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Ultra-Performance Liquid Chromatography (Uplc)

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ABSTRACT

Today's pharmaceutical industry and analytical labs are always looking for innovative solutions to save costs, shorten medication analysis times, and boost product quality. Ultra-performance liquid chromatography is one innovative method of using liquid chromatography. By employing particles smaller than 2 µm, UPLC enhances three aspects of liquid chromatography, namely speed, sensitivity, and resolution of analysis. The mechanism can tolerate large backpressures. UPLC separations consume up to 100 MPa, however neither the analytical column nor other chromatographic system components are affected. UPLC requires less time and solvent than HPLC. Ultra-performance liquid chromatography may reach better resolutions and sensitivities via particle chemistry, system optimization, and data processing. Using sub-2 mm particles at faster linear velocities and equipment that works at higher pressures than HPLC may dramatically enhance resolution, sensitivity, and analysis speed. This new field of analytical separation science maintains the value and concepts of HPLC while offering a step function enhancement in chromatographic performance. This review focuses on UPLC's fundamental theory, instrumentation, and principles. In this overview, the UPLC chromatography technique is explained along with the most current research in the area.

Keywords: HPLC, High separation efficiency, Resolution, Sensitivity, UPLC.

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INTRODUCTION

Chromatography is a method for separating a mixture's components, or solutes, based on how evenly each solute is dispersed between a stationary phase that is adjacent to the mobile phase, a moving fluid stream. While the stationary phase can either be a solid or a liquid, the mobile phase can either be a liquid or a gas. The factors that influence this separation are the molecular characteristics associated with adsorption, affinity, and partition, as well as variations among their molecular weights [1, 2]. As a result, certain components of the mixture travel quickly through the mobile phase and exit the chromatographic system, while others pass slowly through the stationary phase and spend more time there. Based on this methodology, the chromatography technique is composed of three elements.

- Stationary phase: A solid phase or a layer of liquid absorbed on the surface of solid support is always present in this phase.
- Separated molecules.
- Mobile phase: This phase typically contains a liquid or a gas component [3].

An essential liquid chromatography (LC) method for separating various components in mixtures is high-performance liquid chromatography. It has been used for many years to quantify and identify compounds throughout the drug development process all over the globe. Column technology (column dimension and column particle size) and instrumentation have significantly advanced in order to further accomplish the substantial rise in sensitivity, resolution, and speed in LC [4]. Waters developed and patented Ultra Performance Liquid Chromatography (UPLC) in 2004 to accomplish the aforementioned goals. UPLC is based on tiny, porous particles (sub 2micron particles). The underlying theory of this development, which links the relationship between plate height and linear velocity, is the Van Deemter equation. Because the top pressure limit of traditional HPLCs is approximately 6000 psi, the tiny particles need a high pressure to function with UPLC. It was shown that the efficacy increases noticeably when the particle size is reduced below 2.5 µm and that this effect does not decline while the rate of flow or linear speed is increased [5]. The utilization of speed, small-radius particles, and the highest number of peaks (peak capacity) that may be resolved combines efficiency and resolution. In comparison to HPLC, this approach uses less mobile phase volume and has a runtime of around 1.5 minutes. The retention factor of the separation may be increased by the smaller particles alone by increasing the pressure to 1000 bars or greater. For UPLC, a smaller injection volume is needed, increasing both efficiency and resolution. The lower mobile phase viscosity that results from, the higher column temperature produces a flow rate and high diffusion coefficient without suffering a substantial efficiency loss and an increase in column back pressure. Recently developed technologies in system optimization, particle chemistry performance, data processing, detector control and design may be reflected in UPLC, a variant of HPLC [6]. These developments result in a highly noticeable improvement in resolution, sensitivity, and efficiency, as well as quicker

