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# ADVANCEMENTS IN LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY: METHOD DEVELOPMENT AND APPLICATIONS

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## ABSTRACT

Liquid chromatography is an advanced technique used to separate multiple analytes with high precision. Mass spectrometry is known for its precise detection capabilities. In the analysis, the coupling effect of LC-MS has proven to be a highly useful and significant analytical tool. LC-MS is widely used in environmental, biomedical, food and pharmaceutical analysis. It has demonstrated applicability to biochemical screening of inherited diseases, therapeutic drug monitoring, and toxicology. LC-MS can accurately detect steroid hormones, vitamins and their related metabolites from biological materials. The creation of novel LC-MS techniques will serve as a tool to help further analyses. LC-MS method development begins with goal setting followed by research and planning. Development of a validated method requires thermodynamic and kinetic considerations as well as chromatographic development with MS optimization and tuning. The use of silica-based core-shell particles, microcolumns, monolithic columns, and hybrid columns is constructive for the future of LC-MS technology.

**KEYWORDS:** Liquid Chromatography, Mass Spectrometry, Method Development, Biomedical Analysis, Environmental Analysis.

## INTRODUCTION OF LC

Separation techniques based on sample partitioning between mobile and stationary phases are commonly known as chromatography. The stationary phase can be either liquid or solid, whereas the mobile phase can be either gas, liquid, or supercritical fluid. As the mobile phase components pass through the stationary phase, individual analytes from the mixture are

equilibrated or partitioned between the two phases. Separation is based on differences in partition coefficients of individual analytes within the sample. Chromatographic methods can be divided into four categories. The first is based on system geometry and is further subdivided into column and planar chromatography. Classification by operating method includes development chromatography and elution chromatography. A combination of mechanisms, classification based on retention mechanisms is further broken down into linear chromatography, adsorption chromatography, partition chromatography, size exclusion chromatography, and gel filtration chromatography. By phase, such as gas chromatography and liquid chromatography, is the final categorization. Although liquid chromatography employs a liquid as the mobile phase, gas chromatography uses a carrier gas.[1]

**Liquid Chromatography**

Liquid chromatography [LC] is the most used method for separating samples into individual parts. This separation occurs as a function of sample interaction with mobile and stationary phases.[2] In LC, the sample is injected into the mobile phase stream through an inlet. Injection is facilitated by a supported high-pressure pump and transported further through the column or stationary phase. The separation process takes place in a column and is continuously monitored with a flow detector. Pillars are the most important part of the instrumentation. Conventional column lengths vary between 100 and 300 mm. These columns have an inner diameter of 3 to 4.6 mm and an outer diameter of approximately 6.35 mm. short columns, 30-500 mm in length and 3-5 µm id, are used especially in quantitative bioanalysis and LC-MS.

Microcapillary packed and nano LC columns have proven useful whenever higher efficiency LC-MS is required, especially when sample volumes are limited. These columns consist of quartz tubing with an inner diameter of 0.05 to 0.5 mm.[2] Column effluent characteristics are measured by a detector. The detector converts the physical parameters of the wastewater into electrical signals. UV/VIS, diode arrays, rapid scanning detectors, fluorescence detectors, and electrochemical detectors are common detectors used in conjunction with LC. Mass spectrometry [MS] and nuclear magnetic resonance [NMR] are notably selective and detectors in use now a days.[3] The success of LC depends on various techniques used for separation. These separation techniques are classified based on the partition mechanism used and further include adsorption chromatography [normal and reversed phase], partition

chromatography, ion exchange chromatography, ion pair chromatography, and gel permeation/size exclusion chromatography.

**Types of liquid chromatography**

**High performance Liquid Chromatography [HPLC]**

HPLC analyzes samples 1-2 times faster than conventional chromatography. One of the most important factors in its effectiveness is the small pillars of particles. HPLC is well suited for multicomponent analysis of real samples and complex mixtures.[4] Recent developments in particulate bonded phases are efficient and versatile. The analysis of multicomponent mixtures is greatly improved by fine particle bonded phases.[5]

**Ultra-Performance Liquid Chromatography [UPLC]**

UPLC is considered a further development of HPLC. UPLC is based on the relationship between linear velocity and plate height stated by van Demeter. The use of sub-2-micron particles successfully reduces column size. Pressures of up to 1000 bar are then reached, saving time, and reducing solvent consumption.[6]

