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A REVIEW OF ADVANCE STUDY ON NANO LIQUID CHROMATOGRAPHY AND ITS APPLICATION: THE FUTURE OF THE FIELD

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ABSTRACT

Nano liquid chromatography is a kind of microfluidic system used to resolve different pharmaceutical, clinical, biomedical, chiral, metabolic, protein, peptides and enantiomeric compound. It is used to detect compound in nanogram level by using nano liter of solvents hence ensuring low consumption of solvent. Miniaturized separation techniques have emerged as ecofriendly alternatives to available separation methods. Nano-liquid chromatography (nano-LC), microchip devices and nano-capillary electrophoresis are miniaturized techniques that minimize reagent utilization and waste generation. Moreover, high cost of equipment may be some limitation although nano liquid chromatography is a newer technology ensures opening of new bridge to newer application for industry and laboratories. This article reviews the instrumentation used for nano-flow LC, the types of columns employed, and techniques for multidimensionality of separations, which might be key to the future state of the technique to the high-throughput needs of modern bioanalysis. Some theoretical aspects are explained to better explain both the potential and the principal limitations of nano-LC. An update of the current applications where nano-LC is widely used, such as proteomics and metabolomics, is discussed. Recent pharmaceutical and biomedical applications of this separation methods are also presented to signify the high-quality satisfactory performance for complex matrices, especially for proteomic analysis, that is achieve with nano-LC.

[Keywords: Nano liquid chromatography, Miniaturized technique, Van Deemter equation, Chromatographic dilution]

INTRODUCTION [1-5,7,12,13]

Russian botanist Mikhail Semenovich Tswett, invented chromatography in 1903 during his research on plant pigments. Chromatography process can be defined as separation technique involving mass-transfer between stationary phase and mobile phase. The nano-LC technique, as it is currently known, was first introduced by Karlssonand Novotny in 1988, testing packed columns with very small i.d. It is being used as a complementary and/or competitive separation method to a conventional chromatography.

Basically, Nano Liquid Chromatography (NLC) may be defined as 'a modality of chromatography involving samples in nano gram concentration, mobile phase flow in nanoliter per minutes with detection at nano gram or pico gram per milliliter'. This definition is a complete one and all these requirements can be fulfilled on chipbased chromatography. Therefore, mostly a true and complete nano-chromatography is only possible on chip, which is also called as Lab-on-Chip Chromatography.

About three decades ago, as documented by the data reported in the literature in 1978–90s years, researchers introduced miniaturization in the field of Liquid Chromatography (LC). Since that time, several research groups have studied the different aspects of miniaturization achieving interesting results that have contributed to the development of these techniques and to the production and marketing of dedicated instrumentation.

Miniaturization is one of the present trends in science and technology, especially in the field of analytical chemistry. It not only provides a number of advantages over non miniaturized techniques but also technical problems that are going to be solved little by little. Great efforts have been made in order to miniaturize LC instrumentation carrying out theoretical, technological, and methodological studies. Among these techniques, Capillary Electrophoresis (CE) with its different modes, has been widely studied and applied. Nano-LC is an alternative to conventional LC, providing more options for chemical analysis. Virtually all samples analyzed through traditional LC may be analyzed through a miniaturized technique. In this context, capillary electrophoresis and capillary electrochromatography additionally supplement and compete with nano-LC as miniaturized liquid phase separations. Nano High Performance Liquid Chromatography (Nano HPLC) is also mentioned as "Nano-bore HPLC" or "Nano-scale HPLC".

It has been frequently suggested that when the chromatographic separation is performed in capillary columns of ids in the range between 10 and 100 μm, the technique is named nano-LC generating nL/min flow rates, and providing higher sensitivity than that obtained with conventional HPLC. It has been reported that this phenomenon is due to both lower chromatographic dilution and higher efficiency while when making use of higher id columns (100 –500 μm.) the method is called Capillary Liquid Chromatography (CLC). Although a unified class is not available usually, CLC and nano-LC vary from each other by the capillary column i.d. employed as well as through the Mobile-Phase (MP) flow rate. NLC is of utmost importance in drugs design and development by providing pharmaceutical analyses at nano levels. In view of these facts, attempts have been made to describe state-of-art of pharmaceutical analyses by NLC. The analytical instrumentation used in nano-LC is still very expensive, limiting its widespread use. Moreover, significant technical knowledge about nano-LC details is required to prevent experimental difficulties, especially those related to the instrumental arrangement. Additionally, the combination of the CLC/nano-LC technique with mass spectrometry resulted to be a effective tool in the improvement of the method sensitivity. The coupling of both techniques (Nano-LC–MS) was easily achieved because of the relatively low flow rates typical of nano-LC system originated by the employment of small I.D. columns.

Mass spectrometry coupled with chromatographic separation techniques as GC, high-performance and ultra-performance LC (HPLC and UPLC, respectively) can be considered nowadays a leader and powerful tool for the analysis of complex matrices. In particular, HPLC-MS is commonly used for biological and pharmaceutical applications. The advance in technology also allowed to interface the highly performing gas chromatography (GC) and liquid-chromatography (LC) with Mass Spectrometry (MS) detectors. Mass spectrometry is based on the measurement of atomic or molecular mass by ionization of the analyzed compound and detection of the ions after acceleration with an electric field and separation with a magnetic field according to their different m/z value.

This review covers the principal aspects of the nano-LC technique and some recent pharmaceutical and biomedical applications, especially in the most employed area, biological research. Proteomic analysis, which corresponds to the major application of nano-LC, is also presented.

PRINCIPLE [1-3]

A miniaturized chromatographic system is based on the same principles applied to conventional chromatography, e.g., van Deemter equation, injection volume, back-pressure, partition or other mechanisms,

analyte-SP interactions; on the same experimental procedures, i.e., isocratic/gradient elution mode, selection of MP, temperature, and so on.

However, there are major differences regarding the instrumentation employed, which includes pumps, injectors, columns, and detectors. This is mainly due to the reduction of column dimensions (i.d.) and consequently to the working flow rate of the MP. The fundamental principle of this advancement is governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or 1/column efficiency).

$$H = A + B/u + Cu$$

Whereas, A is Eddy's diffusion; B is longitudinal diffusion; C is Concentration and u is Linear Velocity.

table 1: classification of HPLC systems

Description	Internal diameter of Column	Flowrate
Nano LC	10-100 μm	24-4000 nL/min
Capillary HPLC	100-100 μm	0.4-200 μL/min
Micro HPLC	1.0-2.1 mm	50-1000 μL/min
Normal HPLC	4.0-5.0 mm	1.0 -10.0 mL/min
Preparative HPLC	>10 mm	> 20 mL/min

This classification includes separations in microchips because nano-HPLC columns on chips have 20 to 100 mm as the i.d.

