it will advance the efficiency and ability of pharmaceutical research.

References

Acevedo, M. S. F., Gama, M. R., Batista, A. D., & Rocha, F. R. P. (2020). Two-dimensional separation by sequential injection chromatography. *Journal of Chromatography A*, *1626*, 461365. **https://doi.org/10.1016/j.chroma.2020.461365**

Akash, M.S.H., & Rehman, K. (2020). High performance liquid chromatography. In *Essentials of pharmaceutical analysis* (pp. 175-184). Springer.

Alvarez-Segura, T., Torres-Lapasió, J. R., Ortiz-Bolsico, C., & García-Alvarez-Coque, M. C. (2016). Stationary phase modulation in liquid chromatography through the serial coupling of columns: A review. *Analytica Chimica Acta*, *923*, 1-23. **https://doi.org/10.1016/j.aca.2016.03.040**

Aly, A. A., Muller, M., De Villiers, A., Pirok, B. W. J., Górecki, T. (2020). Parallel gradients in comprehensive multidimensional liquid chromatography enhance utilization of the separation space and the degree of orthogonality when the separation mechanisms are correlated. *Journal of Chromatography A*, *1628*, 461452. **https://doi.org/10.1016/j.chroma.2020.461452**

Axnanda, S., Crumlin, E. J., Mao, B., Rani, S., Chang, R., Karlsson, P. G., Edwards, M. O. M., Lundqvist, M., Moberg, R., Ross, P., Hussain, Z., & Liu, Z. (2015). Using "tender" x-ray ambient pressure x-ray photoelectron spectroscopy as a direct probe of solid-liquid interface. *Scientific Reports*, *9788*(5), 1-12. **https://www.doi.org/10.1038/srep09788**

Bautista-Gomez, J., Forzano, A. V., Austin, J. M., Collinson, M. M., & Higgins, D. A. (2018). Vapor-phase plotting of organosilane chemical gradients. *Langmuir*, *34*, 9665-8672. **https://doi.org/10.1021/acs.langmuir.8b01977**

Berna, M. J., Ackermann, B. L., & Murphy, A. T. (2004). High-throughput chromatographic approaches to liquid chromatographic/tandem mass spectrometric bioanalysis to support drug discovery and development. *Analytica Chimica Acta*, *509*, 1-9. **https://doi.org/10.1016/j.aca.2003.12.023**

Blum, F. (2014). High performance liquid chromatography. *British Journal of Hospital Medicine, 75*, 18-21. **https://www.doi.org/10.12968/hmed.2014.75.Sup2.C18**

Buffeteau, T., Desbat, B., & Eyquem, D. (1996). Attenuated total reflection Fourier transform infrared microspectroscopy: Theory and application to polymer samples. *Vibrational Spectroscopy*, *11*(1), 29-36. **https://doi.org/10.1016/0924-2031(95)00054-2**

Cain, C. N., Forzano, A. V., Rutan, S. C., & Collinson, M. M. (2019). Experimental- and simulation-based investigations of coupling a mobile phase gradient with a continuous stationary phase gradient. *Journal of Chromatography A*, *1602*, 237-245. **https://doi.org/10.1016/j.chroma.2019.05.033**

Carr, P. W., Stoll, D. R., & Wang, X. (2011). Perspectives on recent advances in the speed of high-performance liquid chromatography. *Analytical Chemistry*, *83*(6), 1890-1900. **https://doi.org/10.1021/ac102570t**

Castro-Perez, J. M. (2007). Current and future trends in the application of HPLC-MS to metabolite-identification studies. *Drug Discovery Today*, *12*, 249-256. **https://doi.org/10.1016/j.drudis.2006.01.007**

Chewa, Y., Khora, M., & Limb, Y. (2021). Choices of chromatographic methods as stability indicating assays for pharmaceutical products: A review. *Heliyon*, *7*, 1-12. **https://doi.org/10.1016/j.heliyon.2021.e06553**

