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An Overview to the Technique of Reverse-Phase Ultra High Performance Liquid Chromatography and Its Use in Pharmacy

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ABSTRACT
A lot of lipid species in biological samples, especially human plasma and serum, can be quantified using the reversed-phase ultrahigh-performance liquid chromatography-mass spectrometry (RP-UHPLC/MS) approach. Lipids from 23 subclasses are separated using a C18 bridged ethylene hybrid (BEH) column (150 x 2.1 mm; 1.7 µm) with a 25-minute run time. Resolution of isobaric and isomeric lipid forms is made possible by lipid species separation. Multiple reaction monitoring (MRM) in the positive ion mode is employed for targeted lipidomic analysis utilizing a triple quadrupole mass spectrometer. Skyline software analyzes the data, and internal standards for each lipid class are used to calculate the analyte concentrations. The rate of advancement has not slowed ten years after the first commercial ultra-high-performance liquid chromatography (UHPLC) equipment was introduced. Using columns with internal diameters of 2.1 mm, we present recent developments in UHPLC apparatus and columns, with an emphasis on those designed mainly for reversed-phase separations of analytes with molecular weights less than around 5000 Daltons. Additionally, when employing the low ionic strength, acidic mobile phases that are used for electrospray mass spectrometric detection, columns with charged surface particles provide larger peak capacities for the separations of positively charged analytes. These columns provide narrow peaks that call for instruments with very low dispersion. We examine current developments in system dispersion measurement and reduction. The present state, advantages, and drawbacks of UHPLC in technique development are critically reviewed in this article. The brief overview and uses of UHPLC in pharmaceutical analysis are given in this review.

I. INTRODUCTION

Reversed-phase ultrahigh-performance liquid chromatography (RP-UHPLC) is a fast and highly efficient chromatography method that uses a polar mobile phase and a non-polar stationary phase to separate substances according to their hydrophobicity.

Jorgenson first used the term UHPLC, which stands for ultra-high-pressure liquid chromatography, in 1997. The usage of nano-columns filled with non-porous 1.0–1.5 µm silica-based particles on a prototype system that can withstand extremely high pressures (up to 4100 bar in 1997 and 7200 bar in 2003 was originally described by this group. [1]

In addition to Jorgenson's study, Lee et al. verified that UHPLC could handle pressures of up to 3600 bar. These proofs of concept led to Waters' 2004 commercialization of the first chromatographic system that could withstand 1000 bar of pressure under the brand name Ultra-performance Liquid Chromatography (UPLC), along with narrow-bore columns filled with 1.7-µm fully porous particles (FPPs). "Ultra-high-performance liquid chromatography" or "ultra-high-pressure liquid chromatography" are the chosen terms for the technology's name. Alternatively, a few studies have reported on the use of the term very high-pressure liquid chromatography (VHPLC). [2]

LC separations employing columns with particles smaller than the 2.5–5–µm diameters commonly employed in HPLC are referred to as ultra-high-performance liquid chromatography (UHPLC). Greater efficiency per unit time is the advantage of employing columns with smaller particles (usually sub-2 µm). [3] Although UHPLC was first demonstrated with homemade apparatus, the first commercial UHPLC system was introduced in 2004 and allowed for the widespread use of this method. It was necessary to optimize the instrumentation in tandem with the columns in order to reap the benefits of having sub-2-µm particles. The system had to have low extra-column dispersion and a column compartment that minimized radial temperature gradients in addition to being able to operate dependably at pressures of up to 1000 bar. [4] Using columns with internal diameters of 2.1 mm, we present current developments in UHPLC apparatus and columns, with a particular emphasis on those

designed particularly for reversed-phase (RP) separations of analytes with molecular weights less than around 5000 Da. This is currently the largest category of UHPLC applications. [5]

Ultra-high-pressure liquid chromatography (UHPLC) has emerged as the contemporary standard HPLC platform in recent years. UHPLC is perfect for rapid method development because of its shorter analytical time and faster column equilibration.

