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Review Article

LC-MS AS A VERSATILE TOOL FOR DRUG DISCOVERY, DEVELOPMENT, AND CLINICAL DIAGNOSTICS

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ABSTRACT

One of the crucial elements in the analysis of biomolecules is liquid chromatography-mass spectrometry (LC-MS), affording unique sensitivity and specificity to a broad spectrum of applications in the discovery of new drugs, proteomics, and metabolomics. Modern biomolecular science has been reshaped by new techniques in LC-MS technology by enabling the potential to deconstruct increasingly complex systems in biological and life sciences research. A highlight of current trends, hot instruments, and advancements, along with modern approaches for improvements in separations and processing the data for advancement in this sector, will also be addressed herein. Combining high-resolution MS (HRMS) and ultra-high-performance liquid chromatography (UHPLC) marks one of the most meaningful changes since this combination produces the best results, both in terms of better resolution and as regards increasing sample throughput. Many of these previous limitations for biological macromolecule analysis are fast being mitigated because improved ionization technologies, particularly in electrospray ionization and matrix-assisted laser desorption/ionization, expand the breadth of possible LC-MS analyses. Recent improvements in multidimensional separation technology and stationary phase diversity increase chromatographic efficiencies, especially to study more intricate biological matrices. Together with strategies to overcome these limitations, this paper also discusses challenges such as matrix effects, sample preparation challenges, and data quality. Researchers can uncover novel information on biomolecular interactions, disease mechanisms, and therapeutic targets by integrating state-of-the-art LC-MS technologies with innovative computational approaches. As LC-MS continues to evolve, it holds immense potential to shape the future of precision medicine and biomolecular research.

Keywords: Liquid chromatography-mass spectrometry, Electrospray ionization, Artificial intelligence and machine learning, Multidimensional separation technology, Biomolecular research

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INTRODUCTION

Being a basic analytical technique of biomolecular research, liquid chromatography-mass spectrometry (LC-MS) has enabled researchers to extensively study the complexity of biological systems. LC-MS possesses superior information content on the molecular composition of biological samples by combining the resolving power of LC separation and the constraining sensitivity and accuracy of MS. This chapter discusses the history, core concepts, and broad applications of LC-MS (Fig. 1) in biomolecular analysis, particularly highlighting how ground-breaking LC-MS has been for modern research [1]. The current application of LC-MS can be linked to concurrent innovations in MS and chromatographic techniques of the 20th century. Ever since its 1900s release, LC has been a routine procedure for the chemical separation of mixtures on the basis of their chemical properties. At the same time, molecular weights could be determined with a very high accuracy as MS, which was invented in the early 1900s, came into play. As a result of the emergence of suitable ionization techniques, atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) in the 1970s, the integration of the two technologies emerged [2].

These advances allowed the integration of LC and MS, which previously prevented the ionization of non-volatile components present in liquid phases, which would be induced by ionizing agents. Technological advances in hardware, software, and data analysis tools have always played a pivotal role in making LC-MS an indispensable tool in biomolecular research over the decades. Complementary techniques are combined to do LC-MS. One sample mixture is homogenized in the LC phase, according to the affinity of the sample to the stationary phase of the chromatographic column and the nature of the mobile phase. At this point, complex mixtures are separated into their components [3]. The mass-to-charge (m/z) ratios of the eluting compounds are measured after they have been ionized and included in the MS.

The LC-MS method consists of a chromatographic system fitted with a sample, usually liquid. In the context of sample fractionation, the mobile phase transports the sample through a column to which a stationary phase is attached. Compounds are separated by columns using a combination of hydrophobicity, size, polarity, and chemical properties. Hydrophilic interaction chromatography (polar species) and reverse-phase chromatography (RP) (non-polar species) are the kinds of applied methods, for example. Compounds are introduced into the mass spectrometer, where they are ionized as they elute from the LC column. Air pressure chemical ionization (APCI) and ESI are commonly applied methods. Those approaches preserve structural information of neutral molecules by transferring them into charged ions without a large fragmentation (Fig. 2). The mass analyzer decomposes the generated ions on the basis of m/z ratios [4]. High-resolution analyzer formats permit measurement of high accuracy mass and detection of molecular species. The mass spectrum is constructed from the detector(s) output, i.e., the counts/number of positive ions per m/z peak. Based on tandem MS (MS/MS), the spectrum gives the structural and molecular mass data of the analyte. Mass spectra are then further analyzed using the power of the software but can be applied as a tool for structuring characterization, identification, and quantification of samples [5]. The fundamentals and operation of LC-MS are of great importance to the underlying lipidomics – science of lipid composition, to proteomics – science of protein identification and post translational