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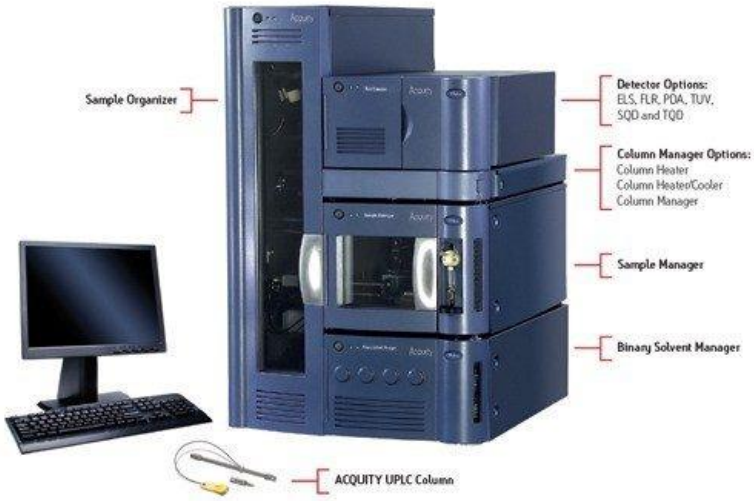
results and reduced solvent usage, which reduces costs and makes the technology more environmentally friendly [7].



Principle

The van Deemter relationship, which describes the link between plate height and flow rate, is the foundation of UPLC (Eqn 1). According to the van Deemter equation, smaller particles have a considerably wider flow range than bigger particles for successful outcomes [8, 9].

$$H = A + \frac{B}{v} + Cv \quad (1)$$

Where H denotes a height equal to the theoretical plate, and A, C, and B are constants, v represents the carrier gas flow rate. To improve column efficiency, HETP should be kept to a minimum. The letter A, which stands for eddy mixing, is independent of velocity. If the columns are full of tiny, uniform-sized particles, it is smaller. The tendency of the particles' natural diffusion is represented by the symbol B. This value is divided by v since this impact is less at high flow rates. The word C denotes the kinetic barrier to equilibrium that exists in all throughout the separation process. Kinetic opposition is the time delay experienced during the transition between the mobile phase and the stationary phase and back again. The flow velocity of the mobile phase increases the propensity of molecules on the carrier material to follow after particles. This indicates an inverse relationship between this term and linear velocity. As a result, throughput can be increased without affecting chromatography performance, and the separation process may be speed up. The development of ultra-performance liquid chromatography has

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forced the improvement of current instrumentation for liquid chromatography, which benefits from extraction efficiency and constant pressures. Efficiency is inversely related to the particle radius and proportional to the length of the column [9]. The column length could be decreased by the same percentage as the particle radius because the resolution is unaffected. Utilizing UPLC has enhanced the efficiency of separation spectra and allowed for the identification of drug metabolites [10, 11].

Table 1: Some parameters on Ultra Performance Liquid Chromatography

S.no.	UPLC	Parameters	Inference
1.	Mobile phase flow rate	Less	Using less mobile phase
2.	Column	ACQUITY UPLC BEH C8 and C18	Possessing great mechanical stability and efficiency while being able to tolerate extreme pressure
3.	Column Temperature	65◦C	Improved selectivity decreased solvent viscosity, and accelerated mass transfer rate.
4.	Maximum back pressure	103.5 MPa	More rapid separation
5.	Column dimension	150 * 2.1mm	Greater resolution
6.	Injection volume	2µl	Can handle samples with even minute traces.
7.	Particle size	Less than 2µm	Shorter analysis time

Instrumentation

Small, pressure-tolerant particles

Smaller particles provide both resolution and speed since they are more effective and can operate at higher linear velocities without losing effectiveness. Since UPLC depends on the same retentivity and selectivity as HPLC, efficiency is the key separation characteristic. Resolution is directly dependent on the square root of N in the base resolution equation (Eqn 1).

$$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k}{k+1}\right) \quad (1)$$

However, since N and particle size (dp) are inversely related, when the size of a particle is reduced by a factor of 3, for example, from 5 m to 1.7 m on UPLC scale, N increases by 3 and resolution by the square root of 3, or 1.7. N is inversely proportional to the square of the peak width as well (Eqn 2):

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$$N \alpha 1/w^2 \quad (2)$$