Examples include automated method optimization, rapid column/mobile-phase screening, and the transfer of current HPLC techniques to speedier analysis. Although we concentrate on the creation of reversed-phase techniques for small-molecule pharmaceutical assay and impurity analysis, our findings and insights can be applied to several sample types and applications. [6] The higher pressure limitations of UHPLC not only produce faster analysis (when used with short, small-particle columns), but they also make it possible to use longer columns for routine analysis of complicated materials that is superior. One important analytical method for both qualitative and quantitative drug analysis is High Performance Liquid Chromatography (HPLC). Over 90% of medications prescribed by recognized pharmacopoeias are subjected to HPLC analysis. [7]

However, because of the stricter regulations, which include more samples, faster processing, and more sensitive analysis methods. The more sophisticated chromatographic techniques are being adopted by the pharmaceutical industry. Based on the idea of using a stationary phase made up of particles smaller than 2 µm, Ultra-high Performance Liquid Chromatography (UHPLC) is an improvement on HPLC. [8] Analysis speed and peak capacity can be increased to unprecedented levels and the sample can be examined faster with smaller particles. A novel method in chromatographic separations, the UHPLC technique has been effectively used for quick, high-resolution separations with the necessary sensitivity. [9]

Because it significantly boosts the throughput of conventional HPLC procedures, ultra-high-performance (pressure) liquid chromatography (UHPLC) has attracted a lot of attention since its commercial release in 2004. Although the primary factors driving the growing usage of UHPLC are its capacity to perform quick separations and high resolution,

In this review, we also describe several other developments, including:

1. Utilizing UHPLC technology to analyze complicated materials with high resolution.
2. Creating columns filled with sub-2-µm particles to create various chromatographic modes (such as chiral LC, SEC, IEX, HILIC, and SFC).
3. To further improve kinetic performance, higher pressure drop, higher temperature, smaller particle sizes, and sub-2-µm core-shell particles are evaluated.
4. UHPLC is used to improve biopharmaceutical characterization. 5. UHPLC-MS is developed for applications such as bioanalysis, multi-residue screening, and metabolomics. [10]

The majority of chromatographic suppliers (at least ten) have marketed their own UHPLC systems (more than 20 distinct systems) that can withstand pressures between 600 and 1400 bar throughout the last ten years. Many detector devices, such as the ultraviolet (UV), UV-diode-array detector (UV-DAD), evaporative light-scattering detector (ELSD), corona aerosol-discharge (CAD), refractive index (RI), fluorescence detector (FD), mass spectrometry (MS), and others, have been improved in design and can all be connected to UHPLC. [11-18]

Fig. UHPLC System

More than 100 supports and more than 10 distinct phase chemistries are available from at least 15 sources, and the number of columns filled with totally porous sub-2-µm particles has also increased. Regarding particle size (1.5-2 µm), pressure tolerance (600-1400 bar), pH, and temperature ranges, none of these stationary phases are comparable. These significant technological advancements have led to an exponential increase in the number of applications.

UHPLC was primarily employed to accomplish quick separations in the early days of its commercialization. Analysis time can be reduced by a factor of 9 while maintaining the same kinetic performance by using a UHPLC column of 50 × 2.1 mm I.D., 1.7 µm rather than a standard HPLC column of 150 × 4.6 mm I.D., 5 µm. [19]

Because of the enormous number of samples, this high-throughput feature is appealing in chemical, food, and environmental studies where productivity must be increased. The pharmaceutical industry is another industry that is pushing for quick separations. In applications like quality control, pharmacokinetics, and drug metabolism, increased productivity and lower costs are especially necessary during the drug-discovery and development process. The application of UHPLC has various additional advantages besides high-speed separations. Using a variety of examples, this review aims to examine the current and upcoming trends in this technique. [20]

CHEMISTRY OF SMALL PARTICLES:

Except for the microscopic particles commonly employed in HPLC, the Van Deemter equation falls short of its expectations. Although creating and engineering sub-2mm particles is a significant challenge, scientists are making headway in this area. Commercially available and very effective, 1.5mm drill particles have a limited loading and storage capacity due to their small surface area. To maintain retention and capacity, UPLC must use perforated new particles that can withstand high pressures, just as HPLC. Despite their great performance potential, silica-based particles can tolerate a variety of challenges, such as limited pH range and basic analyst tailing. [21] Although polymeric columns can overcome pH restrictions, they have disadvantages including low strength and effectiveness. Generating sub-2 mm particles requires a lot of labor, and scientists have been investing their resources in this project for some time. High-quality, non-porous 1.5 mm particles are traded, but their tiny surface area limits their load capacity and retention. To maintain strength and retention, UPLC must employ new porous-resistant particles at high pressures, just as HPLC. Despite their high mechanical strength, silica-based particles can have a number of disadvantages, such as basic analyst tailing and a pH limitation list. Despite the drawbacks of polymeric columns, such as their limited power and low efficiency, they can overcome pH limits. [22]