**Hydrophilic interaction liquid chromatography [HILIC]**

Separation of small polar compounds on polar stationary phases can be effectively achieved by HILIC. This normal-phase liquid chromatography technique has the advantages of ion chromatography as well as reversed-phase liquid chromatography.[7]

**Electrochemically Modulated Liquid Chromatography [EMLC]**

In EMLC, the separation efficacy is enhanced by changing the potential of conductive stationary phase, which results in manipulation of analyte retention. The use of HPLC at higher column temperature is one of the examples of EMLC. For mixture of aromatic sulfonates in mobile phase, high temperature reduces analysis time by more than factor of 20.[8]

**Micellar liquid chromatography [MLC]**

In MLC, a micellar solution is used as the mobile phase for RPLC. A small amount of organic solvent is added to the micellar solution to achieve retention and improve peak efficiency and resolution. MLC has proven useful for the analysis of real-world samples such as physiological and food samples. MLC provides a solution for direct sample injection by solubilizing proteins.[9]

**Perfusion Chromatography**

It is one of the most effective techniques for separating proteins and other large molecules. Two types of pores were used as perfusion media, either pores with sizes of 6000–8000 Å or diffusion pores with sizes of 800–1500 Å. Various types of chromatographic methods are suitable for this technique, including reversed-phase chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, and affinity chromatography. Separation performance is partially dependent on particle size, but completely independent of sample flow rate.[10]

**Immunoaffinity chromatography**

The binding affinity of an antigen for its parent antibody is used as the basis for immunoaffinity chromatography. Various parameters such as biological affinity pairs, matrix support, immobilization binding chemistry and effective elution conditions are optimized to achieve a successful his IAC platform.[11,12]

**Chiral separation**

Separation using a chiral additive in the mobile or stationary phase. Chiral separations by LC are useful for enantiomer studies, including preparative separation and composition determination of various analytes. This method is also useful for monitoring asymmetric reactions. This method has been used in a variety of studies, including analysis of pharmacokinetics, environment, enantioselectivity, and stereochemistry of natural compounds. Recently, several types of chiral stationary phases have been developed and commercialized. The most common types are the Pirkle type, the ligand exchange type, and the molecular imprint. The classification of CSPs by macrocyclic antibiotics has also been focused. Various proteins, synthetic polymers, cyclodextrins, cyclofructans, polysaccharides, and crown ethers are some common variants of the same thing.[13]

**Monolithic column**

Monolithic columns consist of polyetheretherketone tubing packed with rigid, continuous, porous silica. They are mainly used for the analysis of high molecular weight compounds such as proteins and polynucleotides and are of great importance in electro chromatographic techniques.[14] For certain analyte groups, these columns have proven to be faster, more robust and provide better resolution.[15]

**Mass spectrometry**

The principle of mass spectrometry is to separate gaseous ions with electric and magnetic fields according to their mass-to-charge ratio. [16] Quantification of organic compounds can be efficiently performed by MS method. The most important application of MS is to determine molecular weight along with structural properties of individual analytes. Targeted and non-targeted links can be identified simultaneously in an online and real-time manner. [17] Mass spectrometers are essentially three-part sophisticated computerized instruments. The first is analyte ionization to produce gaseous ions from the analyte.[18] Ionization can be produced by electron ionization, chemical ionization, negative electron capture ionization, desorption ionization, and sputtering ionization.[19] These ions are further resolved into characteristic mass components according to their mass-to-charge ratio by the mass spectrometer. The most used mass spectrometers are quadrupole mass spectrometers, time-of-flight [ToF] mass spectrometers, magnetic sector mass spectrometers, electrostatic sector mass spectrometers, and trapped ion mass spectrometers. The last and most important part of the mass spectrometer is the detector. A detector is used to detect the ions and record the composition of each resolved ion species.[20] Faraday cup detectors [or cylinder electrodes], electron multipliers, and photomultiplier tubes [or scintillation counters] in the industry.[21]

