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Review Paper

Instrumentation and Methodology for Nano-Scale Liquid Chromatography

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ABSTRACT

The technology of miniaturized separation procuring grip as a renewable substitute for traditional method of separation which presents an eco-friendly solution. By miniaturizing, Nano Liquid Chromatography and Nano Capillary electrophoresis results in decreased consumption of reagent and generation of waste. This summary examines analytical instruments of nano-LC, discussing practical considerations and differences between traditional and miniaturized instruments. We also discuss the aspects to explain the challenges of nano-LC. Even though Nano Liquid Chromatography features limitations due to high cost of equipment, its creative advance opens door to revolutionize scope in research and industry laboratories. NLC is a modern microfluidic platform which is used for analyzing multiplex mixtures of clinical,chiral, pharmaceutical and enantiomeric substances. Miniaturized columns are also known as nano columns, which work at nanoliter flow rates and are essential in applications of NLC. Nano-LC offers a complementary alternative to traditional LC, providing numerous capabilities and scope for chemical analysis. In the principality of miniaturized liquid analysis separations, Capillary Electrophoresis and nano-LC which offer competing and complementary strengths. Nano-LC is a transforming investigation in chiral, proteomics and biomedical fields. This technique also enhances or improves the resolution, efficiency and sensitivity of separation methods. Additionally, it is well-suited for analyzing trace amounts of molecules.

INTRODUCTION

In 1988, Carlson and Novotny first introduced Nano Liquid Chromatography, by testing packed columns using very small internal diameter. It is a miniaturized technique in which analytes get separated on a capillary column containing the

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selected stationary phase.Generally, Nano Liquid Chromatography is defined as a modality of chromatography which involves the sample in nano gram concentration where mobile phase flows at nano or pictogram per ml. Miniaturization is currently a prominent trend in Science and Technology, particularly in the branch of analytical chemistry. It has several advantages over non-miniaturized techniques, although it also raises technical issues that are being tackled one by one. Nano Liquid Chromatography plays a crucial and vital role in in development and design of drug by facilitating advanced pharmaceutical analysis at nano level. As an alternative to conventional liquid chromatography, Nano-LC provides additional capabilities, enhanced options and broadening scope for chemical analysis. The high cost of analytical instrumentation for NLC limits its widespread application.furthermore, a deep understanding of nano-LC is necessary to avoid experimental issues, particularly those linked to instrumental setup. In nano-LC, parameters associated with HPLC, involves mobile phase, injection of sample and detection levels are measured in nanogram scale. Here mobile phase flow rate is typically expressed in nano milliliter per ml, sample injection volumes are measured in nano liters and detection of analytes are quantified in nanogram per ml. Nano Liquid Chromatography is often referred to as "lab-on-a-chip" chromatography as it is typically conducted on microchips. NLC provides several benefits which includes high sensitivity, faster analysis, enhanced separation efficiency and increased absorption capacity due to reduced particle size.Additionally, combination of Capillary Liquid Chromatography or Nano Liquid Chromatography results in developing method sensitivity. The Van Deemter equation is expressed mathematically which describes the relation between efficiency of column and broadening of band mechanisms which occur during process of separation.

Now the Van Deemter equation is expressed in its simplified form as

$$HETP = A + [B / u] + Cu$$

Where,

HETP = Height Equivalent Theoretical Plate

u= Average mobile phase velocity

C = Resistance to mass transfer

B = Longitudinal diffusion

A = Eddy diffusion

The main goal of Van Deemter equation is to improve efficiency of column which can be enhanced by upgrading the diffusion from various factors like flow rate and pressure flow rate.

Principle:

Columns are the dominant apparatus which are used to carry out separations in chromatography. Columns can be categorized depending on their internal diameter: nano-LC columns ranges from 10 to 1000 mm id, micro-LC columns from 0.50 to 1.0 mm id whereas capillary-LC columns with 100 to 500 mm id. The terminology of micro column, capillary-LC and microbore are Chromatography run on the elemental principle in separation of compounds depending on their interconnection with a stationary phase which uses a smaller column with a narrow diameter and run at much lower flow rates to gain high resolution. Separation occurs because of interaction with stationary phase and analyte leading to various retention times for individual component before eluted by the mobile phase.

Instrumentation:

In Nano-LC,pumps,columns, injection loops, connections and detection interfaces work together to reduce counter-pressure and designed to handle small volumes.