Connolly, D., O'Shea, V., Clark, P., O'Connor, B., & Paull, B. (2007). Evaluation of photografted charged sites within polymer monoliths in capillary columns using contactless conductivity detection. *Journal of Separation Science, 30*, 3060-3068. **https://www.doi.org/10.1002/jssc.200700365**

Currivan, S., Connolly, D., Gillespie, E., & Paull, B. (2010). Fabrication and characterization of capillary polymeric monoliths incorporating continuous stationary phase gradients. *Journal of Separation Science, 33,* 484-492. **https://ww.doi.org/10.1002/jssc.200900720**

D'Atri, V., Fekete, S., Clarke, A., Veuthey, J., & Guillarme, D. (2019). Recent advances in chromatography for pharmaceutical analysis. *Analytical Chemistry*, *91*, 210-239. **https://doi.org/10.1021/acs.analchem.8b05026**

Dawood, M. (2014). Durability of steel components strengthened with fiber-reinforced polymer (FRP) composites. In V. M. Karbhari (Ed.), *Rehabilitation of metallic civil infrastructure using fiber reinforced polymer (FRP) composites* (pp. 96-114). Woodhead Publishing. **https://doi.org/10.1533/9780857096654.1.96**

Dessauer, R. (2006). HABIS as photopolymerization initiators. In *Photochemistry, history, and commercial applications of hexaarylbiimidazoles*. (pp. 123-133). Elsevier Science.

Dolan, J. W. (2013). Gradient elution, part IV: Dwell-volume problems. *LC-GC North America*, *31*(6), 456-463.

Dong, M. W. (2005). HPLC instrumentation in pharmaceutical analysis: Status, advances, and trends. In S. Ahuja & M. W. Dong (Eds.), *Handbook of Pharmaceutical Analysis by HPLC* (1se ed.). (pp. 47-75). Elsevier Inc.

Dong, M. W. (2006). HPLC instrumentation and trends. In *Modern HPLC for practicing scientists* (pp. 77-110). John Wiley & Sons, Inc. **https://doi.org/10.1002/0471973106**

Driskell, J.A. (2003). Vitamin B6: Properties and determination. In L. Truigo, P.M. Finglas (Eds.), *Encyclopedia of food sciences and nutrition* (2nd ed.) (pp. 6012-6020). Academic Press.

El Rassi, Z., & Horváth, C. (1986). Tandem columns and mixed-bed columns in high-performance liquid chromatography of proteins. *Journal of Chromatography A*, *359*, 255-264. **https://www.doi.org/10.1016/0021-9673(86)80079-6**

Engelhardt, H., & Elgass, H. (1975). Reproducibility problems in gradient elution caused by differing equipment. *Journal of Chromatography A*, *112*, 415-423. **https://doi.org/10.1016/S0021-9673(00)99973-4**

Erni, F., & Frei, R. W. (1978). Two-dimensional column liquid chromatographic technique for resolution of complex mixtures. *Journal of Chromatography A*, *149*, 561-569. **https://doi.org/10.1016/S0021-9673(00)81011-0**

Escorihuela, J., & Zuilhof, H. (2017). Rapid surface functionalization of hydrogen-terminated silicon by alkyl silanols. *Journal of the American Chemical Society*, *139*, 5870-5876. **https://doi.org/10.1021/jacs.7b01106**

Ettre, L. S. (2008). M.S. Tswett, and the invention of chromatography part I: Life and early work (1872-1903). In J. V. Hinshaw, *Chapters in the evolution of chromatography* (pp. 49-59). Imperial College Press.