**LC-MS: Coupling of Liquid Chromatography [LC] with Mass Spectrometry [MS]**

LC-MS combines the advantages of versatile LC separations with the sensitive detection of MS. The combination of these two techniques has proven to be a very effective and important analytical tool in environmental analysis. This technique enables the detection of non-volatile, thermolabile, and polar species in the environment.[22] By avoiding antibody cross-reactivity, LC-MS/MS has proven to be an effective tool to analyze different steroid combinations simultaneously.[23] Effective analysis of metabolites by MS builds on previously used separation techniques. MS can be combined with gas, liquid, or capillary electrophoresis [CE]. GC-MS is the preferred tool for detecting volatile and thermally stable analytes, while CE-MS has proven effective for detecting polar and charged molecules. LC-MS is the most versatile tool as it can be used for both polar and non-polar compounds. Among these three tools, LC-MS has the best coverage of metabolomics.[24]

**LC-MS Studies in Recent Past**

**Biomedical Analysis**

LC-ES-MS is a highly recommended technique for metabolic and biochemical studies of endogenous compounds such as proteins, glycoproteins, glycolipids, and peptides. This method can also be used to analyze small compounds such as nucleic acids, vitamins, steroids, and fatty acids. This technique was effective in validating post-translational modifications of proteins and in precisely localizing glycopeptide-containing fractions in proteolytic digests of glycoproteins. Aqueous humor metabolites can be analyzed by LC-QTOF-MS. This method has reproducibly measured thousands of metabolic properties. Of these 1000, perhaps a quarter of the metabolites were identified.[25] On the other hand, MS/MS fragmentation helped identify over 50 metabolic features. A total of 47 metabolic pathways are associated with these identified metabolites. This method helped determine the role of microbial metabolites in ocular disease. In addition, amino acids, lipids, and oxidative stress also play important roles in the pathogenesis of ocular disease.[26] A biomarker for solid tumors. However, large extractions and large preparative C18 bed volumes limit the automation of discovery.[27]

**Environmental Analysis**

Food and environmental safety have become very important in society. The lack of tools to identify and quantify contamination is a serious problem. LC-MS is one of the most effective tools for analyzing and evaluating contaminants in food and the environment. The method has proven effective against a wide range of emerging contaminants, including organic and inorganic nanomaterials, perfluorinated alkylating agents, personal care products, and human and veterinary drug ingredients such as hormones.[28] Advances in LC-MS technology such as untargeted LC-MS and the use of microbore columns have proven useful for environmental analysis.[29] LC-MS is the most affordable technique for detecting and analyzing various pesticide compounds. Steroid estrogens, which interfere with the endocrine system, and nitrosamines in wastewater can be detected with high sensitivity by LC-MS. LC-MS can be used in conjunction with atmospheric pressure ionization [API] techniques to analyze thermally sensitive, polar, high molecular weight ionic compounds. LC-MS with an Orbitrap analyzer aids in target quantification and identification of unknown analytes in wastewater. LC-MS/MS can be used to detect low concentrations of perfluorinated organic compounds [PFOS] in cleaning products, textiles, and photographic materials. Liquid chromatography coupled with high-resolution mass spectrometry [LC-HRMS] is useful for studying various

organic contaminants such as degradation products.[30] The future importance of LC-MS lies in the field of metabolomics, which is widely used for plant improvement. Plant primary and secondary metabolites are involved in highly complex metabolic pathways. The advanced metabolomics tools of LC-MS have proven effective in the qualification and quantification of these metabolites. [31] Secondary metabolites such as vitamins, flavonoids, glucosinolates and carotenoids can be easily detected by LC-MS. Additionally, LC-MS methods can be operated with targeted and untargeted methods. Recently, an online bioinformatics tool for LC-MS programmed data transfer has been prepared. Using such tools in experiments reduces data processing time and increases the effectiveness of online systems.[32] Efficient detection of secondary metabolites with ovicidal properties against H. placei is possible by coupling LC-MS with online bioinformatics tools.