THEORETICAL ASPECTS OF NANO-LC

1. Improving sensitivity reducing the chromatographic dilution [1-4]

Decrease in column inner diameter results in a reduced amount of chromatographic dilution and, as a result, the concentration of the injected sample increased on the LC system.

The use of lower MP volumes is one of the advantages in reducing the column i.d. This is advantageous not only in reducing costs but also in improving the chromatographic performances. In chromatography, after injecting the sample into the column as a small plug, the MP moves analytes toward the detector producing a band enlargement that depends on several experimental parameters. Samples are diluted during the chromatographic separation process. During the chromatographic process, injected analytes can undergo dilution in the column that alters separation efficiency.

This dilution event, called **chromatographic dilution (D),** is expressed by,

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

Where **Co** is the initial concentration and
Cmax is the final concentration of the analyte during the chromatographic process,
dc is the column internal diameter,
1 is the total porosity of the column,
L is the column length,
$\mathbf{V_{inj}}$ is the sample injection volume,
k is retention factor and
H is Plate height.

D increases proportionally with the square of the column diameter and with the square root of the length of the column. The lower internal diameter in nano-LC promotes a high reduction in D value. Thus, downscaling of chromatographic method means less chromatographic dilution, increasing the mass detectability of the separation. 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

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

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.

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.

2. Efficiency and extra column band broadening [2]

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.

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.

$$h = A \cdot v^{0.33} + B/v + Cv$$

$$h = H/d_p$$

$$v = u \cdot d_p / D_m$$

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.

The miniaturization of a conventional analytical LC system utilizes columns of lower i.d. In order to achieve the desired good efficiency, it is necessary to evaluate all parameters influencing the extra column band broadening. Therefore, this effect have to be minimized to avoid loss of resolution due to a diminishing of efficiency.

On the one hand, this approach helps in reducing extra column band broadening; on the other hand, it can generate problems related to sensitivity due to both low injected sample volumes and reduced path length. However, this difficulty can be resolved by increasing the injected volume and focusing analytes either in a precolumn and/or in on column. In addition, or alternatively, sensitivity may be improved by making use of more sensitive detectors.

NANOFLOW MEASUREMENT [11]

Precise and stable mobile phase delivery at a flow rate very often below 1 µl/min is required for most nano LC separations. HPLC solvents do not behave as absolute liquids. Low pressure LC separations are affected by this phenomenon less than high pressure LC applications. The flow rate as well as pressure of the LC system is reached quickly in low pressure nano LC; thus, the effect of system stabilization time is very small and it can be neglected Addition of a nanoflow sensor into the nano LC platform enhances the reproducibility of LC analysis and it can also help to solve issues connected with minor mobile phase leakage in the LC system. The flow measurement in nano LC is based on the thermal mass flow measurement principle and it is realized through the constant power mode, where the two elements act as both, heater and temperature sensor. They are provided with an equal amount of constant power and the change in temperature is proportional to the value of the flow rate. Additionally, the output signal of this type of detector is also a function of the physical properties of the sensed liquid. Thus, an external calibration of a nanoflow sensor is needed for every recommended solvent or solvent mixture. Some configurations utilizing the thermal mass flow measurement principle are used in commercial nanoflow meters. A typical flow rate accuracy of such devices is less than 2 %.

INSTRUMENTATION

In nano-LC, the standard instrumentation is all miniaturized. Pumps, connections, columns, injection loop and detection interface are dimensioned for small amount of volumes and low counter-pressure. These parameters can greatly influence the chromatographic efficiency of nano-LC and need to be controlled for a successful separation.

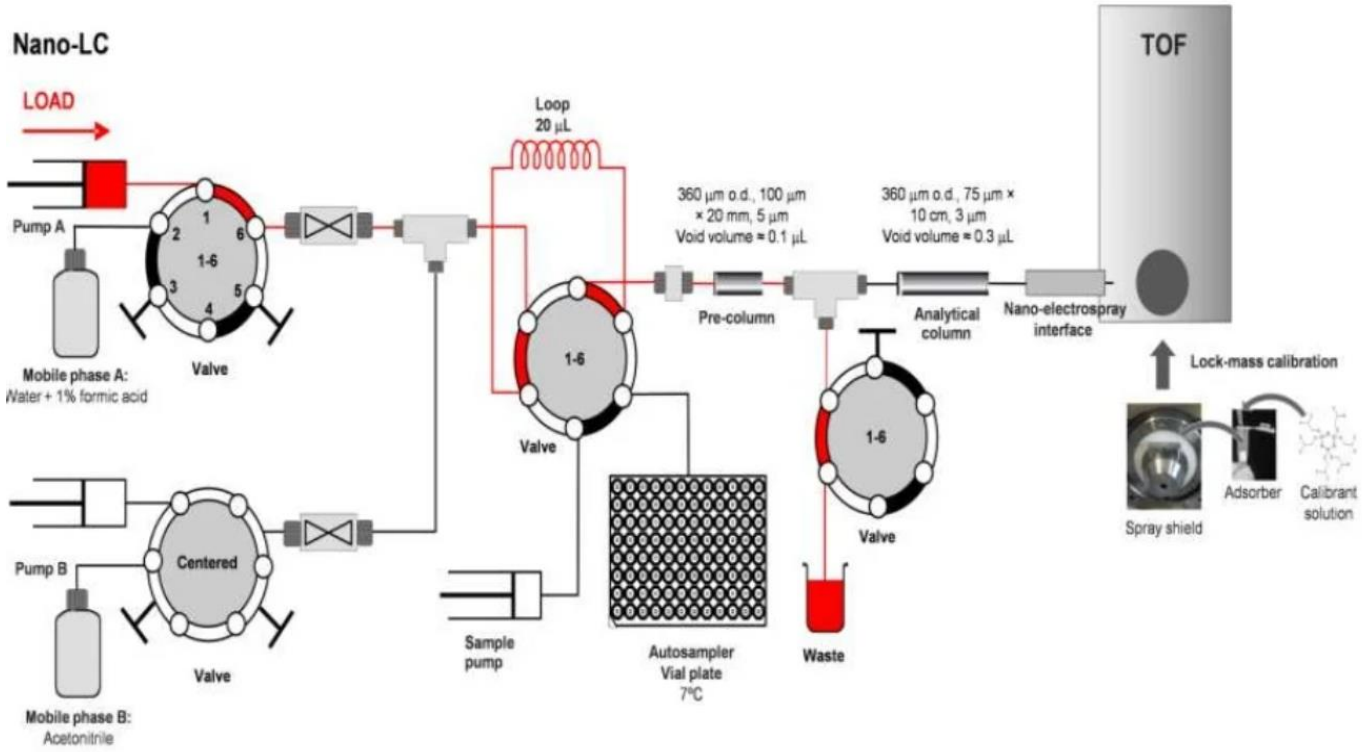

fig 1. schematic diagram of assembled nano-LC instrumentation.