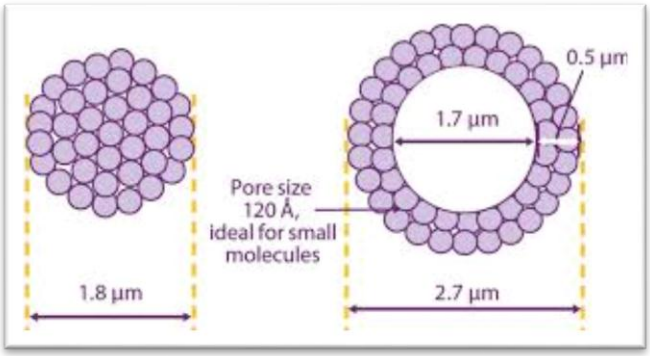

Fig. Size of Small Particles

ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Scientists are constantly forced to choose between speed and resolution when using high performance liquid chromatography, despite the fact that it has proven to be very helpful in many scientific domains. The very effective technique known as ultra performance liquid chromatography (UPLC), which is mainly based on small particle separations, removes the requirement for selection. [23]

One type of column chromatography used for component separation, identification, and quantification is ultra high pressure liquid chromatography, or ultra HPLC (UPLC). It makes it possible to swiftly and efficiently separate and analyze tiny particles. The process of sending a mixture of particles through a column for separation is known as liquid chromatography. This makes it possible to measure the analyte which was extracted from the mixture against other molecules. [24] The stationary phase is the packing material that fills the columns. The mixture, referred to as the mobile phase, is forced through the columns by a pump in UPLC. The retention durations of the various molecules are displayed by a detector as the mobile phase moves through the stationary phase. [25]

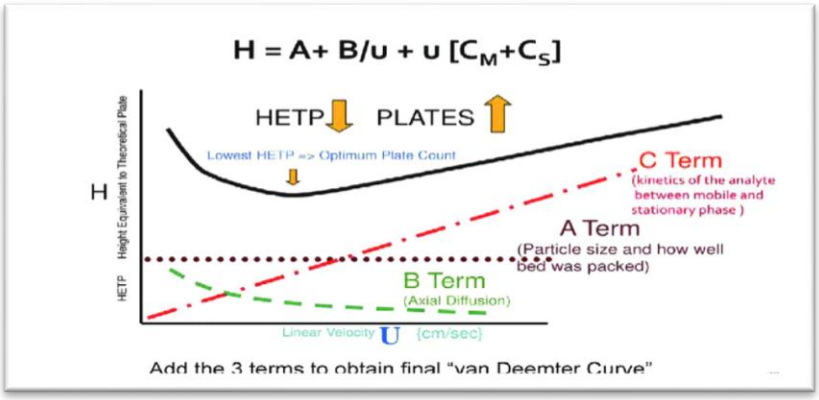

Fig. Van Deemter Equation

The interactions between the solvent, the molecules under study, and the stationary phase all affect retention time. The van Deemter equation serves as the theoretical foundation for the creation of UPLC. An empirical formula known as the van Deemter equation illustrates the connection between plate height, or column efficiency, and linear velocity, or flow rate. The resulting curve can be used to examine chromatographic performance because one of the variables in the van Deemter equation is particle size. [26] Even with higher flow rates or linear velocities, there is a noticeable increase in efficiency as the particle size drops to less than 2.5 µm.

Working with microscopic particles has several benefits, including efficiency and the ability to operate at higher linear velocities. This makes it possible to boost speed and resolution. Particle size and peak width are inversely correlated with resolution, which is proportional to the square root of N. Peak height and peak width are negatively correlated. [27] This implies that higher resolution narrower, taller peaks will be possible with the usage of smaller particles. It's also crucial to remember that the ideal flow rises as particle size falls. However, since pressure and flow rate are related, smaller particles will need more pressure or energy.

Since the new pressures associated with UPLC could not be met by existing HPLC technology, Waters created a new system known as ACQUITY UPLC. Considering all of the benefits that small particle separations currently offer over HPLC, Waters ACQUITY Ultra Performance LC systems were created. It was not an easy task. [28-30]

For the columns, a novel hybrid material known as Bridged Ethyl Hybrid (BEH) was developed. A smoother internal surface was also required for the columns.