modifications (PTMs), and to metabolomics – science of drugs and small molecule compounds. It is a very popular application in environmental studies, biomarker discovery, and medical research. LC-MS has become a workhorse in biomolecular research (Table 1), combining resolution-based fractionation and sensitivity-based molecular interrogation for informative biology-based discovery and advances in scientific discovery [6].

ROLE OF LC-MS IN BIOMOLECULAR RESEARCH

One of the most widely employed analytic techniques in biomolecular science, LC-MS, possesses the unrivaled sensitivity, specificity, and versatility that are applicable to the analysis of biologically heterogeneous systems [7]. LC-MS is available to systematically study biomolecules across a range of species, from large proteins to small metabolites, based on the complementary strengths of resolution of LC and MS [8]. As an all-purpose application, LCD-MS is currently embedded into fields such as clinical diagnosis, drug development, proteome, metabolomics, and lipidome.

LC-MS in proteomics

LC-MS. LC-MS changed proteomics – the field from which systematic studies of proteins are extracted to become a practice. Identification, quantification, and structural characterization of proteins can be performed by the method, which also allows PTM – phosphorylation, glycosylation, and acetylation – to be conducted. Protein peptides are deconstructed using methods such as tandem MS (MS/MS) which allow for detailed analysis of the amino acid sequence of the peptide. Amplification of both throughput and quantitative accuracy has also been achieved by data-independent acquisition and isobaric labeling, which converted the LC-MS technique into a key device system for elucidating what is happening in cells, disease processes, and therapeutic targets [9].

Metabolomics and lipidomics

Small molecules for cellular metabolism that can be used to influence metabolism through LC-MS in metabolomics are explored. Due to the high sensitivity of the method, a very small concentration of metabolites is detectable, which can be used to identify drug metabolism, disease markers, and metabolic pathways. Analogously, LC-MS based on lipidomics allows the detection and quantification of a variety of lipid species, including triglycerides, phospholipids, and sphingolipids. This is highly relevant to the interpretation of membrane dynamics, lipid metabolism, and the role of lipids in disease such as diabetes and cardiovascular disease [10].

Fig. 1: Shimadzu liquid chromatography-mass spectrometry instrumentation used in biomolecular research

Glycomics and glycoproteomics

Analysis of glycans (glycomics) and glycoproteins (glycoproteomics) have also greatly benefited from the advent of LC-MS. These molecules play one of the major roles in immune response, cell signaling, and pathophysiology. The task of labeling the glycan structures and glycosylation patterns of a single level, in relationship to the research question and also over time, at the individual level, is a valid problem in the field of cancer research, vaccine development, and host-pathogen interactions, and LC-MS are attractive platform to pursue those goals [11].

Applications in drug discovery and development

In pharmaceutical research, LC-MS plays a critical role in drug discovery and development. It is widely used for analyzing the absorption, distribution, metabolism, and excretion (ADME) of drugs, ensuring the efficacy and safety of therapeutic agents. LC-MS also supports pharmacokinetics studies by measuring drug concentrations in biological matrices such as plasma and urine. In addition, it aids in the identification of drug metabolites, the elucidation of metabolic pathways, and the development of bioanalytical assays. It is an essential method of pharmaceutical discovery and development in pharmaceutical research. It is widely adopted in ADME assessment of drugs, which aims at the efficacy and safety of medicinal molecules. Pharmacokinetic studies are also aided by LC–MS, which appears to measure levels of drugs in biological fluids, such as urine and plasma. Furthermore, it can be used to design bioanalytical assays, understand metabolic pathways, and identify drug metabolites [12,13].