Additionally, the relationship between peak height and peak breadth is inverse (Eqn 3):

$$H \alpha 1/w^2 \quad (3)$$

Since narrower peaks are higher peaks, an improvement in sensitivity is therefore acquired like the particle size reduces to raise N and consequently resolution. The peak volume per unit of time in gradient separations is greater for narrower peaks, which is desirable for several uses, including peptide mapping. When moving in the direction of smaller particles, yet another equation comes into play (Eqn 4):

$$F_{xxx} \alpha 1/dpc \quad (4)$$

The van Deemter plan also reveals this connection. As particle size decreases, the optimal flow F increases to achieve maximum N. Since back pressure is related to flow rate, smaller particle sizes necessarily require much higher working pressures, a system that can reliably supply such pressures while simultaneously preserving the effectiveness of the microparticle separation with meticulously controlled volumes, and a system designed appropriately to benefit from efficiency advantages. Efficiency is negatively correlated with particle size and inversely proportionate to column length (Eqn 5).

$$N \alpha L/DP \quad (5)$$

It is possible to reduce the column by a similar amount as the particle size without affecting the resolution. When employing a rate of flow that is 3 times greater due to smaller particles and minimizing the column by one-third, the separation is finished in one-ninth of the time while maintaining resolution. Commercially available nonporous 1.5- particles, highly efficient, have a limited absorption rate and retention because of their limited surface areas [12, 13].

Sample injection

A small fraction of the precisely calculated solution containing the sample in the mobile phase is delivered by the injector. The injection must be administered with consistency and accuracy. Conventional injection valves may be manually operated or electronically controlled, and the injection technique must be essentially pulse-free to protect the column from severe pressure fluctuations. The device's swept volume should be as low as practical to reduce the chance of band spreading. To effectively capitalize on UPLC's speed, a short injecting cycle time is necessary. Improved sensitivity requires low volume injections with reduced carryover [14]. The sample volume in UPLC is between 2 and 5 L, and direct injection procedures are employed for biological material [15, 16].

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UPLC Column

Small particles smaller than 2 µm in size make up the UPLC columns. As it was already explained, the UPLC technology depends on tiny particle size. Because the bonded stationary phase is necessary for both selection and retention, the particles are glued together in a matrix. There are four columns made by ACQUITY that are bonded stationary phase columns that may be utilized with the UPLC method.

- BEH C8 and C18 columns, which are straight alkyl chains that may be used over a wide pH range, are the most often used UPLC columns. The trifunctional ligands' poor pH stability is paired with the 1.7 m BEH particles' strong pH stability to provide the largest practical pH working range.
- Columns BEH Shield R18 As a complement to C18 and C8 columns, they provide UPLC selectivity.
- Columns of BEH phenyl Between the silyl and phenyl rings functionality, they have trifunctional C6 alkyl ethyl.
- A remarkable column lifespan is achieved using BEH Amide columns, which combine a trifunctionally linked amide phase with tiny BEH particles. They make it easier to employ a variety of phase pHs, from pH 2 to 11 [17].

Table 2: Based on technology, column differentiation

S.no.	Columns	Description	Reference
1.	Beh shield rp18 columns	Compared to straight chain alkyl columns, Waters shield ligands routinely show improved retention of phenolic substances. When paired with the ultra-efficiency and broad pH range of the 1.7µm BEH particle, the alternate selectivity and great peak shape from the inserted polar group ligand provide a potent and essential tool for UPLC method development.	**[18]**
2.	BEH Phenyl columns	Between the silyl functionality and the phenyl ring, a trifunctional C6 alkyl tether is used. This ligand offers very minimal column bleed, extended column lifetimes, and good peak shape when used in conjunction with the same exclusive end-capping procedures as the ACQUITY UPLCTM BEH C8 and C18 columns.	**[19]**
3.	BEH HILIC Columns	These customized columns have been tested and improved for UPLC HILIC separations. ACQUITY UPLC BEH HILIC columns address HILIC stationary phases' chemical instability. Unbonded silica stationary phases in HILIC	**[20]**