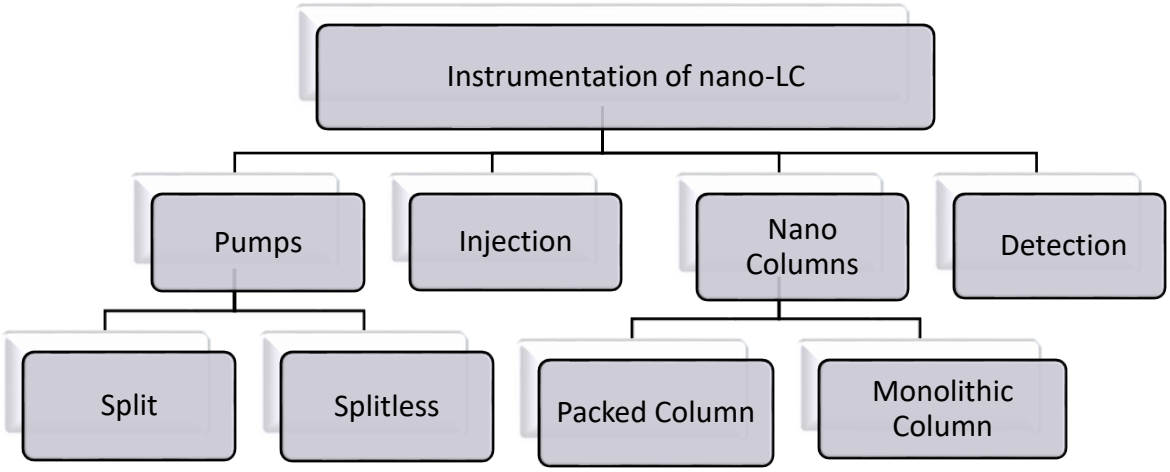

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fig 2. parts of instrumentation of nano-liquid chromatography.

1. PUMPS [1-4]

Nano-LC requires a pump system, which gives reproducible nano flow rates and stability during the separation, and permit gradient elution at nano-scale levels. It requisite flow rates of 500 nL/min or less.

Pumps for nano-LC need to present reproducible nano flow rates and stability during the separation, and permit gradient elution at nano-scale levels. Two main apparatus can be used in nano-LC: split and splitless pumps.

A. Split Pumps: Split systems divide excessive flows (mL/min) from conventional HPLC pumps by use of a flow restrictor between the pump and the miniaturized column. Split systems may lead to variable split ratios and low reliability of the nano flow, decreasing the repeatability of the separation. Spilt systems can be divided into two groups: the passive split system and the active split system. In the passive split system, splitter divides the high flow of the pump between the column and restrictor. The passive split systems are simple and relatively inexpensive, but compromise on flow stability and accuracy. The active split systems have improved flow stability and exhibit better reproducibility than passive split systems, but still the majority of the mobile phase is wasted. The split system has also been used by some commercial instrumentation, e.g., Ultimate 3000 series pumps (LC Packings) where the split was obtained employing dedicated cartridges with workflows between 20 nL/min and 50µL/min.

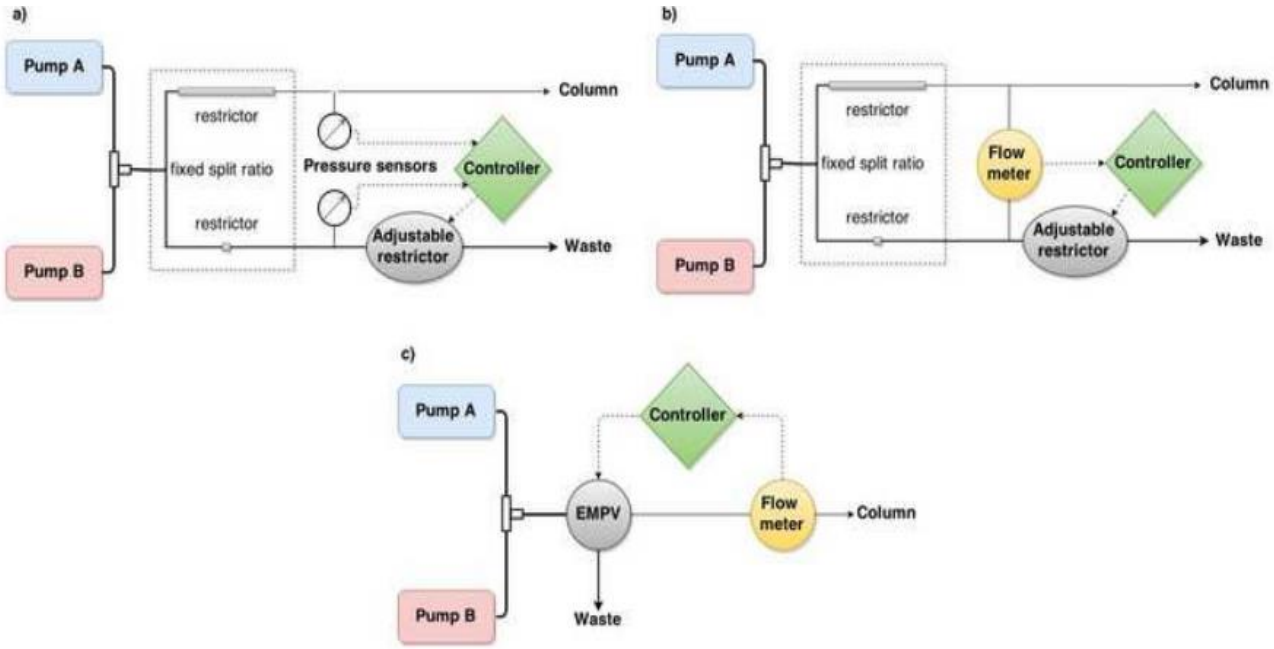

Fig 3. Active flow splitting systems; (a) and (b) represent arrangements with fixed splitter. An adjustable restrictor increases/decreases the resistance of the split line according to the pressure imbalance between the lines detected by pressure transducers (a) or by the flow sensor which connects the 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. [11]

