WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

NANO HPLC: A Review of Current Methodologies

Ceema Mathew[1], Mangam Mamatha [1]*, Katikela Swetha[1], Kolan Shreeya[1], Shashikala Metri[2]

[1]Department of Pharmaceutical Analysis, Gokaraju Rangaraju College of Pharmacy, Osmania University, Hyderabad, Telangana- 500090

[2]Department of Pharmacognosy, Gokaraju Rangaraju College of Pharmacy, Osmania University, Hyderabad, Telangana- 500090

Corresponding Author: Mangam Mamatha

ABSTRACT:

Nano liquid chromatography, a form of microfluidic technology, is employed to segregate diverse chiral, metabolic, pharmacological, therapeutic, biomedical, protein, peptide, and enantiomeric chemicals. It minimizes solvent consumption by utilizing nanoliters of solvents to identify substances at the nanogram scale. Miniature separation techniques have emerged as environmentally acceptable alternatives to existing separation operations. Miniaturized techniques, including nano-liquid chromatography, nano-capillary electrophoresis, and microchip devices, minimize waste generation and reagent use. Additionally, while being a relatively new technology, nano liquid chromatography ensures the opening of new corporate and laboratory applications. The expensive equipment, however, can be a drawback. This article reviews the tools used for nano-flow, the types of columns employed, and the techniques for multidimensionality of separations. In order to fulfil the high-throughput requirements of modern bioanalysis, these elements might be essential to the technique's future growth. A few theoretical aspects are explained in order to better understand the potential of nano-HPLC as well as its primary limitations. A summary of the latest applications of nano-HPLC, such as proteomics and metabolomics, is discussed. Recent pharmaceutical and biological applications of nano-HPLC further illustrate the high-quality, sufficient performance that is attained for complicated matrices, especially for proteome analysis.

KEY WORDS: HPLC, Chromatography, microfluidic technology, nano-capillary electrophoresis, proteome analysis.

INTRODUCTION

High-Performance Liquid Chromatography is referred to as HPLC. HPLC is an analytical method employed for the separation, identification, and quantification of constituents in a mixture. It is employed in analytical and biochemistry to identify, quantify, and purify the constituents of a mixture [1].

Chromatography was invented in 1903 by Russian botanist Mikhail Semenovich Tswett while investigating plant pigments. The term "chromatographic process" refers to a separation method utilizing mass transfer between stationary and mobile phases. Despite its development over a century ago, chromatography continues to be the foundation of separation science and is utilized in research facilities and pharmaceutical firms around. The efficacy of this strategy can be attributed to its uniqueness and modernity. HPLC is an advanced kind of liquid chromatography that use identical principles to separate chemical mixtures. The industry utilizes HPLC to separate and analyze intricate mixtures of chemicals, rendering it an indispensable tool in chemical and biological research [4]. The approach employs a straightforward mechanical separation process that isolates compounds inside a narrow capillary column using mobile and stationary phases. Complex materials can be thoroughly characterized via HPLC, including their size, shape, molecular weight, structure, hydrophobicity, and hydrophilicity. This is among the most adaptable methods for biophysical analysis.

Fig:1. High-performance liquid chromatography

High-Performance Liquid Chromatography (HPLC) comprises a detector, a stationary phase, a mobile phase, and a sample source. The HPLC components are chosen by the analyzer to fulfill the specific requirements of the sample and the analysis objectives [5]. The mobile phase, which can be a liquid, gas, or a combination of both, may display non-polar, moderately polar, or extremely polar properties when analyzing a given sample component. A solid phase, such as cellulose, silica, alumina, or a mixture thereof, often functions as the stationary phase. To prevent the sample components from eluting through the column without engaging with the stationary phase, the stationary phase must have the ability to adsorb or bind these components [3]. The most often employed HPLC detectors are electrochemical, fluorescence, and UV-visible. High-Performance Liquid Chromatography (HPLC) is employed in forensic science, agrochemicals, pharmaceuticals, biotechnology, and environmental research. It is employed to identify biological molecules like DNA, antigens, and antibodies, as well as pollutants in food and certain active pharmaceutical chemicals. HPLC is utilized in the pharmaceutical sector to quantify molecules in biological samples, formulate innovative medications or therapeutic formulations, and purify substances at a process scale. High-Performance Liquid Chromatography (HPLC) is utilized in the biotechnology sector for protein identification and purification, biopharmaceutical development, and the characterization and optimization of biocatalysts. High-Performance Liquid Chromatography (HPLC) is utilized in forensic science to analyze biological specimens and detect compounds present at crime scenes.