Finding biomarkers and clinical diagnostics

LC-MS, a powerful technique in the selection of biomarker candidates for the diagnosis of disease states and physiological conditions, can be used for the identification of disease-related molecular markers. LC-MS offers the ability to detect low-abundance biomarkers by the high sensitivity of LC-MS for complex biological matrices. Monitoring of vitamin hormone levels, newborn screening, and therapeutic drug monitoring can all be performed by use of LC-MS in clinical diagnostics. It is a well-known strategy in personalized medicine, tailoring treatments according to the unique molecular profile of the patient, owing to its accuracy and reproducibility [14].

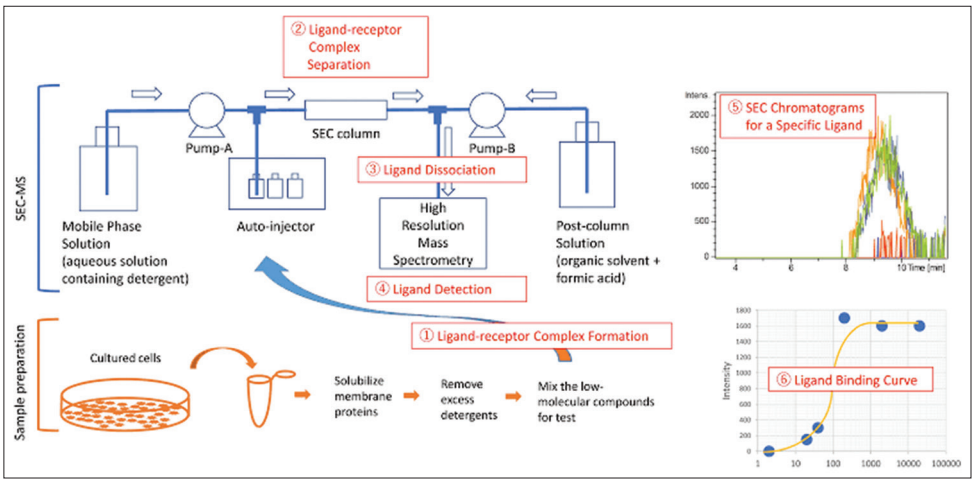

Fig. 2: Schematic representation of the working principle of liquid chromatography-mass spectrometry

DEVELOPMENTS IN BIOMOLECULAR STUDIES USING HIGH-RESOLUTION MASS ANALYZERS

TOF analyzers are widely used in the biological sample processing of small molecules and metabolites for metabolomics. For example, Agilent 6545 Q TOF has been used to conduct cancer metabolomics for finding oncometabolites, which would help us understand tumor metabolism and tumor potential therapeutic targets. One of the most highly resolved and accurately mass-measured high-resolution MS (HRMS) methods is Fourier transform ion cyclotron resonance (FT-ICR) MS (FT-ICR MS) [15]. Ions are immobilized by the force of a high magnetic field, and the signals that arise from the cyclotron motion of the ions are converted into mass spectra. In the lipidomics research field, FT-ICR MS (FT-ICR MS), great at distinguishing slightly shifted isobaric lipids and providing structural over coverage information, can be very powerful. As an example, FT-ICR has been applied to lipidomic studies for the characterization of complex lipid species in brain tissue, which in turn can help to facilitate research into neurodegenerative diseases like Alzheimer's. Multiple mass analyzers are integrated in hybrid systems, which utilize the advantage of each component to realize improved performance. Quadrupole-Orbitrap, quadrupole-TOF, and Orbitrap-FT-ICR setups are a few examples [16]. As deep structural probes, these platforms offer high resolution, high sensitivity, and MS/MS capabilities. Hybrid analyzers are becoming very popular in glycomics and proteomics for the study of PTMs on glycoproteins and peptides. For example, to define glycosylation profiles of antibodies and to enable biopharmaceutical development, Thermo Scientific Fusion Lumos, a quadrupole-Orbitrap hybrid, has been implemented in glycoproteomics [17].