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

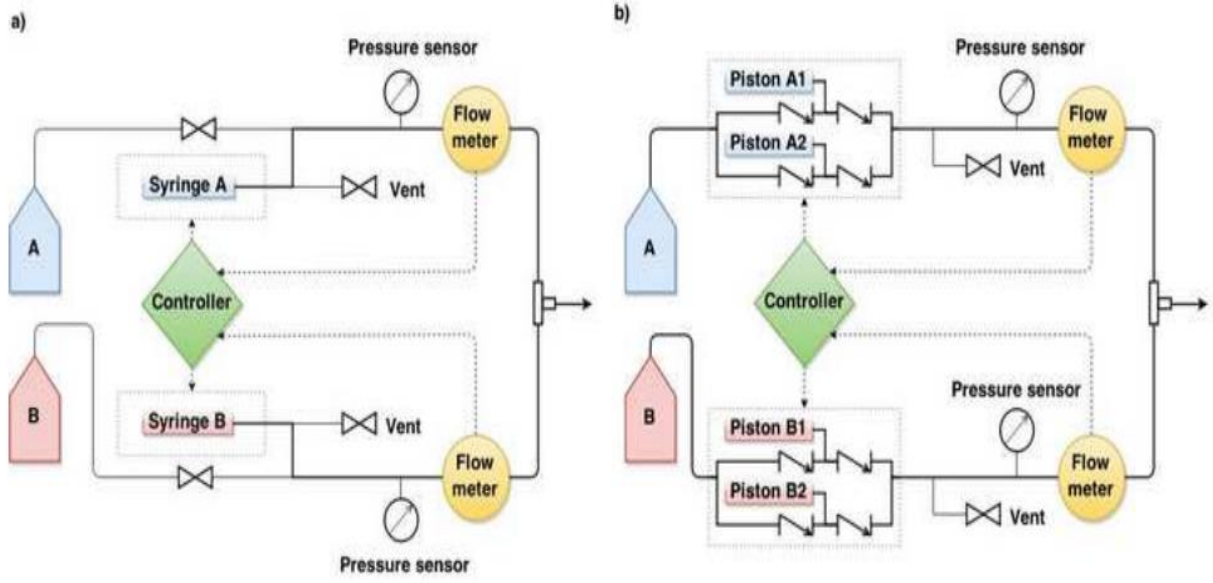

fig 4. splitless nanoflow gradient pump arrangement; (a) solvent refill system; (b) continuous flow system. [11]

2. INJECTION [1,2]

The volume of the injected sample is another key parameter that needs great observation in chromatography since a inappropriate injection volume can cause critical complication, deteriorating the analytes' separation due

to extra column band broadening. Therefore, in any chromatographic system the sample must be introduced as a zone as narrow as possible; the problem is more evident when decreasing the i.d. of the column. The maximum sample volume that can be injected into a column rely upon various parameters such as the length of the column (L), the particle diameter (dp), the column diameter (dc), and the retention factor (k)

$$V_{max} = 0.36 \sqrt{L d_p}\, d_c^2 (k + 1)$$

Typically, the sample is definite, so the injection system should ensure that no sample is misplaced to waste. Direct injection setups can be utilized in nano LC setups. The maximum injection volumes for nano columns may be expressed as a function of the column length, plate number, retention factor or few other different parameters, and are usually a few nanolitres. Small injected volumes are a major issue in nano-LC, causing loss of detectability, however large injected volumes produce a band broadening effect, reducing the efficiency of the separation, specifically for poorly retained compounds. Commercial auto-samplers, which usually work at microliter levels, require an instrument adjustment for use in the nanoliter range. This may be overcome by the use of a split valve between the injector and the column. The sample is injected onto the column at once. Because there is only one column so there may chance of lower risk of losing analytes and making it popular in some proteomics laboratories. The drawbacks are the low injection volume (up to 1 µL), the lack of column protection. Different methodologies can be applied to overcome these problems with direct injection, such as off-line pre-concentration, large-volume injection directly into the separation column using specific injection conditions, and on-line pre-concentration (using a trap column). Off-line pre-concentration may be carried out by reducing the sample volume by evaporating or lyophilizing the sample.

3. NANO COLUMNS [1,4]

Although columns of 10 mm i.d. can be employed, nano-LC columns of 75 mm i.d. are the most frequently used. This i.d. column provides a good compromise between detectability, loadability and robustness in nano-LC separations.

In the early days of nano LC, researchers had to manufacture their own columns because of the lack of commercially available columns. In the 1990s, the first nano LC columns have became commercially available as a result of efficient packing procedures. Throughout the last two decades, the column offering has grown enormously and nano LC columns are now available. Self-packed nano LC columns are used by research laboratories, often for economic at intervals.

A. Packed columns

The packed columns used in the nano LC columns are made of Polyimide-coated fused silica capillaries. that present flexibility, high mechanical resistance and a variety of internal dimensions, but stainless steel and titanium tubes are also used for nano columns. They can be packed with silica-based particles, filled by a monolithic bed or, much less commonly, wall-coated with appropriate organic or inorganic matters. The most common particle sizes for packed nano columns are 3–5 mm. However, particle-filled small i.d. columns are difficult to prepare.

B. Monolithic columns

Monoliths can be processed by usage of various synthesis ways, organic or inorganic-based, and biocompatible substances are interesting alternatives in biospecific analyses. In this type of column, a porous (silica or polymer) structure is formed throughout the column, eliminating the need for frits because the stationary phase is fixed to the column wall. Monolithic stationary phases are single rods of organic or inorganic material which can be produced in the capillary column. No frits are required with monolithic columns and the high porosity of these materials allows higher flow rates of mobile phase, lowering the separation time. Current state-of-the-art nano LC columns are commercially available in lengths up to 50 cm to provide the separation power required for these complex proteomics samples.