The fundamental premise of HPLC is that the differing affinities of molecules for the stationary phase determine their flow rates through it. The varying rates of movement among various molecules result in their separation from one another. The comparative affinities of the analyte molecules for the stationary and mobile phases dictate the velocity of component migration [4]. In contrast to molecules with weaker adsorption, compounds with a high affinity for the stationary phase will demonstrate reduced mobility and enhanced separation. Partition equilibria and retention times are the essential concepts governing the function of HPLC. Compounds will partition between two immiscible phases when in equilibrium. Compounds will cling to the stationary phase of the column, which consists of the adsorbent material, and the contrasting mobile phase in HPLC. The retention time of a specific material indicates the period needed for the sample to pass through the column and get to the detector. A chemical mixture is introduced onto a chromatographic column for the HPLC procedure. The packing material in this column, comprising silica particles, is covered with an absorbent or adsorbent substance.

NANO-HPLC:

The word "NANO" comes from the Greek word "nano," which means "extremely small." sizes range from 10 to 1000 nm. Karlsson and Novotny originally presented nano-HPLC in 1988 [3]. It alludes to a particular kind of High-Performance Liquid Chromatography (HPLC) system that uses extremely low flow rates, usually in the range of nanoliters per minute, and very small cells, or nano-scale columns. This enables the accurate and extremely sensitive separation of analytes in small sample volumes, which is frequently utilized in high-sensitivity applications like proteomics, metabolomics, among other areas of biochemistry and analytical chemistry.

Fig.2 Nano HPLC

Capillary columns were first utilized in gas chromatography in the 1950s by Golay, while Horváth and associates performed liquid chromatography (LC) separations using columns with reduced inner diameters (id) [1]. Karlsson and Novotny first utilized the now-established nano-LC technology in 1988 to evaluate packed columns with remarkably small inner diameters. Recently, biological applications in proteomics research have predominantly driven the advancement of tiny HPLC systems. Conventional high-performance liquid chromatography (HPLC) analysis employs columns with an inner diameter between 3.5 and 4.6 mm. The standard flow rate for these is measured in mL/min for analytical columns. Nanocolumns employed in nano-HPLC are defined by diminished diameters (20–100 µm id) and flow rates quantified in nanoliters per minute [1].

Karlsson and Novotny pioneered Nano Liquid Chromatography in 1988 after implementing minor modifications to HPLC. Nano Liquid Chromatography (NLC) is a chromatographic technique that utilizes samples with nanogram concentrations, a mobile phase flow rate of nanoliters per minute, and detection capabilities at nanogram or picogram per milliliter levels. This definition is comprehensive, and chip-based chromatography can satisfy all of these characteristics. Thus, genuine and comprehensive nano-chromatography, known as lab-on-chip chromatography, is frequently achievable solely on chip [2]. Literature statistics from 1978 to the 1990s reveal that researchers embraced miniaturization in liquid chromatography approximately thirty years ago. Subsequently, other research teams have investigated various facets of miniaturization, resulting in significant findings that have improved these procedures and advanced the creation and dissemination of specialized equipment [2]. A significant progression in science and technology, particularly