COUPLING MULTI-DIMENSIONAL CHROMATOGRAPHY WITH LC-MS FOR ENHANCED ANALYSIS

A leading strategy for biomolecular analysis, the combination of multidimensional chromatography (MDC) and LC-MS provides an efficient means to increase the separation, resolution, and sensitivity of heterogeneous biological matrices. MDC combines two or more chromatographic methods with different separation processes, such as ion-exchange chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography (HILIC), or RP [38]. Because of the scope of the single-component approaches currently available, the integration of those approaches provided herein satisfies the requirement of a more holistic picture of analyte separation, especially for very complex samples such as proteomes, metabolomes, or lipidomes. As an example, in proteomics, HRMS after two-dimensional liquid chromatography is widely employed in large-scale protein profiling. The very successful peptide separation based on charge and hydrophobicity, enabled by the application of both SCX in the first dimension and RP-LC in the second dimension, offers a proof of principle. This method has played a critical role in PTM profiling and low-abundance protein identification in cancer research [39]. Moreover, a unified perspective of cellular metabolic networks can be derived by starting HILIC and RP-LC metabolomics, in which the former provides more separation of polar and non-polar metabolites, respectively. In addition, MDC-LC-MS has contributed greatly to pharmaceutical research, especially drug metabolism discovery and impurity profiling, in which overcoming the challenge with extensive separation of structurally similar molecules is critical [40]. Structural elucidation capabilities have been dramatically enhanced through the combination of MDC and MS/MS or ion mobility spectrometry (IMS), being able to resolve the isobaric samples. MDC-LC-MS has great promise to play a role in precision medicine, drug discovery, and biomolecular research as it develops [41].

INNOVATIVE IONIZATION METHODS FOR GREATER SENSITIVITY IN LC-MS

Ionization techniques in LC-MS are of fundamental importance as it is by these ionization techniques that the analytes are converted to charged species which can be mass analyzed (Fig. 3). As time goes by, many new inventive methods of ionization have been developed and brought into existence, so the detection limit of an analyte in a complex biosample has been optimized, the sensitivity of detection has been enhanced, and a larger variety of analytic analytes has been achieved [42]. These developments have allowed biomolecular research to undergo dramatic change, and the techniques used in biomolecular research include drug discovery, proteomics, and metabolomics, and so on [43].

ESI

ESI is one of the most frequently used techniques to ionize long and polar macromolecules, such as proteins and peptides, with the capability to ionize long and polar macromolecules, such as proteins and peptides due to its ability to ionize long and polar macromolecules, including proteins and peptides. Sensitivity has been enhanced by recent advances in ESI technology (Fig. 4), i.e., nanospray ionization (nESI) and microspray ionization. Using very low flow rates (at the nanometer level), the advantages of this procedure are that the efficiency of ionization is increased, and fewer samples are required. Specifically, nESI has been used to successfully detect and quantify even very low concentrations of proteins in single-cell proteomics, etc. In the proteomics community, the ESI is widely used for the analysis of PTMs and protein complexes. It has made it possible to detect low-abundance metabolites in biological fluids, such as plasma and cerebrospinal fluid as amino acids and nucleotides, based on metabolomics [44].

APCI

APCI is particularly useful in the analysis of medium-to-nonpolar molecules, non-electrophiles, which are poorly ionized by ESI. It is ionized by a corona discharge at air pressure, producing small fragmented ions. Its spectrum of detectable substances has increased due to recent developments, such as the combination of APCI with ESI (dual ionization). Example: For the characterization of lipid-based drug delivery systems, APCI has found a wide use in pharmaceutical science to study lipophilic drugs. It has also been used in environmental aspects, to identify contaminants and pesticides in water samples [45].