4. DETECTION [1-4]

The types of detection for nano-LC are similar to those employed for HPLC separations. Diode array detection (DAD) is typically utilized in nano-LC, due to its low cost, wide range of applicability and use of online detection. However, because of the short path length of the nano column, detectability is finite when on-column detection is applied. This is overcome by the use of specially configured detection cells that provide longer light paths.

Detectors usually employed in nano-LC are the ones applied in other analytical techniques such as HPLC and CE. These detectors are adapted after appropriate modification of the cell or the source (in MS). They include UV-visible, fluorescence, conductivity, and extra frequently, MS. Good sensitivity has been demonstrated using Laser-induced fluorescence (LIF) in nano-LC, being also applied for several separations achieved with microchips but these are not robust enough to be applied for routine analysis. Another parameter related to the detection to be controlled is the cell volume that must be as low as possible to avoid band broadening that can compromise the peak efficiency and resolution. Biomedical and pharmaceutical applications usually require good detectability and a universal detection method, such as that provided by MS detection. The nano flow from the column (frequently, 100–500 nL/min) is appropriate for MS coupling through numerous nanospray interfaces, especially Electrospray Ionization (ESI), which requires only a small quantity of eluent from the LC column to be successful. The necessities needed for the nano ESI are, obtaining a stable spray for flow rates of hundreds of nanoliters per minute and maintaining the separation efficiency. Typical nano ESI sprayers are created from silica capillaries that do not conduct electricity. The simplest and most economical method of applying voltage is through a liquid junction. A substitute to the liquid junction is to use coating (for example, gold) fused-silica emitters. This solution gives the benefit of eliminating the connection used in the liquid junction, but the metal coatings can deteriorate following electrical discharge. A common substitute is packing nano ESI sprayers with the stationary phase.

5. HYPHENATION OF NANO-LC WITH MASS SPECTROMETRY [2,4]

MS is the most frequent nano-LC hyphenation. Coupling nano-LC to MS or tandem mass spectrometry (MS-MS) has been carried out in different areas, which has solved many problems in the analytical sciences. For example, nano-LC separations coupled to online MS (or, less commonly, offline MS) have increased the diagnosis and treatment of several human diseases, promoting better quality of life.

Although in LC several sources have been employed, in nano-LC, electrospray ionization (ESI) was experimented and utilized with excellent results simply due to the relatively MP low flow rates. To accomplish success, a nano-spray interface is used. The interface is very simple; it makes use of a stainless-steel union with zero dead-volume where the capillary column joins the tip typically placed in the front of the MS orifice. The MS spectra collected with an ESI interface is very simple because of the usage of low ionization energy. The ESI ionization may be applied to the evaluation of both small ions and compounds with relatively high molecular weight, e.g., proteins, peptides, polymers, and so on. This can be effortlessly done because these compounds also expose multiple charges. Recently, an Electron Ionization source (EI), originally used in GC, was introduced also in nano-LC.

Hyphenations also may be applied by using two miniaturized techniques, such as biological microanalysis systems or coupling another (orthogonal) nano column in the second dimension of the 2D separation.

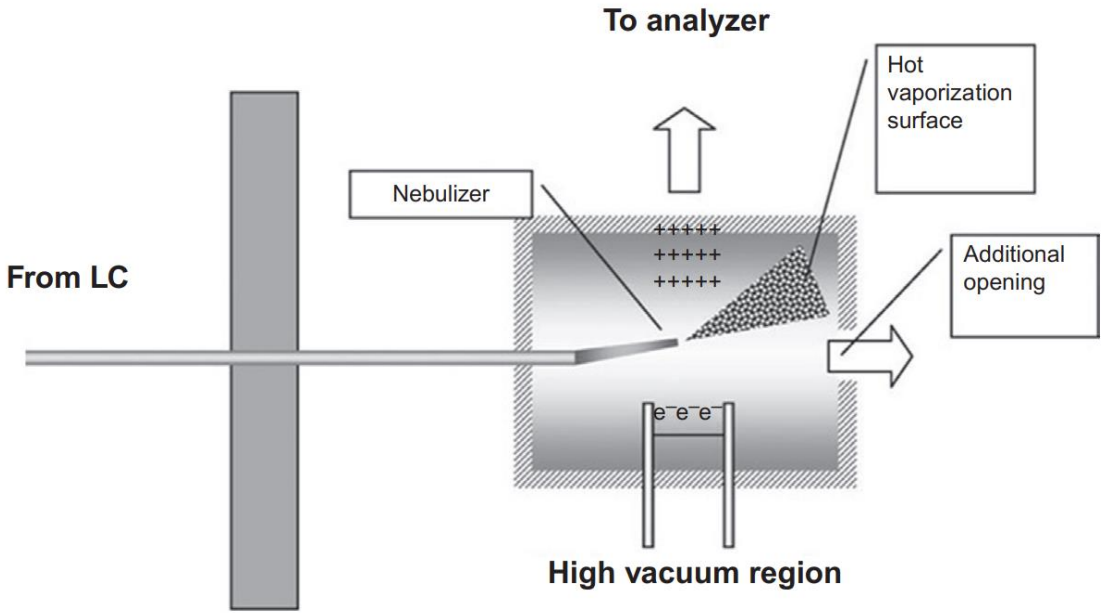

fig 5. scheme of the direct-EI interface. [3]

6. ENRICHMENT IN NANO-LC [4]

Theoretically, the use of nano-LC promotes analyte enrichment more than conventional HPLC. Reduced i.d. columns lower the chromatographic dilution and, consequently, increase the instant concentration of the injected analyte as it passes through the components of the LC instrument. The enrichment factor is attributed to lower dilution factors and is proportional to the square of the column radius and the injected analyte volume The smaller the column radius, the lower the dilution factor, with a resulting increase in analyte detectability this enrichment factor is not easily achieved, because other instrumental factors often decrease the observed analyte concentration, such as excessive connection tubing, dead volumes from connections and disrupted nano flow.

Some experimental observations shows that the small sample volumes injected decrease the detectability of nano-LC in comparison to conventional HPLC, mainly while using of ultraviolet (UV) detection. The application of MS detection, multidimensional (nano)-LC or on-column trapping can greatly increase detectability in nano-LC.

VALIDATION OF LIQUID CHROMATOGRAPHIC METHODS [6]

1. Traditional method validation

The purpose of method validation is to demonstrate that an analytical method is suitable for its intended purpose, and for a quantitative method, it provides a reliable estimate of the actual value of the sample tested. Method validation involves assessing method performance against predefined criteria that are established on the basis of product or sample specifications and the type of measurement to be performed for example, assay, identification or limit test.