Evans, D.R.H., Romero, J.K., & Westoby, M. (2009). Concentration of proteins and removal of solutes. *Methods in Enzymology*, *463*, 97-120. **https://doi.org/10.1016/S0076-6879(09)63009-3**

Felinger, A., & Cavazzini, A. (2017). Kinetic theories of liquid chromatography. In L. R. Snyder, & J. W. Dolan (Eds.), *Liquid chromatography: Fundamentals and instrumentation* (2nd ed.). (pp. 17-37). Elsevier. **https://www.doi.org/10.1016/B978-0-12-805393-5.00002-6**

Forzano, A. V., Cain, C. N., Rutan, S. C., & Collinson, M. M. (2019). In situ silanization for continuous stationary phase gradients on particle packed LC columns. *Analytical Methods*, *11*(29), 3648-3656. **https://www.doi.org/10.1039/c9ay00960d**

Galea, C., Mangelings, D., & Heyden, Y. V. (2015). Characterization and classification of stationary phases in HPLC and SFC—a review. *Analytica Chimica Acta*, *886*, 1-15. **https://doi.org/10.1016/j.aca.2015.04.009**

Ghose, S., & Cramer, S. M. (2001). Characterization and modeling of monolithic stationary phases: Application to preparative chromatography. *Journal of Chromatography A, 928*(1), 13-23. **https://doi.org/10.1016/S0021-9673(01)01115-3**

Gillespie, E., Macka, M., Connolly, D., & Paull, B. (2006). Evaluation of capillary ion exchange stationary phase coating distribution and stability using radial capillary column contactless conductivity detection. *Analyst*, *131*(8), 886-888. **https://doi.org/10.1039/B606942H**

González-González, M., González-Valdez, J., Mayolo-Deloisa, K., & Rito-Palomares, M. (2017). Monolithic chromatography: Insights and practical perspectives. *Journal of Chemical Technology & Biotechnology*, *92*(1), 9-13. **https://doi.org/10.1002/jctb.5040**

González-González, M., Mayolo-Deloisa, K. Rito-Palomares, M. (2020). Recent advances in antibody-based monolith chromatography for therapeutic applications. In A. Matte (Ed.), *Approaches to the purification, analysis and characterization of antibody-based therapeutics* (pp. 105-116). Elsevier Ltd. **https://doi.org/10.1016/C2018-0-02743-7**

Gritti, F., & Guiochon, G. (2013). The van Deemter equation: Assumptions, limits, and adjustment to Modern high performance liquid chromatography. *Journal of Chromatography A*, *1302*, 1-13. **https://doi.org/10.1016/j.chroma.2013.06.032**

Gritti, F., & Guiochon, G. (2014). Band broadening along gradient reversed phase columns: A potential gain in resolution factor. *Journal of Chromatography A, 1342,* 24-29. **https://doi.org/10.1016/j.chroma.2014.03.025**

Hassan, B. A. R. (2012). HPLC uses and importance in the pharmaceutical analysis and industrial field. *Pharmaceutica Analytica Acta, 3*(09), 133. **https://doi.org/10.4172/2153-2435.1000e133**

Hirsh, A. G., & Tsonev, L. I. (2012). Multiple, simultaneous, independent gradients for versatile multidimensional liquid chromatography. Part I: Theory. *Journal of Chromatography A*, *1236*, 52-62. **https://doi.org/10.1016/j.chroma.2012.02.072**

Howard, G. A., & Martin, A. J. P. (1950). The separation of the C_{12}-C_{18} fatty acids by reversed-phase partition chromatography. *Biochemical Journal*, *46*(5), 532-538. **https://www.doi.org/10.1042/bj0460532**

Howard, R. L., Bernardi, F., Leff, M., Abele, E., Allbritton, N. L., & Harris, D. M. (2021). Passive control of silane diffusion for gradient application of surface properties. *Micromachines*, *12*(11), 1360. **https://doi.org/10.3390/mi12111360**

Hsieh, Y., & Korfmacher, W. A. (2006). Increasing speed and throughput when using HPLC-MS/MS systems for drug metabolism and pharmacokinetic screening. *Current Drug Metabolism*, *7*(5), 479-489. **https://doi.org/10.2174/138920006777697963**

Jennings, W., Mittlefehldt, E., & Stremple, P. (1997). The stationary phase. In *Analytical Gas Chromatography* (2nd ed.). (pp. 92-113). Elsevier Science. **https://doi.org/10.1016/B978-0-12-384357-9.X5000-4**