Fig. Acquity UHPLC System

Microchip technology, known as eCordTM, is another feature of ACQUITY UPLC BEH columns that records production data for every column, including quality control tests and certificates of analysis. In order to preserve a comprehensive continuous column history, the eCord database is additionally updated in real time with

technique information, such as the quantity of injections or temperature and pressure data. [31] Several of these benefits are mostly predicated on the theory behind liquid chromatography. Generally speaking, a separation's resolution will rise as its efficiency does. A reduction in particle size will boost efficiency and accelerate the flow rate since efficiency and the ideal flow rate are inversely correlated with particle size. The particle size is reduced to 1.7 um in the ACQUITY system from 3.5 or 5 um. [32] The particles were made especially to have a high load capacity, increase efficiency, and endure a wide range of pH and pressure. Additional advancements in the chromatography technique include specialized detectors with fiber optic flow cells, fast injection cycle sample management, and a high pressure solvent delivery system to account for the reduced particle size. [33] The WATERS corporation is the trademark owner of the phrase UPLC. The method is commonly known as Ultra High Performance Liquid Chromatography (uHPLC) elsewhere. The real cause of uHPLC's higher flow rate and resolution is the smaller bead size. Determinter's equation, which reads $H = A + B/\mu + C\mu$, can be used to mathematically illustrate this. The particle size is μ, while the plate height is H. [34] The so-called 'Eddy diffusion term', or A constant, is unaffected by flow rate. The "analyte mass transfer" coefficient is denoted by C, whereas the diffusion coefficient is represented by the B constant. Higher resolution is made possible by a decrease in u, which also results in a decrease in the A and C values required for a comparable H value. [35] Faster separations for comparable resolutions are also produced by lessening the impact of the C value on the H value. Keep in mind that uHPLC is superior to HPLC in every way and is anticipated to displace HPLC soon. [36]

REVERSED PHASE CHROMATOGRAPHY

Reversed-phase liquid chromatography (RP-LC) is a type of liquid chromatography where organic molecules are separated using polar mobile phases and non-polar stationary phases. In recent years, the reversed phase mode has been used for the great majority of high-performance liquid chromatography (HPLC) separations and studies. [37] The more hydrophobic the sample components, the longer they remain in the system in the reversed phase state.

The following variables impact solute separation and retention in the reversed phase chromatographic system:
a. The stationary phase's chemical makeup, or the ligands that are bonded to its surface, and their bonding density, or how much of the surface they cover.

b. The mobile phase's composition. types of bulk solvents whose mixes alter the mobile phase's polarity, hence the term "modifier" for a solvent added to alter the mobile phase's polarity.

c. Additives, like buffers, alter the mobile phase's pH, which in turn alters the solutes' polarity and ionization state. [38-40]

The stationary phases, which are packed inside columns, are made up of hydrophobic substrates that are bonded to the surface of porous silica-gel particles in a variety of geometries (spheric, irregular) at different diameters (sub-2, 3, 5, 7, 10 um), and with a range of pore diameters (60, 100, 150, 300, A) in order to preserve the organic components in mixtures. Chemically bound hydrocarbons, including C3, C4, C8, C18, and others, blanket the particle's surface. The components of the sample will be kept for a longer period of time if the hydrocarbon linked to the stationary phase is longer. [41] For methods that use mobile phases with extremely high pH, some stationary phases are also composed of hydrophobic polymeric particles or hybridized silica-organic groups. C-18 columns, commonly known by trade names like ODS (octadecylsilane) or RP-18, are used in the majority of modern biological material separation techniques.

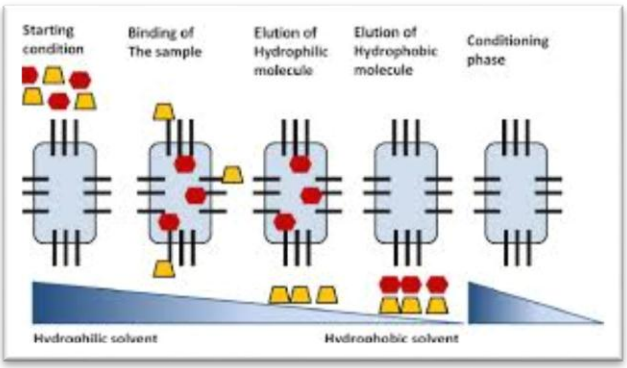

Fig. Steps of reversed phase chromatography

Methanol and acetonitrile make up the great bulk of the polar organic solvents that are mixed with water to form the mobile phases. These mixtures typically contain a variety of chemicals, including specific additives (EDTA), surfactants (alkyl amines or alkyl sulfonates), and buffers (acetate, phosphate, and citrate). Increasing efficiency, selectivity, and controlling solute retention are the objectives of employing supplements of any form. [42-45]

Reversed-Phase Columns

- C8: Octyl Columns.
- C18: Octadecyl Columns.
- C1: MEB1 Columns.
- C2: MEB2 Columns.
- C3: Propyl Columns.
- C4: MEB4 Columns.
- C5: Pentyl Columns.
- C6: Hexyl Columns. [46]