**Biochemical Screening for Genetic Disorders**

LC-MS methods have now proven effective for the detection and analysis of small DNA fragments. This LC-MS in negative ion ESI mode is used for this purpose. This procedure has proven to be less time consuming than traditional techniques. The entire screening process required several days for primer extension assays and cleavage of extension products. LC-MS can then be completed within hours. The maximum time required to complete the LC-MS process is 1-2 days, depending on the complexity of the analytes. [33] Other than LC-MS-based proteomics, there is no comprehensive way to analyze alterations in mutant proteomes. The success of this method relies on charge- or hydrophobicity-based separation of the peptide mixture. Cation exchange chromatography or reversed-phase chromatography can be used for this method.[34] LC-MS/MS has greatly expanded the feasibility of screening for Niemann-Pick C1 disease in neonates.[31] LC-MS has proven applicability in high-throughput laboratories by detecting multiple diseases in a single injection. LC-MS can be used to detect up to 45 genetic disorders by measuring various amino acids and acylcarnitines.[35] Broad cytotoxic and genotoxic properties.[36] LC-MS/MS and polymerase chain reaction [PCR] fill the need for easy and sensitive diagnosis of multiple nucleic acid aberrations and their modifications. Because these changes are associated with cancer, this technology allows continuous monitoring of disease progression.

**Pharmaceuticals**

HPLC-MS or HPLC-MS-MS is the most preferred analytical technique used during various stages of drug development.[38] In the drug discovery stage, it is necessary to screen lead

molecules. LC-MS analysis is actively used for protein identification, natural product identification, metabolic stability profiling, and molecular weight determination to support combinatorial or medicinal chemistry. Evaluation of products for IND/CTA filling is required during preclinical development. During this phase, LC-MS supports the analysis of impurities, degradation products, and metabolites. During the clinical stages of drug development through quantitative bioanalysis and structural identification, LC-MS supports registration of NDA/MAA submissions. At the end of manufacturing, LC-MS is used for compliance testing by identifying impurities and degradation products.[39] Furthermore, the LC-MS method can be applied to ensure the safe use of APIs in clinical therapy. A sensitive and reliable LC-MS/MS method was developed and validated for the quantitative analysis of six potentially genotoxic impurities in pantoprazole sodium starting material. This method can also be applied for in-process monitoring of impurities during pharmaceutical manufacturing.[40] Detection of unknown enantiomers in real samples is difficult. High-throughput derivatization-based bioanalysis using LC-MS has proven useful to solve this problem. Amine and carboxylic acid derivatizing reagents have been developed and used to detect potential disease-related biomarkers.[41]

**Therapeutic Drug Monitoring and Toxicology**

Current routine applications of LC-MS in clinical diagnostics include multiple analytes for drug monitoring and endocrinology. It is also useful for newborn screening and toxicology research. The application of these techniques in the in vitro diagnostic [IVD] industry is now becoming a priority market as innovative assay kits have greatly automated his LC-MS instruments.[42] Gemifloxacin, a quinolone antibiotic of many drugs in plasma and urine samples, was quantified from human urine samples using isocratic elution and tandem positive ionization MS. This method was rapid, sensitive, selective, and robust compared to conventional methods.[43] One LC-MS/MS method was found to be suitable for the quantification of rifampicin in plasma samples from 340 patients.[44] LC-MS/MS combined with iTRAQ8-Plex labeling was shown to be a reliable method for quantitative proteomic analysis of letrozole in human plasma compared to protein abundance. This technology allows access to personalized cancer care.[45,46] In the context of COVID-19, accurate detection of the monomeric SARS-CoV-2 S protein was only possible by LC-MS. [47] An LC-MS method was developed for the determination of one of the most common illicit drugs, methamphetamine, and its isomer N-isopropylbenzylamine. This procedure was performed with positive electrospray ionization [ESI] using multiple reaction monitoring mode. This

method has been useful for routine analysis of methamphetamine and n-isopropylbenzylamine in questionable samples in laboratories and forensic departments.[48] LC-MS methods are also useful for the quantification of commonly used drugs. Sodium glucose cotransporter 2 inhibitors [SGLT2i] are widely used in diabetic disease. Quantification of SGLT2i, including canagliflozin, dapagliflozin, and empagliflozin, in plasma and urine by LC-MS/MS methods is useful for pharmacokinetic studies and biomedical analyses. A short run time of 1.0 min allows rapid analysis of many samples. [49] The UHPLC-MS/MS method is applied for initial studies of intracellular drug concentration and distribution. Cellular levels of pemetrexed can be rapidly determined using a sensitive and reliable UHPLC-MS/MS method.[50] UPLC-MS/MS has proven to be a sensitive method for identifying drug-drug interactions. This technique was used to quantify salvianolic acid B, salicylic acid, and acetylsalicylic acid concentrations in human plasma. This method has helped detect pharmacokinetic changes caused by drug interactions.[51]