Generally, the instrumentation of Nano-LC includes the following components:

- Pumps

- Injectors
- Nano columns
- Detectors

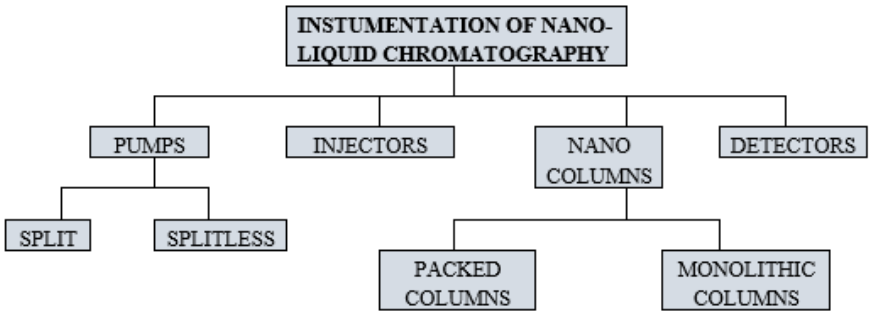

Figure1: Instrumentation of Nano Liquid Chromatography

Pumps: To facilitate Nano Liquid Chromatography, a sophisticated pump system must be able to provide consistent nanoliter flows and, maintain stability during separation process with precise control over nano-scale levels. These pump systems are versatile and are designed to function in both isocratic and gradient modes which are capable to operate at high pressures up to 1000 bar with precision and reproducibility. In Nano-LC typically two types of pumps are used: split pumps and split lesspumps, where split less pumps are dominant and mostly preferred.

Splitless pumps - These systems are designed to prevent solvent losses and deliver highly reproducible Nano flowrates. These systems are categorized into two distinct groups: “Solvent refill” systems which involve periodic refilling & “Continuous flow” systems which provide a steady flow. Syringe pumps with a single reservoir offer benefits over split systems with limited volume but continuous flow systems which utilize reciprocating pumps with dual pistons per channel are mostly preferred model. Continuous flow pumps can operate in both isocratic and gradient modes over Nano flowrates.

Split pumps - Split systems, incorporates a flow restrictor between pump and miniaturized column which are capable to split excessive flow from conventional HPLC pumps. Split systems are classified into two types:“Passive” split system and “Active” split systems. Passive split systems use a splitter divides high flow rate from pump to column and a flow restrictor. Although these systems are simple and low cost but often sacrifice accuracy and flow stability. Active split systems demonstrate enhanced stability and reproducibility but they result in wastage of mobile phase.

Injectors: To determine optimal sample volume for column injection, various parameters are considered which includes column length [L], diameter of column [dip], column diameter [da] and retention factor [k]. Direct injection methods are well suited for nano-LC systems to introduce efficient sample. In nano-LC, utilization of small injected volumes results in difficulty to detect and quantify the compounds. Utilization of large injected volumes results in band broadening effect and reduced separation efficiency, particularly for poorly retained compounds. The sample is injected at once into column, since there is only one column results in decreasing the risk of losing eluates and contributing particularly for proteomics. Researchers like Heron et al has revealed that using a weak solvent for injection of sample leads to increase in effect of enrichment and promotes concentration of sample when introducing a strong

mobile phase. To achieve chromatographic performance, the injection of sample must be as narrow as possible.

Nano Columns: In nano-LC, columns can have internal diameter [id] up to 10mm but columns with a 75mm id are most commonly employed and widely used. Columns with 75mm id are mostly adopted in nano-LC due to its capability of robustness,load ability and detectability in nano-LC applications. Nano-LC columns are basically fabricated from polyimide-coated fused silica capillaries or metal tubes manufactured from titanium or stainless steel, offering an ideal characteristic of durability, flexibility and mechanical strength. In 1990s, commercially available nano-LC were first emerged, marking a significant forward in improvement of nano-scale chromatography. The design of nano-LC columns come in several formats which includes open tubular columns, monolithic and pillar array columns, providing to select the best column to to optimize the analytical needs. Packed columns remain the preferred choice for nano-LC separations, but open tubular columns and monoliths are relatively less established for future growth. To meet the demands, researchers manufacture nano columns in their laboratories, allowing for requirements of particle size and stationary phase. Nano-LC columns are broadly categorized into two types which are described in detail below.