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		dissolve at neutral pH. The robust BEH particle's broad pH range overcomes this pH constraint, resulting in long column lifetimes. ACQUITY UPLCTM BEH HILIC columns provide advantages over RPC, including retention and separation of highly polar basic compounds, alternate selectivity compared to RPC, enhanced ESI-MS sensitivity, simplified sample preparation, high efficiency, and long column lifetimes.	
4.	BEH C8 columns	Unsurpassed efficiency, asymmetry, and chemical stability. ACQUITY UPLC BEH C, 1.7 µm Columns are a ubiquitous C column option for many analytes. Mobile-phase pH [1-12] and temperature [80 °C] compatibility. This trifunctionally-bonded alkyl column may improve retention, selectivity, and sensitivity of ionizable chemicals (with mobile-phase pH) while giving superior low- and high-pH stability for all analyte types.	**[21]**

Column Manager and Heater Cooler

The automatic column switching ACQUITY Ultra Performance Liquid Chromatography Column Manager is developed for large volume UPLC sample preparation, as well as its Column Cooler/Heater facilitates laboratories to use temperature as a method parameter. Users may take full advantage of the performance, mechanical strength and variety of stationary phases that ACQUITY UPLC BEH Columns have to offer by using the ACQUITY UPLC Column Manager. The Column Manager provides automatic switching for a maximum of four columns with internal diameters of 150 mm, and 2.1 mm temperature control from 10°C to 90°C, and a bypass channel for flow injections. the ACQUITY UPLC System is made up of a sample manager with a column detector, binary solvent manager, heater and an optional sample organizer. Two different serial flow pumps are used by binary solvent management to supply parallel binary gradients. There are integrated valves that enable the selection of up to four different solvents. The pressure must not exceed 15,000 psi (1000 bar) in order to fully use the sub-2 mm particles. Additionally, the sample manager utilizes a variety of technological advancements. Low dispersion is maintained throughout the injection process by using pressure-aided sample introduction, and self-monitoring and diagnostics are made possible by a set of pressure transducers. A needle calibration sensor increases accuracy, while needle-in-needle sampling increases longevity. To further reduce carryover, the injection process duration is 25 seconds without any need for wash and 60 seconds with a dual wash. In a thermostatically regulated environment, a number of microtiter plate configurations (mid-height, deep well, or vials) may also be supported. The sample manager can inject as many as 22 microtiter plates by

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using the optional sample organizer. The column heater is similarly managed by the sample manager. As much as 65° can be reached in columns [22].

Detector

The detector used for UPLC should have a high sampling rate, tiny peaks that can be produced and low peak dispersion. This will reduce the amount of wasted separated solute on the colµmn. Due to the technology used for detection, the UPLC methodology offers separation sensitivity that is two to three times more than the HPLC analytical approach that was previously used. The Tunable Vis-UV and Acquity photodiode array detectors used in Ultra Performance Liquid Chromatography have internally reflecting surfaces made of Teflon AF, which improves the efficiency of transmittance by removing inner absorptions. These have acquisition rates of 20 (PDA) and 40 (TUV) points, route lengths of 10 nun, and total internal volµmes of 500 nL. Additionally, UPLC has been utilized with mass spectrometric detection [23, 24].

Types of Detectors

Ultra-violet/visible (UV): This detector is utilized for organic substances that absorb light between 190 and 800 nm. For detection, this detector may be set to a certain wavelength in the UV or visible spectrµm. For regular and sophisticated analysis in life science, pharmaceutical, environmental, agricultural, and petrochemical applications, it offers performance advantages. The Accuracy of an optical fiber-like light-guided flow cell makes up the tunable UV Visible detector cell. With a modest 500 mL volµme, the flow cell's intrinsic reflectance method efficiently transmits light along its 10 mm channel length. In order to ensure limited distribution and to start taking usage of drip detectors that communicate with the software to warn the user of possible issues, the system's tubing and connections are routed effectively. The sensitivity and specificity provided by mass spectrometry are best accessed by UPLC. The performance qualities of UPLC combined with the low dispersion performance of Waters MS Technologies with high-speed detection may significantly enhance detection capability [25].

Photodiode Array (PDA) detector: The detector provides simultaneous, enhanced optical detection in the 190–800 nm wavelength range. With its spectrµm analysis capabilities, it offers unheard-of trace impurity identification and quantification. Detection of co-elution and accurate chemical identification while simultaneously operating in 2D and 3D. This detector has a lot of use in pharmaceutical research and development [26].