In general, an analytical method is validated before use. In addition, it is a good practice to periodically re-evaluate the metho. d itself as well as the associated validation criteria and data to ensure that the method remains appropriate

Parameter of validation:

1.**Specificity / Selectivity:** Ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc.

2.**Accuracy / Trueness:** Expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

3. Precision: The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

3 a. Repeatability: Expresses the precision under the same operating conditions over a short interval of time. Also called intra-assay precision.

3 b. Intermediate Precision: Expresses within-laboratory variations: different days, different analysts, different equipment, etc.

3 c. Reproducibility: Expresses the precision between laboratories.

4. Detection Limit (DL)/ Limit of Detection (LOD): The lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

5. Quantitation Limit (QL) or Limit of Quantificaiton (LOQ): The lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

6. Linearity: Ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

7. Range: The interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

8. Robustness & Ruggedness: A measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides indication of its reliability during normal usage.

These validation approaches provide reasonable assurance that a method will perform as needed. However, these validation studies frequently occur as a single event early in the lifecycle of a method and may not always predict performance during routine use that may take place over years.

2. Enhanced approaches

Enhanced approaches, also called the Lifecycle approach rely on a combination of scientific knowledge and risk management to understand and control sources of variability. They are aligned with Quality by Design (QbD) principles, which are described for the pharmaceutical industry in ICH guidelines Q8-Q10. These concepts can also be applied to provide a framework for analytical methods. In addition, in an enhanced approach, greater emphasis is placed in the importance of connecting all developmental activities and ensuring this knowledge remains available throughout the method lifecycle.

Enhanced approaches to the method lifecycle emphasize the following:

A. Establishing method criteria that are linked with the purpose of the method. The criteria has been termed the analytical target profile (ATP)

B. Taking a systematic approach to method development that relies on sound scientific and experimental design principles.

c. Applying a risk-based approach to prioritize experiments designed to understand the effect of method conditions and sample preparation on chromatographic behavior

D. Developing a good understanding of how method parameters affect chromatographic behavior through experimentation, application of fundamental knowledge

E. Establishing meaningful method controls (e.g., system suitability) based on an understanding of the relationship between method conditions and method performance.

F. Periodically reviewing the analytical method to ensure that it remains appropriate for its intended use and updating or replacing it as appropriate.

The extent of application of these concepts relies upon how the test results are used, that is, the decisions that will made with the test results.

APPLICATIONS [2,4,7]

Nano-LC has been applied to the separation of a wide number of compounds in different areas such as proteomic, pharmaceutical, or environmental fields. Most recent applications of the use of nano-LC in analytical chemistry, those related with proteomics being the most common.

1. Proteomics

One of the main areas in which nano-LC has found its major applicability is by far the proteomic field, especially in peptide mapping, protein sequencing, or determination of post-translational modification of proteins. The main reason is surely the limited amount of these types of samples (particularly when working with primary cells) and thus the fact that in these cases it is of special importance to maximize the number of protein identifications. In a high number of these works of nano-LC is only used for certain specific purposes, which represent a small part of the whole work (cell fractionation, protein extraction, and digestion, data analysis, etc.)

Protein sequencing of complex biological samples is important for biomarker identifications, disease control and clinical treatments, principally from plasma and tissue samples. HPLC-based methods overcome the classical problems of protein analysis, like gel electrophoresis and immunoanalysis, which are both limited by many steps before analyses. The range of proteome complexity requires fast and unquestionable identification techniques, promoted by the emergence of nano-LC coupled to MS and MS-MS. These have allowed the precise determination of amino acid sequences from proteins or peptides, which is assisted by a full identification database. However, classical methods are still used with nano-LC–MS, because much information about protein sequencing and peptide mapping is obtained by a mixture of two or more identification strategies.

Methods to get quantitative information from proteomic studies based on 2D-LC-MS have also been developed. Other strategies to get quantitative information from LC-MS have been developed. The groups of Yates and Mann reported approaches supported metabolic labelling of cells in culture using heavy nitrogen and heavy amino acids, respectively. Others employed isotopically labelled peptides that were used as internal standards to accurately quantify gel separated proteins.

A great number of nano-LC columns for peptides separation are commercially convenient with different i.d. and SPs. Most of them are full of silica particles with C18 covalently linked. However, also partially porous particles, Hydrophilic Interaction Liquid Chromatography (HILIC), and monolithic columns have also been employed. Monolithic capillary columns (100 μm) with surface bound lectin affinity ligands were wont to perform lectin chromatography by nano-LC with diode array detection (DAD). During this case also two polymethacrylate monoliths were prepared to yield a neutral and a cationic macroporous polymer and two lectins, including Concanavalin and nutriment agglutinin, were immobilized onto the monolithic columns.

Proteomic analyses have been performed for synovial fluid from rheumatic patients by using nano-LC–MS-MS. Osteoarthritis and rheumatoid arthritis are both destructive articular diseases, characterized by a gradual degradation of the cartilage tissues by defense cells, followed by inflammation disturbances. Mateos et al. identified peptides related to both articular diseases and other peptides exclusive to each one. Knowledge of the proteome from synovial fluids was important to detect protein fractions that acted as biomarkers and promoted an efficient clinical control of patient treatments.

The use of 2-D nano-LC methods is also becoming popular, especially in proteomic applications to scale back the complexity of the samples as well as the dynamic range of the peptide abundance although in some cases only one of the analytical columns is really a nano-LC column. 2-D LC for peptide analysis is usually performed by using ion exchange chromatography to create fractions of peptides.

A nano-LC system including a high-resolution MS operating at very low flow rate (20nL/min) was employed for routine analysis of proteins and peptides. An analytical column (25 cm length×25μm i.d.) full of 2μm porous particles was used for peptide separation.

2. Analysis of enantiomers

Separation of chiral compounds is an interesting and challenging topic of research in many analytical chemistry areas as, for instance, pharmaceutical, biomedical, and environmental fields where bioactivity of many molecules is related to their chirality and pure enantiomeric forms are often required. For the pharmaceutical field as an example, almost half of the drugs in use are chiral and very frequently the pharmacological effect is restricted in most of the cases to one of the enantiomers. Traditionally, GC, HPLC, or supercritical fluid chromatography (SFC) have been the techniques most commonly used for chiral separations. Chiral stationary

phases (CSPs) (or chiral mobile phase additives) are quite expensive materials and the small amounts requested for nano-LC as well as the shorter analysis times make this technique very attractive for enantiopurity assessment.