in analytical chemistry, is miniaturization. In addition to offering certain benefits over non-miniaturized techniques, it also addresses technological problems that will be gradually overcome. Nano-HPLC serves as an alternative to traditional HPLC, offering enhanced capabilities for chemical analysis. A miniaturized technique can be utilized to analyze almost any specimen usually assessed by conventional liquid chromatography (LC). "Nano-bore HPLC" and "Nano-scale HPLC" are synonymous terms for Nano High Performance Liquid Chromatography (Nano HPLC). The chromatographic separation method known as nano-LC is often recommended for achieving nL/min flow rates and offering enhanced sensitivity relative to traditional HPLC when performed on capillary columns with inner diameters between 10 and 100 μm [4]. The approach, employing bigger columns (100–500 μm), is termed Capillary Liquid Chromatography (CLC), and it is claimed that this phenomenon arises from less chromatographic dilution and improved efficiency. The prohibitive cost of the analytical equipment used in nano HPLC hinders its wider use. Furthermore, to avert experimental problems, particularly with instrument configuration, a thorough technical understanding of nano HPLC features is necessary. Furthermore, the CLC/nano-LC technology, in conjunction with mass spectrometry, has demonstrated effectiveness in enhancing the procedure's sensitivity. The integration of Nano-LC and MS was readily accomplished owing to the exceptionally low flow rates characteristic of nano-LC systems employing short inner diameter columns [3]. Gas chromatography (GC), high-performance liquid chromatography (HPLC), and ultra-performance liquid chromatography (UPLC), when integrated with mass spectrometry, are regarded as leading and potent instruments for the analysis of complex matrices currently utilized. HPLC-MS is frequently employed in pharmaceutical and biological applications.

PRINCIPLE:

The fundamental principle of this advancement is governed by the van Deemter equation [2].
which is an empirical formula that describes the relationship between linear velocity (flow
rate) and plate height (HETP or 1/column efficiency) [2]

Whereas,

A= Eddy's diffusion
B=longitudinal diffusion
C=concentration
μ = linear velocity

$$H = A + \frac{B}{u} + Cu$$

The nanoflow The great concentration efficiency provided by HPLC chromatography allows for high analytical sensitivity when separations are carried out with flow rates in the low nanoliter per minute range. [2]

THEORY:

1. Improving sensitivity reducing the chromatographic dilution.

The concentration of the injected material rose on the HPLC system as a result of less chromatographic dilution caused by a decrease in column inner diameter [1]. One benefit of lowering the column i.d. is the utilization of smaller MP volumes. The MP transports analytes toward the detector in chromatography after injecting the sample into the column as a tiny plug, resulting in a band enlargement that is dependent on a number of experimental conditions [2]. This dilution event, called chromatographic dilution (D), is expressed by,

$$\mathbf{D = Co\ Cmax = \epsilon\ \pi\ r\ 2\ (1+k)\ \sqrt{2\pi LH\ Vinj}}$$

The lower internal diameter in nano-LC promotes a high reduction in D value [3]. Thus, downscaling of chromatographic method means less chromatographic dilution, increasing the mass detectability of the separation [2]. Increase in sensitivity (f) resulting from the use of a LC column with a smaller inner diameter can be approximated by the subsequent relation: Correction factor (f) = (Diameter of std column/ Diameter of narrow column) ² the flow rate (F) in a column is given by

$$\mathbf{F = u\varepsilon\pi dc\ 2\ /4}$$

where u is the linear velocity of the mobile phase. The reduction of dc leads to a large reduction in the flow rate of the mobile phase, decreasing solvent consumption and waste production in nano-LC separations [4]. Theoretically, the miniaturization of LC systems is very advantageous for liquid phase separations. However, some practical separation aspects must be considered, because they contribute to losses in separation efficiency [4].

2. Efficiency and extra column band broadening

The efficiency of a capillary column can be described considering the van Deemter equation where the height equivalent to a theoretical plate (H) vs the linear velocity is plotted. H= A + B/u + Cu Where A, B, and C are parameters related to "eddy diffusion", molecular diffusion in the longitudinal direction, and mass transfer between the Mobile Phase and the Stationary Phase, respectively [2]. When making resemblance studies regarding packed columns in different MP conditions and particle diameter, the decreased equation proposed by Kennedy and Knox is often used.

$$\mathbf{h = A\ v\ 0.33 + B/v + Cv\ h = H/dp\ v = u\ \ dp\ /\ Dm}$$

where h is the reduced height equivalent to a theoretical plate, v the reduced linear velocity, dp the particle diameter, and Dm the diffusion coefficient of the sample in the MP [3]. When a traditional analytical LC system is reduced in size, columns with a lower i.d. are used. It is essential to assess every

factor affecting the additional column band broadening in order to attain the intended high efficiency. In order to prevent resolution loss as a result of decreasing efficiency, this effect must be reduced [2]. While this method reduces the amount of unnecessary column band broadening, it can also lead to sensitivity issues because of the shorter path length and low injected sample volumes. Increasing the injected volume and concentrating the analytes in a precolumn or on a column, however, can overcome this challenge. Additionally, or as an alternative, using more sensitive detectors can increase sensitivity.