1. Packed Columns
2. Monolithic Columns

1. Packed columns**:** To fabricate packed columns, fused silica capillaries are commonly used which is coated with a layer of polyimide to enhance mechanical strength. Besides being flexible, these columns possess resistance and mechanical strength. These columns are modified in various ways, which includes monolithic bed of silica-based particles and packed with silica-based particles, less commonly coated with organic or inorganic materials. In packing nano columns, particle size frequently employed in the range from 3 to 5 micrometers. The preparation of columns with small internal diameter filled with particles is a complex and difficult task. Commercially packed columns with id of 50 & 75 mm are available from leading manufacturers which includes ThermosFisher, Sigma-Aldrich and Agilent.
2. Monolithic columns**:** Monoliths can be prepared by utilization of various synthetic ways which includes organic, inorganic materials and biocompatible compounds which is especially advantageous in bispecificanalysis. In monolithic columns, a porous structure of polymer or silica is created throughout the column which eliminates the requirement for frits, since the stationary phase is covalently attached to column wall. In monoliths, stationary phase is composed of solid and single rods of organic or inorganic material which are designed to facilitate capillary column. A key parameter of monolithic columns is that they eradicate the need for frits and their high porosity permits speedy flow rates of mobile phase which reduces the separation time. In latest generation of nano-LC columns offers separation power by proteomic sample which can now be commercially available in lengths up to 50 cm. Monolithic columns are broadly categorized into three distinct types based on their constitution like silica-based, organic polymer-based and organic-silica hybrid monoliths.

Detectors: The detection types used in nano liquid chromatography are same to those methodologies used in separation of HPLC. Diodearray detectors [DAD] are commonly occupied in nan0-LC as they offer advantages like online detection capabilities, cost effective and versatility. The

short path length in nano-LC is limited due to detectability of on column detection methods. By employing specialized detection cells, results in longer path length in which the problem or issue is solved. Another important parameter in detection is volume of cell, which should be kept minimum to avoid broadening of band thereby conserving resolution and peak efficiency. Biomedical and Pharmaceutical fields, constantly require high demand in detectability methods in which mass spectrometry [MS] appropriate to use. Inductively coupled plasma MS and laser-induced fluorescence are alternative methods which are employed in nano-LC, but they lack robustness to supply the needs of routine analysis. Also infrared, fluorescent and electrochemical detection are also engaged in nano-LC applications. The applicability pf electrochemical detection is limited to components that possess electrochemical activity.Similarly,fluorescent detection is restricted to fluorescent compounds only. Non-fluorescent compounds can also be detected by employingfluorescence detection through online post-column derivatization.Commonly, this process can be performed on a microfluidic chip, which is connected to a bubble cell. Combining ESI-MS with nano-LC is a presiding approach for detection in nano-LC. ESI-MS instrumentation has become a commonplace in laboratories and is appropriate for countless applications in medicine,pharmacology, proteomics and metabolomics.

APPLICATIONS:

- In pharmaceutical analysis especially in the field of proteomics like proteins sequencing protein mapping place a crucial role also in other fields like pharmacokinetics chiral purity etc.
- In environment analysis a nano-LC MS method was employed for estimation of target compounds in mineral water which results in certificate of proficiency
- In food analysis nano liquid chromatography isutilized to analysesanthocyanin's in red fruit juice which is comparedto those results using conventional HPLC in recent years has gained detection and quantification of harmful substance and it also allows determining of aflatoxins in peanuts
- In biological samples phospholipid in human urine is determined by performing NLC-ESI-MS/MS and also determines oligosaccharide in ovarian microfluidic chip based nano hock with tandem mass spectrometry is used to determine abused drugs like cocaine and amphetamine etc. in human hair
- In cases of biomedicine, biomarkers play a vital role in diagnosing and understanding the disease and health conditions
- In forensic analysis toxic substances and their metabolitesin wastewater are analyzed to estimate population exposure and guide public health needs to control the risk
- In herbal analysis when manner liquid chromatography is coupled with Fourier transform mass spectrometry [FTMS] results in analysis of cycloids in plants like viola ignoble.

Theoretical Aspects of Nano-Lc:

In the process of chromatography, the injected analytes experience dilution within the column, potentially resulting in decreased efficiency of separation. The utilization of smaller chromatographic systems reduces the dilution of chromatography, which in turn improve the mass detectability of the separation. From a theoretical viewpoint, miniaturizing LC systems provides numerous advanced field in liquid-phase separations.Although, various practical related issues can influence efficiency of separation and it is necessary to continue optimal performance of separation.

Extra column and efficiency of band broadening:

To evaluate the efficiency of capillary column, the Van Deemter equation is utilized, where HETP is plotted against linear velocity. For resemblance studies, concerning packed columns under several mobile phase conditions and particle of diameter, the reduced plate height equation is often suggested by Kennedy and Knox. By miniaturizing traditional analytical LC systems requires columns with small internal diameter. Attaining an optimal efficiency involves a comprehensive evaluation of parameters / factors influencing extra column broadening of band. As a result, this effect should be reduced to avoid loss of efficiency. One upper hand with this approach is it helps to decrease extra column broadening of band.However, this approach can lead to sensitivity because of limited injection of sample volumes and reduced detection of path lengths. This difficulty can be eliminated by injection of large volumes and employing pre-column / on column fastening techniques to enhance concentration of analyte.Additionally, employing more sensitive detectors may result in improved sensitivity.