STATIONARY PHASES

Majors, Dolan, Carr, and Snyder's essay provides a detailed account of the development and history of reversed phase stationary phases. The majority of liquid chromatography runs in the 1970s used solid particles, such as alumina or unaltered silica gel, as the stationary phases. These days, this kind of method is called normal-phase chromatography. [47] Biomolecules with hydrophilic characteristics in the sample firmly adhere to the stationary phase since the stationary phase in this approach is hydrophilic and the mobile phase is non-polar (made up of organic solvents like hexane and heptane). Furthermore, they were difficult to dissolve in the solvents of the mobile phase. [48] Hydrophobic molecules, on the other hand, elute through the polar stationary phase early with insufficient retention because they have less affinity for it. This is the reason why, in order to accept biological chemicals, the silica-based particles were treated with hydrocarbons throughout the 1970s, immobilized or bonded on their surface, and the mobile phases were changed to aqueous and polar in nature. [49]

The term "reversed-phase chromatography" refers to the employment of polar mobile phases and a hydrophobic stationary phase, which is basically the opposite of normal phase chromatography because the polarity of the stationary and mobile phases has been inverted. Consequently, hydrophilic molecules in the sample move through the column and are eluted first, while hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase. By utilizing an organic (non-polar) solvent to decrease the polarity of the mobile phase, which lessens hydrophobic interactions, hydrophobic molecules can be eluted from the column. A higher concentration of organic solvent will be needed to elute the molecule because hydrophobic molecules will bond to the stationary phase more firmly. [50]

The selectivity factor, chromatographic resolution, plate count, and other experimental factors employed in other chromatographic techniques, as well as many of the mathematical parameters of the theory of chromatography, also apply to RP-LC. A large range of molecules can be separated using it. Since many proteins can be denatured by the organic solvents used in normal-phase chromatography, it is commonly utilized for protein separation. RP-LC is a commonly used analytical method nowadays. The development of separation techniques is made possible by the enormous range of stationary phases that can be used in RP-LC. [51]

Silica-based stationary phases

For a number of reasons, silica gel particles are frequently employed as a stationary phase in high-performance liquid chromatography (HPLC). These include:

1. High surface area: Because silica gel particles have a large surface area, they can interact directly with solutes or, after the bonding of different ligands, they can interact with sample molecules in a number of ways, improving separations.
2. Inertness and chemical and thermal stability: Because silica gel typically does not react with the mobile phase solvents or the substances being separated, it is chemically stable and produces precise, reproducible, and trustworthy analysis.
3. Broad applicability: Silica gel is adaptable and can be altered with different functional groups, which makes it appropriate for a variety of analytes and uses.
4. Effective separation: Silica gel particles' special qualities, such as their large surface area and regulated average particle diameter pore size, enable accurate and effective compound separation in HPLC.

5. Repeatability: High batch-to-batch repeatability is a feature of silica gel particles that is essential for dependable and repeatable HPLC analyses over many years.
6. Control of particle diameter and pore size: It is possible to precisely manage separation depending on molecule size by engineering silica gel to have particular pore diameters.
7. Cost-effectiveness: Because silica is the most plentiful element in the world, its gel is an affordable option for HPLC applications, which is why labs use it extensively. [52-56]

HPLC columns have been categorized by L# kinds according to the US Pharmacopoeia (USP). An octadecyl carbon chain (C18)-bonded silica (USP classification L1) is the most widely used column in this classification. Pure silica (L3), cyano-bonded silica (CN) (L10), C8-bonded silica (L7), and phenyl-bonded silica (L11) come next. While CN columns can be employed in a reversed-phase manner based on analyte and mobile phase conditions, C18, C8, and phenyl are designated reversed-phase stationary phases. [57] Not every C18 column has the same retention characteristics. Silica can be surface functionalized in a monomeric or polymeric reaction, and the leftover silanol groups can be covered in a second phase by various short-chain organosilanes (end-capping). While the overall retention mechanism stays the same, small alterations in the surface chemistries of distinct stationary phases will lead to changes in selectivity. [58]

Modern columns have varied polarity depending on the ligand attached to the stationary phase. Pentafluorphenyl is known as PFP. Cyano is CN. NH2 is an amino acid. Octadecyl, or C18, is ODS. C18 and nitrile make up the mixed mode column known as ODCN. Using a variety of stationary phase geometries, recent advancements in chromatographic supports and instrumentation for liquid chromatography (LC) enable quick and extremely effective separations. Numerous analytical techniques have been put forth, including the use of columns filled with sub-3 µm superficially porous particles, silica-based monolithic supports, and high mobile phase temperatures or with completely porous particles less than 2 µm for use in ultra-high-pressure LC systems (UHPLC) (fused or solid core). [59]