INSTRUMENTATION:

1. PUMP:

A pump system is necessary for nano-LC in order to provide stable separation and repeatable nano flow rates, as well as to enable gradient elution at nanoscale levels. 500 nL/min or less is the minimum required flow rate [5]. Nano-LC pumps must allow gradient elution at nanoscale levels and exhibit repeatable nano flow rates and stability throughout the separation process. Split and splitless pumps are the two primary pieces of equipment that can be utilized in nano-LC.

A. Split Pumps:

Split systems use a flow restrictor between the pump and the miniature column to separate excessive flows (mL/min) from traditional HPLC pumps [5]. The repeatability of the separation may be lowered by split systems that result in fluctuating split ratios and low nano flow dependability. The two types of split systems are active split systems and passive split systems. The splitter in a passive split system distributes the pump's high flow between the restrictor and column. Although passive split systems are straightforward and reasonably priced, they sacrifice precision and flow stability [6]. Even while active split systems are more reproducible and have better flow stability than passive split systems, most of the mobile phase is still squandered. Some commercial instrumentation has also used the split system. For example, the Ultimate 3000 series pumps (LC Packings) used dedicated cartridges to achieve the split, with rates ranging from 20 nL/min to 50µL/min.

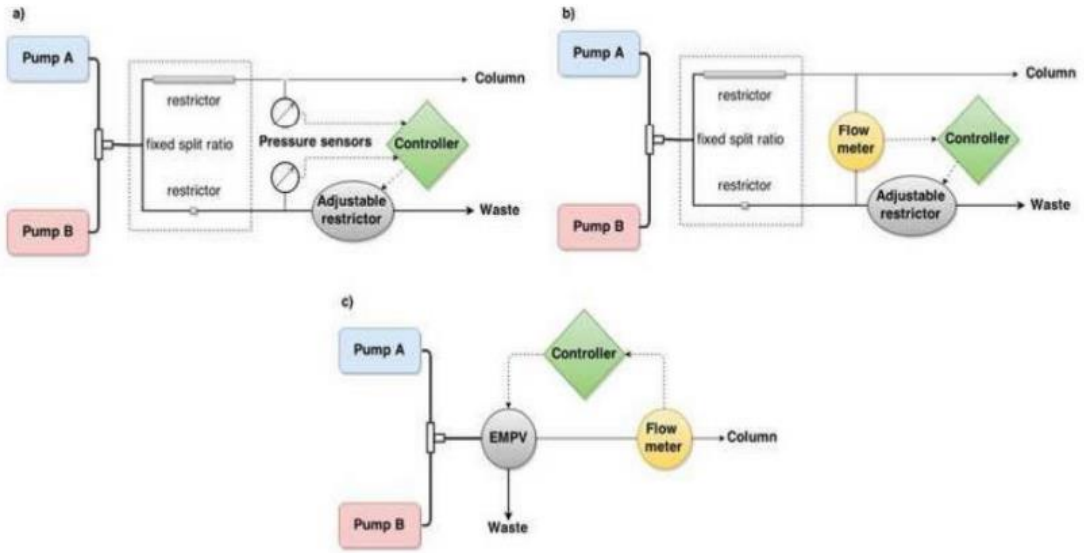

Fig.3 Active flow splitting systems; (a) and (b) represent arrangements with fixed splitter. An adjustable restrictor increases/decreases the resistance of the split line according to the pressure imbalance between the lines detected by pressure transducers (a) or by the flow sensor which connects the lines (b). (c) shows a configuration with an adjustable splitter where the electronic controller systematically drives the EMPV according to the flow detected by the calibrated flow meter.