Glycopeptides antibiotic based stationary phases were also successfully employed in nano-LC, as showed by Fanali et al., for the separation of both basic and acidic chiral compounds. The high enantio resolution capability of this type of chiral selector enabled the separation also working with short packed columns. The developed and optimized methods were also applied to the analysis of real samples, like pharmaceutical formulations, human serum, or urine, and coupling with MS was arranged.

3. Biomarkers

Biomarkers are defined as endogenous indicators of a selected biological state, usually a peptide or a carbohydrate. They will be experimentally measured and evaluated for normal or disordered processes. within the biomedical sciences, biomarkers are especially related to healthy or diseased states. A biomarker also can be a substance introduced into an organism to estimate its normal or diseased function. Nano-LC plays a crucial role in biomarker analyses. The low analyte concentration from biological samples requires sensitive separation techniques and nano-LC coupled to MS or MS-MS easily presents this characteristic. Garcı´a-Villalba et al. evaluated polyphenol metabolism in human carcinoma cells using nano-LC –MS. The polyphenols were found in extra virgin vegetable oil and their metabolites are proven to have anti-tumor activity. The authors quantified the polyphenol metabolites consistent with uptake time by the cancer cells and concluded that these biomarkers were easily measured by nano-LC –MS.

4. Pharmaceutical analysis

Pharmaceutical analysis may be a crucial topic of wide application in different fields, like analysis of the chiral purity and quality of pharmaceutical formulations, drug control, Pharmacokinetic studies, and so on. Although HPLC remains far and away the more used technique in industrial quality control, in chiral analysis nano-LC and CLC are preferred.

Nowadays, HPLC remains the analytical technique of choice in pharmaceutical analysis for both drug discovery and development. Furthermore, it are often advantageously used for studying drug degradation and metabolization, enantiopurity controls, etc. This tendency also seems to be followed by nano-LC (although in pharmaceutical analysis the sample amount is typically not limited) mainly for chiral applications, which are already described. For nonchiral pharmaceutical nano LC applications, Fanali et al. evaluated the use of nano-LC-MS for the analysis of basic compounds of pharmaceutical interest such as nadolol, oxprenolol, alprenolol, and propranolol (using terbutaline because the interior standard).

Separation of chiral compounds is of eminent interest, since most of the bioorganic molecules are chiral. The chirality of drugs is a major concern in the modern pharmaceutical industry, since different enantiomers of the identical drug may have different pharmacological activities, also as pharmacokinetics and pharmacodynamics effects on the physical body. Thus, an isomer can make desired therapeutic activity, while the other may not have any effect, or even produce undesirable consequences. Nano-LC and CLC are suitable techniques for preliminary small-scale chiral analytical studies, particularly for the evaluation of latest SPs often synthetized in small amounts. Nowadays, chiral selectors in CSPs are supported mimic complex biomolecules, like proteins, peptides, and carbohydrates. during this respect, the foremost popular CSPs for LC are those supported polysaccharides coated onto silica particles.

5. Food analysis

The applications of miniaturized LC techniques to the analysis of foodstuffs are scarce with reference to other fields The analysis of anthocyanins in commercial red fruit juices was assessed by nano-LC, and therefore the method was compared with conventional HPLC by Fanali and coworkers. A C18 capillary column of 100μm i.d. and MS was employed within the first case, while a 2.1mm i.d. narrow-bore C18 column and diode array detector

(DAD) was utilized in the second. Both methods were fully validated to achieve higher sensitivity with HPLC. However, nano-LC offered good quantitative leads to a shorter analysis time. The column, full of fully porous particles, allowed the separation of all studied compounds in but 7min and gave better results with respect to the column containing partially porous particles. On the opposite hand, the nano-LC system provided higher separation efficiency and worst peak symmetry and determination than conventional C18 column by HPLC. The developed nano-LC method was applied to wine pomace samples and represented a suitable system for the analysis of anthocyanin dyes.

Beside the control of food quality and nutraceutical analysis, food safety has experienced an excellent rising concern. Food products reach the buyer through human handling and action, which generally results in the introduction of exogenous molecules that could modify and accelerate food deterioration or even endanger human health. In recent years, nano-LC and CLC are applied for the determination of harmful compounds in food matrices such as antibiotics/drugs, pesticides, mycotoxins, and endocrine disrupting compounds (EDCs) like phthalates.

6. Environmental analysis

The use of nano-LC for the analysis of compounds of environmental interest has not been so widely extended up to now, although HPLC is one of the major techniques for the analysis of pollutants and their metabolites. In fact, very few works deal with the application of nano-LC in environmental analysis.

MS detection was coupled through a nanospray interface, obtaining good intra- and interday precision and complete separation of zeralanol (ZAL) and E2 isomers in less than 20min. The nano-LC-MS method was applied for the detection of the target compounds in mineral waters extracting the analytes by SPE with commercial zearalenone (ZEN)-molecular imprinted polymer (MIP) cartridges, obtaining LODs. The method was compared with CEC-MS, but less satisfactory LOD and limit of quantification (LOQ) results were achieved due to the lower injection with respect to nano-LC and the MS signal suppression caused by the presence of the buffer in the MP.

Also, regarding monolithic columns, Rahayu and coworkers in situ prepared a 300μm i.d. monolithic column attaching polyethylene glycol (PEG) groups into a glycidyl methacrylate monolith polymer for the separation of inorganic anions (IO3-, BrO3-, NO3-, Br-, NO3-). It constituted a very simple and fast method to analyze seawater and public drinking water, in which a simple filtration was needed before injection.

7. Forensic analysis

The analyses of medicine of abuse and their metabolites in wastewaters can determine the access of the population to these substances and the public health requirements for their control. Steroid hormones, hallucinogens, cannabinoids, opioids and various prescribed drugs are listed by US National Institutes on Drug Abuse as commonly used drugs of abuse. Urine, sweat, blood (plasma) and saliva are often analyzed for current drug use; however, hair appears to be the simplest specimen, because it requires noninvasive sample collection. Compared to other specimens, a hair sample has little or no possibility of adulteration and informs a longer detection period, revealing a history of substance abuse, if present.