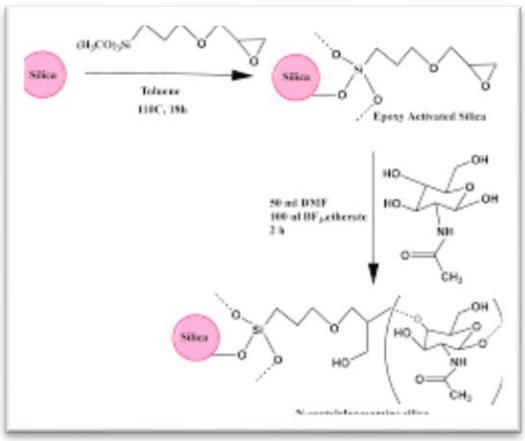

Fig. Silica based stationary phase

MOBILE PHASES

Boyes and Dong released a thorough paper on the most recent developments and industry best practices for mobile phase selection in reversed-phase chromatography. In reversed-phase chromatography, a mobile phase is made up of aqueous buffers or water mixes to which organic solvents are added in order to selectively elute analytes from a reversed-phase column. Acetonitrile and methanol are the two most often used organic solvents, and they must be miscible with water. Other solvents like ethanol, 2-propanol (isopropyl alcohol), and tetrahydrofuran (THF) can also be utilized. Since the organic solvent is added to the aqueous solution in the mobile phase to change the polarity of the mobile phase, it is also known as a modifier. [60]

Since water is the most polar solvent in the reversed phase mobile phase, adding modifiers to the mobile phase to reduce its polarity strengthens its elution. Methanol and acetonitrile are the two most commonly utilized organic modifiers, while acetonitrile is the more preferred option. Because of its potent eluting qualities, isopropanol (2-propanol) can also be utilized; however, its high viscosity, which raises backpressures, limits its application. Methanol and acetonitrile are both less viscous than isopropanol, however a 50:50 methanol:water mixture is also highly viscous and results in large backpressures. [61-65]

Acetonitrile is more transparent than the other solvents in the low UV wavelength range, which makes it the most popular choice for separating molecules with weak or nonexistent chromophores (UV-VIS absorbing groups), like peptides. Acetonitrile offers much lower background absorbance at low wavelengths than the other common solvents, and most peptides only absorb at low wavelengths in the ultra-violet spectrum (usually less

than 225 nm). All three solvents are essentially UV transparent, which is key for common reversed phase chromatography because sample components are usually detected by UV detectors.

The pH of the mobile phase can alter the selectivity of some analytes and have a significant impact on analyte retention. Mobile phase buffers can be used to control the ionization of solutes with ionized functional groups, such as amines, carboxyls, phosphates, phosphonates, sulfates, and sulfonates, found in samples. [66]

As the pH of the mobile phase rises over the pKa of the carboxylic groups in solutes, for instance, the entire molecule becomes more polar and is less maintained on the a-polar stationary phase. In this instance, increasing the phase mobile's pH over 4-5 = pH (the usual pka range for carboxylic groups) causes them to become more ionized, which in turn reduces their retention.

On the other hand, employing a mobile phase with a pH below 4 will improve their retention since it will lessen their degree of ionization, making them less polar. Because they are less ionized and therefore less polar, compounds with basic functional groups, like amines, whose pka ranges are about 8 and above, are retained more when the pH of the mobile phase rises, reaching 8 and above. [67] Control over the retention of amines in this range is constrained, though, because the majority of conventional silica gel-based reversed phase columns are typically only suitable for use with mobile phases that have a pH of 8 and above.

The retention, selectivity, and resolution of the target analytes can all be impacted by the kind of buffer used, making it a crucial consideration in the creation of the RP-LC technique. There are several things to think about while choosing a buffer for RP-HPLC, such as:

1. The mobile phase's ideal pH is: Selecting a buffer with a pKa that is around the required mobile phase pH is crucial because buffers work best around their pKa value.
2. The buffer's solubility in the organic solvent: The buffer needs to work well with the organic solvent being used in the mobile phase, primarily the typical organic solvents acetonitrile, methanol, and isopropanol that were previously discussed.
3. The buffer's UV cut-off: If UV detection is used, the buffer's UV absorption should be below the wavelength at which the analytes of interest are detected. By doing this, the buffer won't interfere with the analytes' detection.
4. The buffer's compatibility with the detector: The buffer needs to work with the mass spectrometry (MS) apparatus if it is being utilized for detection. Certain buffers, such those that contain phosphate salts, are incompatible with MS detectors because they are not volatile enough and prevent the analytes from being detected by MS by suppressing their ionization. [68]