B. Splitless Pumps:

Splitless systems are widely used in nano-LC [7]. These systems prevent solvent losses and have more reproducible nano flow rates. The split less systems can be divided into two groups:

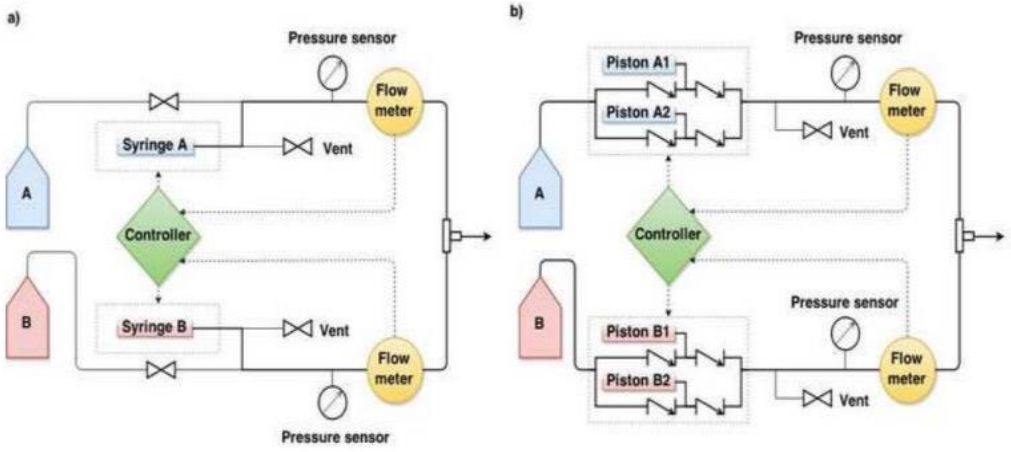

Fig 4. Splitless nanoflow gradient pump arrangement; (a) solvent refill system; (b) continuous flow system.

"Solvent refill" systems and the "continuous flow" systems. Syringe pumps using a single reservoir with a limited volume are better than split systems, but continuous flow pumps, similar to conventional reciprocating pumps with two pistons per channel, are currently the most widely used pump model.[8] Continuous flow pumps may be utilized in both isocratic and gradient elution at nano flows and adjustment of the desired nano flow rates are easily carried out.

2. COLUMNS:

75 µm is the generally recognized standard internal diameter for nano HPLC columns [7]. A nice balance of robustness, loadability, and sensitivity is offered by this column structure.

Two types of columns are used in nano HPLC:

A. Packed columns

B. Monolithic columns

C. Capillary columns

A. Packed columns:

Polyimide-coated fused silica capillaries are utilized to create the packed columns used in nano HPLC columns. The stationary phase is maintained by using frits that are resilient to pressure. The separation efficiency will be hampered by the excessive volume provided by the frit frequently seen in typical columns, which is a stainless-steel mesh in a connection. Thus, it is necessary to create frits inside the fused-silica capillary for usage in nano HPLC columns [5]. Nano HPLC columns have stationary phase particle sizes that are comparable to those of regular HPLC columns: 5 µm, 3 µm, and currently less than 2 µm.

Fig.5 Packed columns

B. Monolithic columns:

Because the stationary phase in this sort of column is cemented to the column wall, a porous (silica or polymer) structure forms throughout the column, negating the requirement for frits [6]. Single rods of inorganic or organic material that are created inside the capillary column are known as monolithic stationary phases. Monolithic columns eliminate the need for frits, and its high porosity permits faster mobile phase flow rates, which shortens the separation time. These columns are mostly utilized in proteomics to analyze highly intricate tryptic digests. Nano-HPLC can be used for a variety of analyses due to the stationary phase chemistry. Depending on the target analytes, different separation methods are used, such as chiral selection, size

exclusion, ion exchange, reversed-phase, hydrophilic interaction chromatography (HILIC), and others. The most advanced nano HPLC columns now on the market come in lengths of up to 50 cm and offer the separation pow

Fig.6 Monolithic columns

C. Capillary columns:

Capillary columns are useful for injections with modest volumes. Non-standard injection techniques are necessary for capillary columns in order to maintain high efficiency zones and peaks and avoid overloading the column's mass capacity [10]. Traditional volume injection techniques typically cause significant band broadening and are incompatible with capillary columns. Band broadening from dead volume between connections and repeatability concerns are the main causes of injection volume problems in the nanoliter regime. 20 Common injection techniques, including as gas pressure driven injections, continuous split flow and static21 injections, and trap columns, are employed to get around these problems. Capillary columns offer higher sensitivity for ESI-based MS but present sensitivity issues for pathlength-dependent UV-Vis detection. Reduced flow rates improve ESI-MS. 9, 10 Silica capillary can be utilized to create emitter tips with dimensions ranging from micrometer9, 11, 12, to nanometer13-16 (nano-ESI), which decreases flow rates and improves efficiency for the electrospray process shown in Figure 2. 17, 18 Less analytes per droplet due to the smaller droplet size is also believed to lessen competitive ionization and clustering. 9, 11, and 19 When compared to traditional ESI, these enhancements lead to a higher ionization efficiency.