John, H., Walden, M., Schafer, S., Genz, S., & Forssmann, W. (2004). Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry*, *378*, 883-897. **https://doi.org/10.1007/s00216-003-2298-y**

Kannan, B., Dong, D., Higgins, D. A., & Collinson, M. M. (2011). Profile control in surface amine gradients prepared by controlled-rate infusion. *Langmuir*, *27*, 1867-1873. **https://www.doi.org/10.1021/la104448n**

Kannan, B., Marin, M. A., Shrestha, K., Higgins, D. A., & Collinson, M. M. (2011). Continuous stationary phase gradients for planar chromatographic media. *Journal of Chromatography A, 1218*(52), 9406-9413. **https://doi.org/10.1016/j.chroma.2011.10.075**

Klages, C., Raev, V., Murugan, D., & Sai, V. V. R. (2019). Argon-water DBD pretreatment and vapor-phase silanization of silica: Comparison with wet-chemical processes. *Plasma Processes and Polymers*, *17*(7), 1-10. **https://doi.org/10.1002/ppap.201900265**

Kobayashi, H., Ikegami, T., Kimura, H., Hara, T., Tokuda, D., & Tanaka, N. (2006). Properties of monolithic silica columns for HPLC. *Analytical Sciences, 22*, 491-499**.** **https://doi.org/10.2116/analsci.22.491**

Krupczyńska, K., & Buszewski, B. (2004). Characterizing HPLC stationary phases: Chromatographic methods can characterize stationary phases without destroying them. *Analytical Chemistry*, *76*(13), 226-234. **https://doi.org/10.10121/ac041583o**

Liang, Z., Li, K., Wang, X., Ke, Y., Jin, Y., & Liang, X. (2012). Combination of off-line two-dimensional hydrophilic interaction liquid chromatography for polar fraction and two-dimensional hydrophilic interaction liquid chromatography × reversed-phase liquid chromatography for medium-polar fraction in a traditional Chinese medicine. *Journal of Chromatography A*, *1224*, 61-69. **https://doi.org/10.1016/j.chroma.2011.12.046**

Llovet, X. (2019). Microscopy: Electron probe microanalysis. In P. Worsfold, A. Townshend, C. Poole, & M. Miró (Eds.), *Encyclopedia of analytical science* (3rd ed.). (pp. 30-38). Elsevier Ltd. **https://doi.org/10.1016/B978-0-12-409547-2.14369-0**

Mageea, M. H., Manulika, J. C., Barnes, B. B., Abate-Pellaa, D., Hewitta, J. T., & Boswella, P. G. (2014). "Measure Your Gradient": A new way to measure gradients in high performance liquid chromatography by mass spectrometric or absorbance detection. *Journal of Chromatography A*, *1369*, 73-82. **https://doi.org/10.1016/j.chroma.2014.09.084**

Maruška, A., Rocco, A., Kornyšova, O., & Fanali, S. (2007). Synthesis and evaluation of polymeric continuous bed (monolithic) reversed-phase gradient stationary phases for capillary liquid chromatography and capillary electrochromatography. *Journal of Biochemical and Biophysical Methods*, *70*(1), 47-55. **https://doi.org/10.1016/j.jbbm.2006.10.011**

Miller, J. M. (2009). Liquid chromatography in columns. In *Chromatography: Concepts and contrasts* (2nd ed.). (pp. 184-277). John Wiley & Sons, Inc.