By reducing the dilution of chromatography and improving sensitivity:

The utilization of narrower columns results in reduced dilution of chromatography. One of the advantage of decreasing column id is the ability to use lower volumes of mobile phase [MP]. Reducing the volume of mobile phase has a two benefits, one is enhanced performance of chromatography and another one iscost savings. In the process of chromatography, the sample is incorporated into the column as a tiny plug, now thaw mobile phase proceeds the analyte to the detector which may result in band broadening due to abundant key experimental parameters. The process of chromatography can result in analyte dilution within the column, which reconstruct / change the separation efficiency. This dilution event is the term used to outline the Chromatographic Dilution [D].

Enrichment:

From a theoretical consideration, the utilization of nano-LC is enhanced to promote effective enrichment compared to conventional HPLC. Reduction in internal diameter columns in nano-LC decreases the chromatographic dilution resulting in increased instant concentration of analyte as they elute or flow through the instrument. The enrichment outcome is due to reduced factors of dilution which is directly proportional to square of radius of the column and injected volume of sample. The dilution factor decreases due as radius of the column decreases which in turn increases the analyte detectability. Achieving the enrichment factor is complicated because of instrumental limitations like lengthy connection tubing, irregular flow patterns and dead volumes. Experimental observations reveal that injection of small sample volumes results in decreased detectability and sensitivity of nano-LC when compared to conventional HPLC, particularly when employing UV detection. The manifestation of mass spectrometry [MS], on-column trapping and multidimensional nano-LC results in effective detectability in nano-LC.

Hyphenation:

Mass spectrometry is frequently used method of detection for separation of nano-LC. Combining nano-LC with MS or MS-MS has proven to be a powerful platform in numerous fields to solve challenges in analytical science. Combining nano-LC with online MS has substantially enhanced the aspect of diagnosis and treatment of disease which leads to improve quality of life and health outcomes. By incorporating nano columns along with secondary separation techniques to give enhanced capabilities of separation in 2-dimensional chromatographic systems. Scientist Luo et al have suggested a 2D-separation method

for complex analysis of proteomics of cancer cells. Electrospray ionization has been explored in nano-LC because of its rapid compatibility with low flow rates of mobile phase. To achieve success, the performance of nano spray interfaces must be taken into consideration. The characterization of interface is very simple which incorporates stainless steel with zero-dead-volume that connects the capillary column to the tip, which is located adjacent to MS orifice. Electrospray ionization [ESI] is a dominant device to analyze a wide range of substances and is suitable for both high and low molecular weight ions and components that includes peptides, proteins and polymers and other related molecules. In recent years, an electron ionization source is traditionally designed for gas chromatography and has also implemented in nano-LC.

Table 1:Difference between high performance liquid chromatography(HPLC), Ultra liquid chromatography(UPLC) and Nano liquid chromatography(NLC).

HPLC	UPLC	NLC
Low sensitivity (3 or higher)	More sensitivity (0.9 to 1.2µg/ml)	High sensitivity (<50 µm)
Particle size of stationary phase is between 3 to 5µ	Particle size of stationary phase is < 2µ	Particle size of stationary phase 2 to 5 µm
Inner diameter of column is 3 to 10mm	Inner diameter of column is 0.75 to 1.8mm	Inner diameter of column is <100µm
Injection volume large are (5 to 25µl)	Injection volume small (2 to 20µl)	Injection volume small (1 to 10µl)
20mm-500mm in length and 1mm to 100mm inner diameter	30-150mm in length and <2mm in inner diameter	<30cm in length and 10 to 75 mm in inner diameter

Figure2: HPLC, UPLC, NLC

CONCLUSION:

In conclusion, nano-scale liquid chromatography has emerged as a powerful tool for the separation, identification, and quantification of complex mixtures at the nanoscale. Recent advances in instrumental and methodological developments have significantly improved the sensitivity, resolution, and throughput of nano-LC systems. This review has highlighted the current state-of-the-art in nano-LC instrumentation, including the development of novel column technologies, nano-flow pumps, and sensitive detection systems. Overall, nano-scale liquid chromatography has the potential to revolutionize various fields, including proteomics, metabolomics, and pharmaceutical analysis. As researchers continue to push the boundaries of nano-LC technology and methodology, we can expect to see significant advances in our understanding of complex biological systems and the development of new diagnostic and therapeutic tools.

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