Fluorescence (FLR) Detector: This detector is used for fluorescence-based applications because of its selectivity and sensitivity. The advantages of UPLC technology are expanded to include the study of vitamins, drugs of abuse, and polynuclear aromatic hydrocarbons (PAHs), as

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well as any substance having chemilµminescent features like fluorescence or phosphorescence [27].

Evaporative light scattering detector: A general-purpose, universal detector called an evaporative light scattering detector may even recognize materials that don't absorb UV light, like lipids, artificial polymers, synthetic polymers, and carbohydrates. ELSD is a specialized approach that uses UV radiation to detect non-UV-absorbing chemicals separated by liquid chromatography [28].

Refractive index (RI) detector: A universal detector called RI is employed when a substance has little or no UV absorbance. These consist of raw materials such as fatty acids, alcohols, sugars, excipients, and pharmaceutical medicinal preparations. UPLC also uses low-molecular-weight polymers for characterization. This detector's major flaw is its lack of sensitivity [29].

Softwares

Through a graphical system console interface, ACQUITY Ultra Performance Liquid Chromatography Systems may be easily diagnosed, controlled, and monitored with the aid of the EmpowerTM and MassLynxTM software. Both Mass Lynx and Empower provide information management capabilities and the dynamic data processing required to transform the result of the ACQUITY Ultra Performance Liquid Chromatography System into meaningful information [30].

Equipment's

Waters is constantly increasing the ACQUITY Ultra Performance Liquid Chromatography System's capabilities: All ACQUITY UPLC colµmns include eCordTM technology, which keeps track of colµmn history. Sample Organizer that boosts capacity of the system by more than ten times. The FlexCart platform enhances usability, accessibility, and practicality [31].

Connection INSIGHTTM Service

Connections INSIGHTTM provides diagnostic data for the ACQUITY UPLC Systems using Intelligent Device Management technology. Waters may create a virtual technical support presence in the lab with the use of Connections INSIGHT and provide consumers the highest degree of proactive care, promptness, and assistance.

Method optimization guidelines and observations

The following suggestions were given when the UPLC technique was being optimized in order to hasten method transfers in the future: Utilizing the high-resolution potential of UPLC columns, increase the strength of the elution solvent to shorten run durations. To enhance longer column lifespan, increase the mobile phase flow rate subsequent to solvent strength. It takes time for mobile phase programmed adjustments to reach the column. The original experiment might

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have been somewhat shortened due to the tiny UPLC dwell volume (110 L, 15% of the HPLC's). The next sample is loaded into the UPLC once the column has been re-equilibrated, thus boosting throughput. To create nice peak forms, reduce injection volumes in a manner suitable for the column diameter. Peak splitting happens when a powerful sample solvent bolus is too big to overcome the packing at the column head. Although this test technique is considered acceptable for injection quantities of up to 5 L, more usual starting points are volumes of 1-3 L. Low carryover from the UPLC injector and increased peak height from using high-resolution columns may make up for smaller injection volumes. Even at quantities up to 80% of the entire volume of the loop, partial loop-fill accuracy was excellent. It is a standard laboratory procedure to keep the injections of sample volume to no more than 50 percent of the overall loop volume. It is not always necessary to employ the full loop-fill mode when using the UPLC injection system, which makes better use of the sample loop and greater injection accuracy possible by sandwiching the sample in an air gap. Full loop fill, when taking overfill functions into account, practically demands much more sample movement. This probably results in more subsequent needle cleaning, may slow down sample processing, and shorten the lifespan of the washing equipment. The extremely small-bore tubing of the UPLC injector creates a sizable velocity of laminar flow difference in the loading sample among its center and wall interface. It was found that the sample loop needs to be overfilled by at least 4 loop volumes to fully discharge the wash fluid from the 5 Liter injector loop. For each sample loop size for this instrument, the manufacturer has estimated the ideal overfill volume with common sample solvents and set it as the default. For atypical sample compositions, operators may specify different overfill quantities. To get a decent peak shape, use the right weak sample wash volume and composition [32].