Moldoveanu, S.C., & David, V. (2022). Introductory information regarding HPLC. In *Essentials in modern HPLC separations* (2nd ed.). (pp. 3-20). Elsevier Inc. **https://doi.org/10.1016/B978-0-323-91177-1.00006-5**

Moldoveanu, S.C., & David, V. (2022). Parameters for the characterization of HPLC separation. In *Essentials in modern HPLC separations* (2nd ed.). (pp. 63-105). Elsevier Inc. **https://doi.org/10.1016/B978-0-323-91177-1.00006-5**

Nesterenko, P. N., & Palamareva, M. D. (2019). Liquid chromatography: Principles. In P. Worsfold, A. Townshend, C. Poole, & M. Miró (Eds.), *Encyclopedia of analytical science* (3rd ed.). (pp. 231-237). Elsevier Ltd. **https://doi.org/10.1016/B978-0-12-409547-2.14215-5**

Nikolin, B., Imamovic, B., Medanhodzic-Vuk, S., & Sober, M. (2004). High performance liquid chromatography in pharmaceutical analyses. *Biomolecules & Biomedicine*, *4*(2), 5-9. **https://doi.org/10.17305/bjbms.2004.3405**

Nyiredy, Sz., Szucs, Z., & Szepesy, L. (2006). Stationary-phase optimized selectivity LC (SOS-LC): Separation examples and practical aspects. *Chromatographia Supplement*, *63*, S3-S9. **https://doi.org/10.1365/s10337-006-0833-7**

Ortiz-Bolsico, C., Torres-Lapasió, J. R., Ruiz-Ángel, M. J., & García-Álvarez-Coque, M. C. (2013). Comparison of two serially coupled column systems and optimization software in isocratic liquid chromatography for resolving complex mixtures. *Journal of Chromatography A*, *1281*, 94-105. **http://dx.doi.org/10.1016/j.chroma.2013.01.064**

Pandey, S., Pandey, P., Tiwari, G., & Tiwari, R. (2010). Bioanalysis in drug discovery and development. *Pharmaceutical Methods*, *1*(1), 14-24. **https://www.doi.org/10.4103/2229-4708.72223**

Petruczynik, A., Wroblewski, K., Wojtanowski, K., Mroczek, T., Juchnowicz, D., Karakula-Juchnowicz, H., & Tuzimski, T. (2020). Comparison of various chromatographic systems for identification of vortioxetine in bulk drug substance, human serum, saliva, and urine

samples by HPLC-DAD and LC-QTOF-MS. *Molecules*, *25*(11), 2483. **https://doi.org/10.3390/molecules25112483**

Petter, C. H., Heigl, N., Bonn, G. K., & Huck, C. W. (2008). Fast, non-invasive and simultaneous near-infrared spectroscopic characterisation of physicochemical stationary phases' properties: From silica particles towards monoliths. *Journal of Separation Science, 31*(14), 2541-2550. **https://doi.org/10.1002/jssc.200800274**

Pucci, V., Raggi, M. A., Svec, F., Fréchet, J. M. J. (2004). Monolithic columns with a gradient of functionalities prepared via photoinitiated grafting for separations using capillary electrochromatography. *Journal of Separation Science*, *27*(10), 779-788. **https://www.doi.org/10.1002/jssc.200401828**

Purnell, J.H. (1960). The correlation of separating power and efficiency of gas-chromatographic columns. *Journal of the Chemical Society*, *1*, 1268-1274. **https://doi.org/10.1039/JR9600001268**

Quarry, M. A., Grob, R. L., & Snyder, L. R. (1984). Measurement and use of retention data from high-performance gradient elution: Contributions from “non-ideal” gradient equipment. *Journal of Chromatography A*, *285*, 1-18. **https://doi.org/10.1016/S0021-9673(01)87732-3**

Robards, K., & Ryan, D. (2022). High performance liquid chromatography: Separations. In *Principles and Practice of Modern Chromatographic Methods* (2nd ed.). (pp. 283-336). Academic Press. **https://doi.org/10.1016/C2019-0-03803-4**

Robards, K., & Ryan, D. (2022). High-performance liquid chromatography: Instrumentation and techniques. In *Principles and practice of modern chromatographic methods* (pp. 247-282). Elsevier Ltd. **https://doi.org/10.1016/B978-0-12-822096-2.00009-8**