3. INJECTION:

The injection system should make sure that no sample is wasted because the sample is usually limited. Nano HPLC setups can make use of direct injection setups [15]. The maximal injection volumes for nano columns are typically a few nanoliters and can be expressed as a function of the retention factor, plate number, column length, and other parameters [14]. Larger injected volumes result in a band broadening effect, which lowers the separation efficiency, particularly for poorly retained substances. Small injected volumes are a significant issue in nano-HPLC, causing loss of detectability. To operate in the nanoliter range, commercial auto-samplers, which typically operate at microliter levels, need an instrument adjustment. A split valve between the injector and the column could be used to get around this [11]. Direct injection of the sample onto the column occurs. It is used in some proteomics labs because there is only one column, which reduces the possibility of losing analytes. The modest injection volume (up to 1 µL), the potential for salts to enter the mass spectrometer, and the absence of column protection are the disadvantages [10]. These issues with direct injection can be addressed by a variety of techniques, including on-line pre-concentration (using a trap column), large-volume injection straight into the separation column under particular injection conditions, and off-line pre-concentration. By lyophilizing or evaporating the sample, the volume can be decreased for offline pre-concentration. Additionally, a pipette-based desalting phase can be included.

3.DETECTOR:

The detection methods used for HPLC separations and nano-LC are comparable. Because of its low cost, broad range of applications, and online detection capabilities, diode array detection (DAD) is commonly used in nano-HPLC. However, when on-column detection is used, detectability is limited due to the nano column's short route length [15]. Longer light routes are provided by specially designed detecting cells, which get around this. The detectors used in other analytical methods like HPLC and CE are typically used in nano-HPLC. Following the proper alteration of the cell or the source (in MS), these detectors are modified. These consist of conductivity, fluorescence, UV-visible, and, more commonly, MS. Laser-induced fluorescence (LIF) in nano-LC has been shown to have good sensitivity. It is also used for a number of separations made with microchips, but these are not reliable enough to be used for routine analysis. Cell volume is another detection-related parameter that needs to be managed; it should be as small as possible to prevent band widening, which could jeopardize peak efficiency and resolution [16]. Good detectability and a universal detection technique, like those offered by MS detection, are typically needed for biomedical and pharmaceutical applications. The column's nano flow, which is typically between 100 and 500 nL/min, is suitable for MS coupling through a variety of nano spray interfaces, particularly Electrospray Ionization (ESI), which only needs a little amount of eluent from the LC column to work. Typical nano ESI sprayers are made from electrically non-conductive silica capillaries. A liquid junction is the

most straightforward and cost-effective way to apply voltage [4]. Coating (for instance, gold) fused-silica emitters can be employed as an alternative to the liquid connection. The advantage of this technique is that the connection used in the liquid junction is eliminated; but, after an electrical discharge, the metal coatings may erode. Packing nano ESI sprayers with the stationary phase is a popular alternative.

Fig.7 Diode array detector (DAD)

APPLICATIONS:

1.Proteomics

2.Histamine determination [2]

3.Enzyme analysis

4.Forensic analysis

5.Environmental analysis [3]

6.Pharmaceutical analysis

7.Biomarkers

8.Analysis of enantiomers [4]

9.Food analysis

ADVANTAGES OF NANO-HPLC:

1. Reduced Sample Volume [1]
2. Enhanced Resolution
3. Improved Peak Capacity [2]
4. Lower Solvent Consumption
5. Better Compatibility with Mass Spectrometry [3]
6. Faster Analysis
7. Precision and Reproducibility
8. Optimized for Complex Samples [4]

DISADVANTAGES OF NANO-HPLC:

1. Lower Sample Throughput
2. Complexity and Maintenance [2]
3. Higher Initial Cost
4. Limited Column Capacity [5]

CONCLUSION:

A highly specialized and sophisticated method, nano-HPLC offers remarkable advantages in applications that call for precise separation of complicated mixtures, small sample quantities, and great sensitivity. It is especially useful in domains such as proteomics, metabolomics, and small molecule analysis because of its benefits, which include improved resolution, decreased sample quantities, greater sensitivity, and compatibility with mass spectrometry. For studies and applications requiring high-resolution, low-volume analysis, nano-HPLC is the best option. Nano-HPLC provides performance that is unmatched. Its usefulness should be evaluated in light of the lab's resources and particular analytical needs. We came to the conclusion that, in terms of solvent consumption, speed, sensitivity, resolution, and other benefits, Nano-HPLC has proven to be noticeably better than its conventional counterpart.

REFERENCES:

1. Ettre LS. M.S. Tswett, the invention of chromatography, LC-GC, 2003; 21:458-67.
2. http://www.chromatographyonline.com/lcgc/data/articlestandard/lcgceurope/382003/6971 8/article.pdf.
3. Andrea Weston and Phyllis R. Brown, HPLC and CE Principles and Practice, academic press, 1997: 01.
4. Dr. Michael E. Swartz, Ultra Performance Liquid Chromatography (UPLC): An Introduction, SEPARATION SCIENCE REDEFINED, 2005; 8-14.www.chromatographyonline.com.
5. Mariana Roberto Gama, Carol H. Collins and Carla B.G. Bottoli, Nano-Liquid Chromatography in Pharmaceutical and Biomedical Research, Journal of Chromatographic Science, 2013; 1–10.
6. Chervet, J.P. Ursem, M. Salzmann. J.P., Instrumental requirements for nano-scale liquid chromatography. Anal. Chem., 1996; 68: 1507-12.
7. Saito.Y, Jinno.K, Greibrokk T. Capillary columns in liquid chromatography: Between conventional columns and microchips. J. Sep. Sci, 2004; 27: 1379-90.
8. Hernandez-Borges J.H, Aturki Z, Rocco A, Fanali S. Recent applications in nano-liquid chromatography. J. Sep. Sci, 2007; 30: 1589-1610.
9. He Qing. Integrated nano liquid chromatography system on a chip. Ph.D. Thesis, California Institute of Technology, Pasadena, California, USA, June 21, 2005.
10. Available on URL: http://www.justchromatography.com/nano-hplc/nano-lc-definition.
11. Available onURL: http://www.justchromatography.com/hplc/nano-liquid-chromatography
12. Tatsunari Yoshida, Ronald E. Majors and Hiroki Kumagai, High−Speed Analyses using Rapid Resolution Liquid Chromatography on ZORBAX column packed 1.8 µm Particles, Chromatography, 2007; 28(2): 81-87.
13. Van Demeter JJ, Zuiderweg EJ, Klinkenberg A. Longitudinal diffusion and resistance to mass transfer as causes of non-ideality in chromatography. Chem. Eng. Sci., 1956; 5: 271-89. 14. Pedro R. Cutillas, Principles of Nanoflow Liquid Chromatography and Applications to Proteomics, Current Nanoscience, 1(1): 65-71. Available on URL: http://www.eurekaselect.com/61610/article/principles-nanoflow-liquid-chromatography-and-applications-proteomics. Kiran Karhale.
15. Nitin B and Suraj B, International Journal of Humanities Social Science and Management (IJHSSM) Issue 2, Mar.-Apr. 2023.
16. Garcia Villalba R, Carrasco Pancorbo A, Zurek G, Behrens M, Bässmann C, Segura Carretero A, FernándezGutiérrez A., Nano and rapid resolution liquid chromatography-electrospray ionization-time of flight mass spectrometry to identify and quantify phenolic compounds in olive oil., J Sep Sci, 2010; 33(14): 2069-78.
17. HosseinHashempour, AlirezaGhassempour, Norelle L. Daly, Bernhard Spengler and Andreas Rompp, Analysis of Cyclotides in Viola ignobilis by Nano Liquid Chromatography Fourier Transform Mass Spectrometry, Protein & Peptide Letters, 2014; 18(7): 747-52.