**Steroid Hormones**

The LC-MS/MS assay outperforms the immunoassays evaluated for the analysis of six serum steroid hormones, including cortisol, aldosterone, testosterone, dehydroepiandrosterone sulfate [DHEAS], 17-hydroxyprogesterone [OHP], and progesterone. was excellent. As assessed, immunoassays generate significant analytical bias and fail to meet method comparability. LC-MS/MS assays have the advantage of being able to analyze low concentration samples.[52] Endogenous steroid hormones and endocannabinoids [EC] are measured by LC-MS/MS as stress markers from hair analysis. Picogram- or microgram-level quantification can be achieved through a combination of surrogates and background correction.[53] LC-MS methods can be used to detect 14 hormones of natural and synthetic origin. The method can also be validated for liver, bile, kidney, and hair matrices. Estimated daily intakes, including hazard ratios and indices, can be calculated by analysis of matrix data. Because there is an association between elevated serum PdG/creatinine and pregnancy, and between low serum PdG/creatinine and thyroid cancer, this method was used to determine pregnancy risk and thyroid cancer in early-stage female candidates. You can identify risks.[54,55] Analysis of plasma and urine for metabolomics and lipidomics by LC-MS has helped control menstrual rhythm. Interacting levels of estradiol, progesterone, follicle-stimulating hormone, and luteinizing hormone were observed at her four time points during her menstrual period. Using LC-MS techniques, a total of 74 different amino acids and

biogenic amines in urine and plasma were analyzed via the amine platform, and 185 different compounds of 9 lipid classes were analyzed in plasma via the lipid platform.[56]

**Vitamins and Related Metabolites**

Till date, an immuno-enzymatic technique is the most preferred method for vitamin D analysis. However due to high specificity and selectivity, LC-MS/MS method is now preferred for analysis of vitamin D metabolites in various biological materials such as serum and dry blood spot.[57] 25-hydroxyvitamin D quantification using LC-MS/MS methodology showed a higher value in plasma compared to serum.[58] LC-MS/MS was found to be highly specific and sensitive tool for quantification of vitamin D levels. Due to prevention of interference from nonspecific reactions and cross-reactivity, LC-MS became most reliable and accurate method for measuring vitamin D. While another accurate and reliable LC-MS/MS method was developed for establishing vitamin A and E reference intervals that can be used in the clinical settings.[59] The UPLC-MS/MS method with reverse phase gradient chromatographic technique was successfully used for determination of total vitamins B1 [thiamine], B2 [riboflavin], B3 [niacin], and B6 [pyridoxine] in infant formula and supporting nutritionals. The method used methanol and 20 mM ammonium formate in water as mobile phase and C-18 column as stationary phase.[60] The LC-MS/MS method is successfully used for detection and quantification of the of vitamin K1 in sixteen fat containing food materials like soybean, rapeseed, and olive oil.[61] There are several HPLC methods available for quantification of vitamin K in serum. Using the post-column reduction method together with electrochemical or fluorescence detection showed limitations due to interference with triglycerides. The LC-MS/MS method has been shown to be highly specific.[62] Recently, a method was developed for the quantification of ascorbic acid in human plasma using LC-MS. This method was further tested with human plasma and white blood cell samples.[63]

**Food Analysis**

A method has been developed for the rapid and economical detection of ivermectin residues in milk using a positive ESI LC-MS/MS technique. This method can detect, quantify, and confirm trace levels of ivermectin residues. This is a highly accurate, precise and sensitive method for routine analysis. This method gives excellent results even when using simple extraction procedures. A simple extraction process was used for ivermectin, and no interferences were detected in the analysis, demonstrating the accuracy of the developed method.[64] Targeted compound screening techniques are used to determine known analytes,

and non-targeted compound screening techniques for unexpected or true unknowns have greatly contributed to the quantification of food contamination.[65] MS can accurately determine nitroimidazole and quinolone residues in honey.[66] In some Asian countries, products derived from soft-shelled turtles [freshwater turtles] are classified as important health foods. Nitrofuran metabolite contamination can be rapidly measured by real-time LC-MS/MS.[67] A single LC-MS/MS technique can be used to measure multiple compounds in fungi. Hericium erinaceus is he one of the edible and medicinal mushrooms used in traditional Chinese medicine. Ultra-performance, high-resolution liquid chromatography-tandem mass spectrometry [UPLC-Q-Exactive-MS/MS] integrated with a standard compound database tentatively identified 102 compounds in Hericium erinaceus.[68]