Several of the most widely used buffers in RP- HPLC consists of:

- Phosphate buffers: Because of their 3 pKa values, phosphate buffers are adaptable and can be employed to achieve a broad range of pH levels. For UV detection, they also have a very low UV background. They are not suitable for MS detection, though.
- Acetate buffers: Often employed in RP-LC, acetate buffers are also adaptable and can be utilized to attain a range of pH levels. It is not very good for UV detection at wavelengths shorter than 220 nm. With MS, the ammonium acetate buffer works well.
- Formate buffers: These buffers have a limited UV detection range below 225 nm and are comparable to acetate buffers in terms of the pH range they can be utilized. Additionally compatible with MS is its ammonium acetate.
- Buffers made of ammonium: These are volatile and frequently employed in LC-MS techniques. Additionally, its low UV detection capabilities are limited. [69,70]

UHPLC vs HPLC

A comparison between Ultra High Performance Liquid Chromatography and High Performance Liquid Chromatography is shown in the table below:

Attribute	**HPLC**	**uHPLC**
Pressure	6000 Psi	100,000 Psi
Particle Size	5µm	1.7µm
Flow Rate	Mililiters per minute	Microliters per minute
Max Resolution	Relatively low	Relatively high

Recent Innovations in UHPLC columns

The earliest commercial columns made for UHPLC were 2.1 x 50 mm and 2.1 x 100 mm in size, and they were packed with 1.7-µm particles. The particles were composed of an ethylene-bridged organic/inorganic hybrid derivatized with C18 groups, and they were totally porous with an average pore size of 130 Å. For RP

separations of analytes with molecular weights under around 5 kDa, the columns were designed. After accounting for system dispersion, these columns have been claimed to have maximum efficiency of 280,000 plates/m. Numerous new surface chemistries have been applied to hybrid and silica sub-2-µm particles throughout the last ten years. [71]

Fig. UHPLC Columns

Apart from the RP mode, UHPLC columns are available for sale for ion-exchange, size-exclusion hydrophilic interaction, and normal-phase chromatography. Analytes up to about 2 MDa, such as industrial and biopolymers, can currently be separated using UHPLC columns. Commercial UHPLC columns come in internal diameters ranging from 0.075 to 4.6 mm. Selectivity and application diversity have increased significantly, but UHPLC column efficiency per unit length—a key indicator of the column's capacity to generate narrow peaks—has not increased as much. With the recent introduction of columns containing sub-2-µm solid-core particles (SCPs), this has started to change. [72]

Recent innovations in UHPLC instrumentation

Utilizing columns filled with particles smaller than 2 µm inevitably raises the backpressure at which UHPLC systems function. Numerous companies now offer UHPLC systems with pressure restrictions of up to 1300 bar. There are two types of pumping technologies: low-pressure mixing and binary high-pressure mixing. [73] While low-pressure mixing pumps offer more flexibility in technique development and support gradients involving more than two solvents/buffers, binary high-pressure mixing pumps often have lower dwell volumes and are better suited for very fast gradients at lower flow rates. The smaller injection-volume requirements of UHPLC columns are accommodated by sample injectors.

To reduce radial temperature gradients inside the UHPLC column, column ovens should refrain from actively circulating the heating fluid (air). The current range of detectors includes circular dichroism, electrochemical (amperometric and coulometric), charged aerosol, fluorescence, refractive index, multi-angle light scattering, evaporative light scattering, absorbance (tunable and photodiode array), and various mass spectrometers. Compared to their HPLC counterparts, these detectors typically have higher data-sampling rates and lower volume flow cells. [74-79]

Detection requirements for UHPLC

There are two main ways that detectors affect a UHPLC system's extra-column dispersion. First, $\sigma v^2 = V2/12$, where V is the flow-cell volume, can be used to estimate the dispersion related to the detector's volume. Total internal reflection-induced light guidance has made it possible to reduce flow-cell capacities in commercial instruments to as little as 250 nL while maintaining enough light throughput to guarantee acceptable noise performance for absorbance detection. To guarantee peak fidelity, the flow cell's volume must be decreased, but the sampling rate must also be increased and the level of electronic filtering must be decreased. The sampling interval was considered by Fountain et al. to be the primary cause of temporal peak broadening.