Robards, K., & Ryan, D. (2022). Theoretical considerations. In *Principles and practice of modern chromatographic methods* (2nd ed.). (pp. 41-96). Academic Press. **https://doi.org/10.1016/B978-0-12-822096-2.00002-5**

Rohr, T., Hilder, E. F., Donovan, J. J., Svec, F., & Fréchet, J. M. J. (2003). Photografting and the control of surface chemistry in three-dimensional porous polymer monoliths. *Macromolecules*, *36*, 1677-1684. **https://doi.org/10.1002/jssc.200401828**

Schellinger, A. P., & Carr, P. W. (2006). Isocratic and gradient elution chromatography: A comparison in terms of speed, retention reproducibility and quantitation. *Journal of Chromatography A*, *1109*(2), 253-266. **https://doi.org/10.1016/j.chroma.2006.01.047**

Schoell, S. J., Oliveros, A., Steenackers, M., Saddow, S. E., & Sharp, I. D. (2012). Multifunctional SiC surfaces: From passivation to biofunctionalization. In S.E. Saddow (Ed.), *Silicon carbide biotechnology: A biocompatible semiconductor for advanced biomedical devices and applications*. (1st ed.). (pp. 63-117). Elsevier Science. **https://doi.org/10.1016/B978-0-12-385906-8.00003-9**

Scott, R. P. W. (Ed.). (1986). The selection of the appropriate detector, In *Chromatographic detectors: Design, function, and operation* (pp. 235-261). Elsevier Science. **https://doi.org/10.1016/S0301-4770(08)70046-0**

Siddiqui, M. R., AlOthman, Z. A., & Rahman, N. (2017). Analytical techniques in pharmaceutical analysis: A review. *Arabian Journal of Chemistry*, *10*, 1409-1421. **https://doi.org/10.1016/j.arabjc.2013.04.016**

Smith, K. C. A., & Oatley, C. W. (1955). The scanning electron microscope and its fields of application. *British Journal of Applied Physics*, *6*(11), 391. **https://www.doi.org/10.1088/0508-3443/6/11/304**

Snyder, L. R., & Dolan, J. W. (2007). Gradient Equipment. In V. L. McGuffin (Ed.), *High-performance gradient elution: The practical application of the linear-solvent-strength model*. (pp. 133-151). Wiley-Interscience. **https://doi.org/10.1002/0470055529**

Stauffer, E., Dolan, J. A., & Newman, R. (2008). Gas Chromatography and Gas Chromatography—Mass Spectrometry. In *Fire Debris Analysis* (pp. 235-293). Academic Press. **https://doi.org/10.1016/B978-0-12-663971-1.X5001-5**

Swartz, M. (2010). HPLC detectors: A brief review. *Journal of Liquid Chromatography & Related Technologies*, *33*, 1130-1150. **https://doi.org/10.1080/10826076.2010.484356**

Usher, K. M., Simmons, C. R., & Dorsey, J. G. (2008). Modeling chromatographic dispersion: A comparison of popular equations. *Journal of Chromatography A*, *1200*(2), 122-128. **https://doi:10.1016/j.chroma.2008.05.073**

Uyama, Y., Inoue, H., Ito, K., Kishida, A., & Ikada, Y. (1991). Comparison of different methods for contact angle measurement. *Journal of Colloid and Interface Science, 141*(1), 275-279. **https://doi.org/10.1016/0021-9797(91)90322-Y**

VanMiddlesworth, B. J., & Dorsey, J. G. (2011). Reequilibration time of superficially porous silica based columns in gradient elution reversed phase liquid chromatography. *Journal of Chromatography A*, *1218*(40), 7158-7165. **https://doi.org/10.1016/j.chroma.2011.08.030**

Wixton, R. L. (2001). The beginnings of chromatography: The pioneers (1900-1960). In C. W. Gehrke, R. L. Wixom, & E. Bayer, *Chromatography a century of discovery 1900-2000: The bridge to the sciences/technology* (1st ed.). (pp. 1-38). Elsevier Science Ltd.