Applications

With the advancement of parameters like retention duration and mobile phase consumption, this approach has been effectively used to analyse different medications for the pharmaceutical industry [33-35]. And with aim of cutting down on analysis time and maintaining high efficiency, In order to determine beta-blockers and diuretics in pharmaceutical formulations simultaneously, Narasimham and Barhate designed and validated a Ultra Performance Liquid Chromatography method [36]. In under 2.6 minutes, a Waters Acquity BEH C18, 50 multiplied by 1.7 m, 2.1 mm UPLC column was able to separate all of the medicines chromatographically.

Pharmaceutical analysis

Drug substance and drug product analysis methods need to be thoroughly designed and verified, and these procedures take a long time using traditional HPLC. With UPLC, analytical technique development and validation may happen more quickly. Impurity profiling, a key activity in drug development, involves identifying and quantifying impurities in drug substances and drug products. Because UPLC offers strong resolving power, repeatability, efficiency, and quick turnaround times, it is a viable choice for this task. As UPLC offers quicker sample analysis, this

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benefit may be used to monitor in-progress and real-time reaction samples when quick process control is needed to reduce the cost of failure. Dissolution testing, one of the crucial tests in the development of pharmaceutical products, is another area where UPLC is put to use [37].

Environmental analysis

Innovative methods are needed to locate and identify chemical contamination in environmental samples. Less time, money, and more information about the sample composition are provided during the analysis of these samples using UPLC. Analysis of organic components in drinking water, soil, air, hazardous wastes, wastewater, pesticide residue, and perfluorinated compounds are a few uses of UPLC [38].

Forensic and Toxicological

When identifying drugs of abuse from blood, urine, and oral sample, UPLC finds excellent use. UPLC is capable of identifying and analysing a variety of cannabinoids, opioids, and barbiturates. The unique advantage of drug screening with outstanding sensitivity and accuracy at the trace level is made possible by the combination of UPLC with different equipment [39].

Advantages

For many separations, the analysis time is split in half compared to traditional chromatographic methods [40].

1. As UPLC run times are shortened, very little solvent is used.
2. UPLC has better resolution and sensitivity than other traditional methods.
3. Sample output is improved, allowing manufacturers to make more goods in accordance with necessary specifications and reducing batch failure.
4. The overall cost of operating is decreased.
5. Multi-residue approaches have a wider use.
6. UPLC characteristics that are different from HPLC and will work in favour of chromatographic procedures.

Disadvantages

1. This kind of colµmn's lifespan is shortened due to greater pressure, which also requires additional maintenance. Until yet, without the negative effects of high pressure, performances comparable to or even greater have been achieved employing stationary phases with a size of around 2 m.
2. Additionally, wavelengths shorter than 2 metres are frequently non-generable and only have a limited function.
3. The life of these colµmns is shortened as a result of higher pressure, which also requires additional maintenance. By utilising stationary phases with a size of around 2 m so far,

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performance comparable to or even greater has been achieved without the negative consequences of high pressure.

4. In UPLC analysis, the life of the colµmns is the primary drawback. The high pressure created during the analysis due to the particle size. Increased pressure shortens the colµmns' life span. The life of these sorts of colµmns is shortened as a result of greater pressure, which also requires additional maintenance. Perform superior analyses without the negative effects of high pressure by using stationary phase with particle sizes of 2 µm [42].

CONCLUSION

One of the most useful techniques in analytical chemistry is ultra-performance liquid chromatography (UPLC), which improves chromatographic analysis in terms of speed, resolution, and sensitivity while reducing the amount of time and solvent needed. UPLC generates peaks with lower background noise and a higher signal-to-noise ratio. The pharmaceutical business can benefit much from the UPLC technique, and it is also the preferred method. This instrµment will show to be indispensable in boosting both the precision of pharmaceutical analysis and the efficiency of scientists conducting such analysis. It's a crucial technique for creating and evaluating methods in the lab, as it saves money and maximizes efficiency. With UPLC, the separation can be completed more quickly and more efficiently, leading to the rapid development of techniques that open up a new window of opportunity for business profitability and facilitate the product's quicker introduction to the market.

Conflict of Interest; The authors declare that the review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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