**Points to Consider during Method Development for LC-MS[69–72]**

Creating a set of experimental conditions to properly study a particular sample is called method development. Relying at the complexity and motive of the approach, it could have several stages and take a long term to complete.[69] The approach improvement manner includes the subsequent steps

**Method Goal**

This is one of the often overlooked but critical steps to success. Objectives of the analysis should be established.

1. Detection: The process of detecting compounds in a sample. A suitable detection technique should aim to achieve this goal.
2. Quantification: This is the process of analyzing the amount of a compound in a sample. The method of quantitation, concentration range, amount of sample required, and level of accuracy/precision should be considered.
3. Identification: Analysis is performed to identify a compound or moiety in a sample. Detection techniques and purity analysis should be considered.
4. Characterize: an analysis is performed to validate the connection properties. Be careful with property or property level decisions.
5. Purification/Isolation: The purpose is to collect compounds for further use. Separation of purified material or 100% recovery of samples is analyzed.[69]

**Research and Planning**

Although the method development process is the same for most compounds, there are some considerations at each stage. This step requires investigating the chemical nature of the specimen. The data required for the procedure is categorized as follows:

a. Specimen chemical and structural data
b. Information on sample types and conditions required for analysis
c. Literature review of previous studies on specific analytes
d. sees regulations and industry guidelines.[70]

**Approaches**

There are two approaches to method development

1. Stepwise approach [one factor at a time]: This approach mirrors previous experimental results.
2. Systematic Screening: This is a protocol-based approach. This approach requires evaluating several factors.

Proposed stationary phases, mobile phases, pH requirements, and column specifications are evaluated beforehand. Evaluation leads to fine-tuning of selectivity and retention resulting in good separations.

**MS initial Tuning**

Before starting chromatographic method development, it is important to check whether the analytes ionize in the MS. A syringe infusion pump has proven helpful in this step. The analyte is pumped into the ion source at constant current. Variables such as gas flow and ionization voltage are adjusted to the analyte. The tuning process creates optimal conditions for generating ions. Then the process becomes more sensitive. In this step, positive or negative ionization modes are selected and different types of adducts are recorded.[71]

**Chromatographic Development[72]**

Chromatographic development is based on separation effectiveness and also depends on the following factors:

**Thermodynamic considerations**

Thermodynamic considerations in process of separation are based on solute retention on stationary phase. Multiple parameters such as time required for retention, volume of solute retention and capacity factors of distribution coefficient are considered.

**Kinetics Considerations**

Kinetic considerations are essential for proper peak width or to avoid band broadening. This method is considered effective when band broadening is minimal. The factors that contribute to the band broadening phenomenon are:

1. Resistance to mass transfer: related to the rate at which analyte molecules are exchanged between the stationary and mobile phases. This is believed to be the main reason for the increased bandwidth.
2. Eddy Diffusion: Changes the speed at which a solute move through the stationary phase, which in turn changes the distance traveled by the solute. Solutes flow through different paths as a function of the reduced resistance that contributes to the overall velocity. Retention times are based on global solute velocities, so unfavorable fluctuations in velocities will broaden the bands.
3. Longitudinal Diffusion: As the solute band travels through the stationary phase, the solvent of higher concentration diffuses into the lower concentration according to the diffusion coefficient. Fast moving mobile phases result in shorter diffusion times and less band broadening.
4. Extra-column effect: The extra-column effect is more important for the early narrow peaks in the chromatogram than for the late, broad peaks. Additional column efficiency can be obtained with injectors, connecting tubes and detector flow cells.

**Resolution**

Peak resolution is one of the key factors to consider in method development. Peaks are separated by the property that single components are selectively retained on the stationary phase. Good resolution is highly dependent on column length, particle diameter packed in the column, and analyte flow rate. However, updating these factors is more expensive and time consuming. Alternatively, using a different stationary phase in the column, controlling the pH of the mobile phase, or adding a complexing agent to the mobile phase can be very effective.