Detectors that are sold commercially can sample at up to 200 Hz. It should be mentioned that detector noise rises with increasing sampling rate and therefore sampling rates higher than 40 Hz are rarely necessary. [80] Reduced signal averaging at higher sample rates and a rise in the relative contribution of the so-called "read noise" related to measuring the photocurrent at the photo diode(s) are the causes of this noise increase. Generally speaking, the sample rate should be selected to guarantee that 100 points are gathered for merging peaks and 20 points are gathered throughout a single peak. [81]

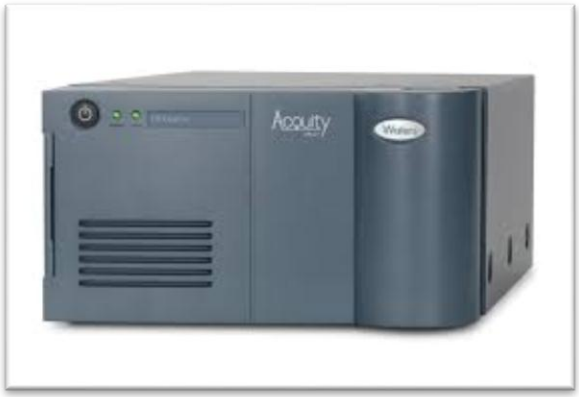

Fig. UHPLC Detector

Compared to standard HPLC flow cells, fluorescence detection for UHPLC necessitates a reduction in flow-cell volume; nevertheless, the smaller space in which the emitted photons are detected results in a loss in detector response. A mercury-doped xenon emission lamp, which raises the excitation energy at the mercury lines, is used in one fluorescence detector for UHPLC to make up for the decreased sensitivity caused by flow-cell volume reduction. [82]

Spaggiari et al. examined the extra-column dispersion of UHPLC/MS systems for a number of UHPLC instruments connected to quadrupole time-of-flight, triple quadrupole, time-of-flight, and quadrupole ion-trap instruments. Band broadening at the mass spectrometer was significantly impacted by the length and i.d. of the tubing used post-column, the presence or absence of an absorbance detector and/or a divert valve, and other factors. All of these dispersive aspects were observed to occur post-column and are not lessened by sample concentrating that takes place with gradient separations.

The extra-column variance obtained from direct injection without a column rises with decreasing sampling rate at flow rates higher than 200 µL/min. Cone-gas flow rates, capillary voltage, and desolvation, however, had no effect on band broadening. The extra-column variations for the four systems' conventional configurations ranged from about 25 to 95 µL2 at 600 µL/min, which was significantly more than the variances for the UHPLC/UV systems displayed in Table 1. However, for all systems under study, the extra-column variations may be reduced to approximately 17-19 µL2 at 600 µL/min by shortening the connecting tubing's length. [83]

Advantages:

- High Efficiency: UHPLC uses smaller particles and higher flow rates, leading to faster and more efficient separations.
- Increased Sensitivity: The shorter analysis times and improved peak shapes in UHPLC lead to increased sensitivity for detection.
- Versatility: RP-UHPLC is widely used in various applications, including biomedical, pharmaceutical, and environmental analysis. [84]

Applications of RP-UHPLC:

1. Lipid Analysis: RP-UHPLC is used for the separation and identification of lipids in biological samples.
2. Peptide and Protein Analysis: RP-UHPLC is used for the analysis of peptides and proteins, including their identification and quantification.
3. Small Molecule Analysis: RP-UHPLC is used for the analysis of small molecules, such as drugs and metabolites.
4. Enantiomeric Separation: RP-UHPLC can be used for the separation of enantiomers, which are mirror-image molecules [85]

II. CONCLUSIONS

The majority of manufacturers and practitioners now use UHPLC instrumentation as their mainstream LC platform. Since its commercial release in 2004, UHPLC technology has been effectively applied to a variety of substances and settings, in conjunction with spectroscopy and MS, as this study demonstrates. The primary benefit of UHPLC is the ability to use columns loaded with sub-2-um particles to do ultra-fast and/or high-resolution separations while using less solvent. The quest for increased column efficiency has become a major theme as UHPLC has developed during the last ten years. Compared to columns with sub-2-µm FPPs, the creation of

columns with sub-2-µm SCPs has allowed for significant efficiency gains (more than 40%). With pressures comparable to those used for columns with 1.7-µm FPPs, this efficiency boost might be achieved for columns with 1.6-µm or 1.7-µm SCPs. On the other hand, columns with 1.3-µm SCPs have pressures that are approximately 100% higher, which limits their use with the UHPLC devices that are currently available. Furthermore, when employing the low ionic strength acidic mobile phases that are preferred for electrospray mass-spectrometric detection, charged surface particle technology provides the opportunity to achieve high efficiencies for both positively-charged analytes and neutral molecules.

It has become necessary to decrease the extra-column dispersion of UHPLC devices in order to fully benefit from these better efficiency columns. Even though this effort has achieved great strides, there is still room for growth in this area.

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