Hair specimens from patients of a detoxification center were collected for the analysis of cocaine, amphetamine, morphine and related drugs. The authors developed an easy and validated nano-LC method as an alternative to inconclusive immuno-assay techniques, using special nanochip-LC instrumentation. They also significantly reduced the sample preparation steps and therefore the amount of sample required (less than 10% of usual quantity).

Although it's an excellent tool for monitoring, nano-LC isn't usually applied for the identification and measurement of drugs of abuse, probably thanks to the lack of nano-LC equipment in routine analysis laboratories. Gas chromatography and traditional LC are the principal instrumental choices because of their wide distribution in forensic centers, whereas immunoassay tests are the foremost common analytical strategies for initial drug detection in biological samples, thanks to their fast and easy execution.

8. Histamine determination

Yaru Song et al., (2004) was determined histamine by using nano-liquid chromatographic/tandem mass spectrometric (nano HPLC/MS/MS). The method involved pre-column derivatization of histamine with 7-Fluoro-4-nitrobenzoxadiazole (NBD-F). Sodium 1-heptanesulfonate, added as an ion-pairing reagent in the mobile phase for sample loading, was found to facilitate greatly the analyte retention. This method was applied to determine histamine in water, alcoholic beverages and rat brain tissues.

9. Enzyme analysis

Nano-LC remains not often used for enzyme analysis. Often, the stationary phase of nano-LC changes the enzyme conformations and their catalytic activity is reduced. Other miniaturized techniques, like capillary electrophoresis (CE), are preferred over nano-LC, because they are doing not promote alteration of the real form of the enzyme. However, reproducibility in nano-LC is above in CE, probably because the pressurized flow is more stable than the electroosmotic flow generated inside the capillary in CE.

Krizek and Kubickova reviewed the foremost recent methods for kinetic enzyme assays and showed that CE and its modes of separation were widely used for enzyme analysis, whereas nano-LC was utilized in only a few papers in recent years. One possibility to beat the limitations of enzyme analysis in nano-LC is the use of bioaffinity columns. These special particulate or monolithic stationary phases immobilize the enzyme in an accessible conformation without a big loss of the original enzymatic activity. consistent with Tetala and van Beek, bioaffinity columns for nano-LC can easily be preared from organic or inorganic-based materials, not just for enzymes but also for other biomolecular analyses related to the immobilized enzymes.

CONCLUSION

Nano-LC is a modern separation technique exhibiting great potentiality, especially in the field of analytical scale. Today, the miniaturization of analytical instrumentation provides an important role in the development of analytical sciences, which is determined by studies in many different areas. It has been applied in various field such as pharmaceutical, food, agro-chemical, environmental, and biomedical interest. Currently, analyses of several pharmaceuticals and drugs can be carried out in small volume samples or samples having very low concentrations of analytes. It has a good scope in drugs development, design and analysis specially in determining in vivo pharmacodynamics and kinetics of drugs. Several column containing either monolithic or particulate material, mainly studied in HPLC, are currently used. Nano-LC experiments can be carried out using either commercial or laboratory assembled instrumentation.

The principal limitation at the present to wider use of nano-LC is the high value of the analytical instrumentation. However, the rapid improvement of new equipment is overcoming this limitation, expanding nano-LC to routine laboratories and industries. The nano chromatographic modalities are not fully developed; however, NLC will become more effective and highly sensitive and selective technique when hyphenated with mass spectrometric detection.

REFERENCES

1. Narayudu Y, SathisKumar D ,“Advanced study of nano liquid chromatography and its application-a review.” *World J. Pharm. Res.* **2015**, 4(4),1355-1367.

2. Maria Asensio-Ramos, Chiara Fanali, Giovanni DO, Salvatore Fanali, “Chapter 27 - Nano-liquid chromatography.” *Elsevier,* **2017**, 2, 637-695.

https://doi.org/10.1016/B978-0-12-805393-5.00027-0.

3. Imran A, Hassan YA, "Role of Nano Liquid Chromatography in Pharmaceutical Analyses."*Curr. Pharm. Anal.* **2009**, 5, 367-380.

4. Mariana RG, Carol HC, Carla BB "Nano-Liquid Chromatography in Pharmaceutical and Biomedical Research." *J. Chromatogr. Sci.* **2013**, 51, 694–703.

doi:10.1093/chromsci/bmt023

5. Rita P, Isabella C, et al. "High Performance Liquid Chromatography Coupled with Mass Spectrometry for/and Nanomaterials: An Overview." *AIP Conf. Proc.* **2020**, 2257, 020002.

6. Kimber L. Barnett, Brent Harrington, Timothy W. Graul, "Chapter 22 - Validation of liquid chromatographic methods." *Elsevier,* **2017**, 2, 533-552.

https://doi.org/10.1016/B978-0-12-805393-5.00022-1.

7. Javier HB & Zeineb A, et al. "Recent Applications in nanoliquid chromatography." *J. sep. sci.* **2007**, 30,1589-610.

doi: 10.1002/jssc.200700061.

8. Cutillas PR, "Principles of nanoflow liquid chromatography and applications to proteomics." *Curr. Nanosci*.**2005,**1, 65 –71.

9. Saito Y, Jinno K and Greibrokk T, "Capillary columns in liquid chromatography: between conventional columns and microchips." *J. Sep. Sci.***2004** , 27, 1379-1390.

https://doi.org/10.1002/jssc.200401902

10. Noga M, Sucharski F, et al. "A practical guide to nano-LC troubleshooting." *J. Sep. Sci* . **2007,** 30, 2179–2189.

11. Sestak J, Moravcova D, Kahle V, "Instrument platforms for nano liquid chromatography." *J. Chromatogr. A.* **2015**,1421,2-17.

doi: 10.1016/j.chroma.2015.07.090. Epub 2015 Jul 29. PMID: 26265002.

12. Katia B, Giovanni DO, et al. "Separation of organophosphorus pesticides by using nano-liquid chromatography." *J. Chromatogr. A.* **2009,** 1216(18),3970-3976. ISSN 0021-9673.

https://doi.org/10.1016/j.chroma.2009.03.005

13. Salvatore F, Zeineb A, et al. "Separation of basic compounds of pharmaceutical interest by using nano-liquid chromatography coupled with mass spectrometry." *J. Chromatogr. A.* **2007,**1150,252-258. ISSN 0021-9673.

https://doi.org/10.1016/j.chroma.2006.10.021.