**Time Considerations**

Time is an important factor to consider to achieve effective peak separation. Analysis time can be shortened by shortening the column length. However, efficient techniques are required to separate the components with the desired resolution.

**Peak Capacity**

If the maximum number of components is resolved with a resolution of 1 between the inactive peak and the terminal peak, this phenomenon is called peak capacity. Higher sample complexity reduces the ability of a given separation method to separate all components.

**MS optimization**

Optimal sensitivity and reproducibility can be achieved with a suitable ion source. The composition of the mobile phase and its flow rate determine the ion source by spray output.[71]

**Sensitivity assessment**

Each analyte concentration has a specific signal-to-noise [S/N] ratio. Sensitivity is dependent on the concentration of each sample and can be concentrated or diluted according to signal-to-noise ratio. An acceptable signal-to-noise ratio of 10 is considered, although lower values may be acceptable depending on the needs of the assay.[71]

**Method Validation**

Validation is performed to minimize the impact of variations on developed methods. This step validates the comprehensive performance of the technology. Performance is validated in terms of precision, precision, linearity, reproducibility, limit of detection [LOD], and limit of quantification [LOQ].[69]

**Advanced Trends in LC-MS**

With recent advances and technologies, LC-MS promises a bright future. Here we discuss some of the current trends in LC-MS.

**Core-Shell Columns**

Core-shell particle columns have particle sizes less than 2 µm. These columns can be used with regular HPLC instruments and require minimal adjustments for better resolution and faster analysis. These columns improved analytical performance without the expense of UHPLC.[73]

**Microcolumn- and nano-liquid chromatography**

Compared to conventional LC, microcolumns and nano LC have proven to support and compete in separation technologies. The inner diameter [ID] of these columns varies from 10 µm to 1.0 mm. Microcolumns are applicable for microbore, microcolumn, capillary, and nanoscale liquid chromatography. Microcolumns have an ID of 0.50–1.0 mm, capillary columns have an ID of 100–500 µm, and nanoscale columns have an ID of 10–100 µm. [74]

**Advanced in monolithic silica columns**

Monolithic columns are packed with continuous porous material compared to traditional beads. These columns can be modified and optimized for different geometric element sizes. Chromatographic separation is through pores, pores and domains through mesopores. These columns are highly permeable and are useful for running chromatography at flow rates as high as 10 mL/min. These columns enable rapid separation of complex mixtures.[75]

**Use of pure water as mobile phase**

Green chromatography can be achieved using pure water as the mobile phase. This can be achieved by changing conditions such as increasing the temperature, changing the composition of the stationary phase, or adding less additive to the mobile phase. Acids or pH-adjusting compounds [buffers] can be added to the mobile phase to achieve selective separation in shorter times.[76]

**Use of drift gas for Low-Vacuum Quadrupole Mass Filter**

In mass spectrometry, ions must have sufficient kinetic energy to reach the detector axially. In a low-vacuum environment, we faced the challenge of providing the necessary kinetic energy. The use of drift gas is popular as a solution in this situation. The drift gas constantly energizes the ions in the quadrupole mass filter, allowing them to be further analyzed in a low-vacuum environment.[77]

**Second-generation hybrid silica stationary phase**

The use of second-generation hybrid silica has opened the way to explore a wide range of experimental designs, including temperature and pH variations. This stationary phase is highly effective for successful impurity profiling in genetic gradient programs, drug discovery, and pharmaceutical analysis. Solvent-based alkaline eluents that could not be used before can now be used as mobile phases. Along with this hybrid silica stationary phase,

stationary phases coated with PGC, type C silica, and zirconia polymers have been shown to be very effective.[78]

## CONCLUSION

Liquid Chromatography-Mass Spectrometry LC-MS has emerged as a powerful analytical tool with a wide range of applications in various fields. Its versatility and precision make it an ideal choice for environmental, biomedical, food, and pharmaceutical analysis. The method development of LC-MS involves goal setting, research, planning, and optimization. The technique has shown promising results in the detection and analysis of complex compounds, including steroid hormones, vitamins, and their related metabolites. The future of LC-MS technology looks promising with the advent of novel techniques and its increasing applications in metabolomics, genetic disorder screening, pharmaceuticals, therapeutic drug monitoring, and toxicology.

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