Matrix-assisted laser desorption/ionization (MALDI)

MALDI is a strong ionizing technique that ionizes molecules adsorbed into a crystalline lattice by laser. Recent developments have greatly enhanced spatial resolution and sensitivity, e.g., MALDI-imaging (MALDI-IMS). Applications: MALDI is the method of preference in proteomics for the characterization of intact biomolecules and long proteins. Spatial distribution of proteins and metabolites in tumor tissues, as determined by MALDI-IMS, has been used by cancer researchers to address tumor heterogeneity [46].

Ambient ionization techniques

Without needing much sample preparation, allow direct ionization of materials in their native matrices. These techniques include Direct Analysis in Real Time (DART) and Desorption Electrospray Ionization (DESI). DESI: Real-time analysis can be carried out using charged droplets which are able to release analytes from the sample surface. It has been used in biomolecular studies for lipid analysis and also in forensic sciences for analysis of drug residue on surfaces. DART: Applied to food safety to identify contaminants, such as mycotoxins in grain, DART ionizes analytes using a hot gas [47].

ADVANCES IN MINIATURIZED AND PORTABLE LC-MS TECHNOLOGIES

Miniaturization, portable, and miniaturization of LC-MS technologies have opened the door to perform superior, rapid, and on-site with no need for sophisticated infrastructure, thanks to the revolution of the novel technological instruments developed in the 21st century. LC-MS systems were bulky, cumbersome, and lab-confined in the recent past. However, through advances in ionization, instrument hardware, and microfabrication technology, these have been reduced in size, robustness, and scaled up to be usable by the user [48]. Thanks to these handheld LC-MS instruments, MS has the potential to be applied to a broad spectrum of applications, including the drug development

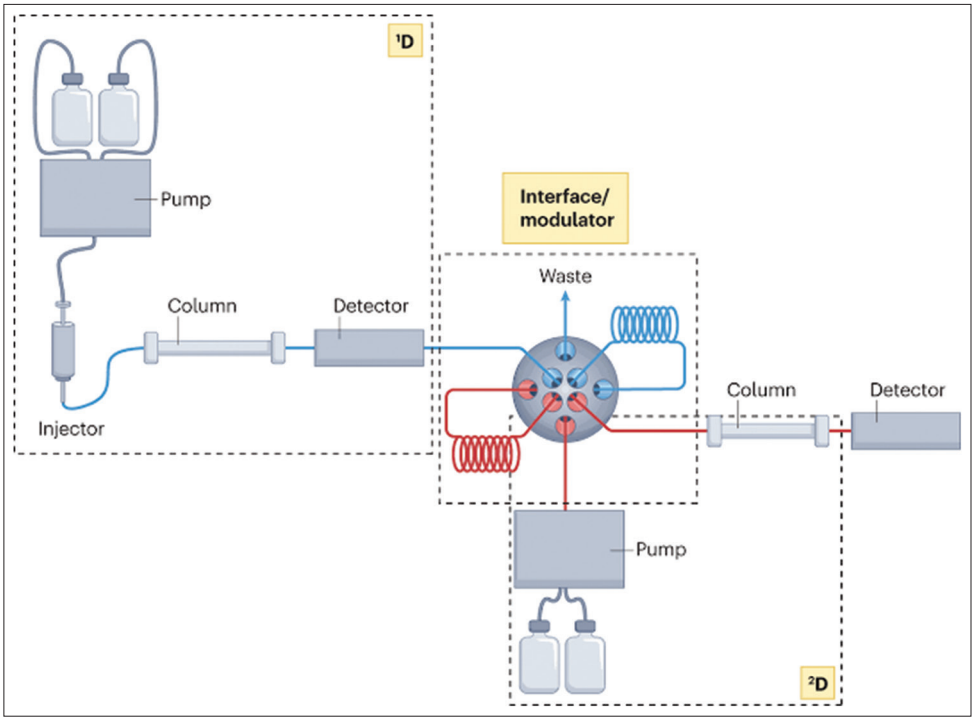

Fig. 3: Illustrates two-dimensional liquid chromatography separation enhancing liquid chromatography-mass spectrometry biomolecular analysis efficiency [42].

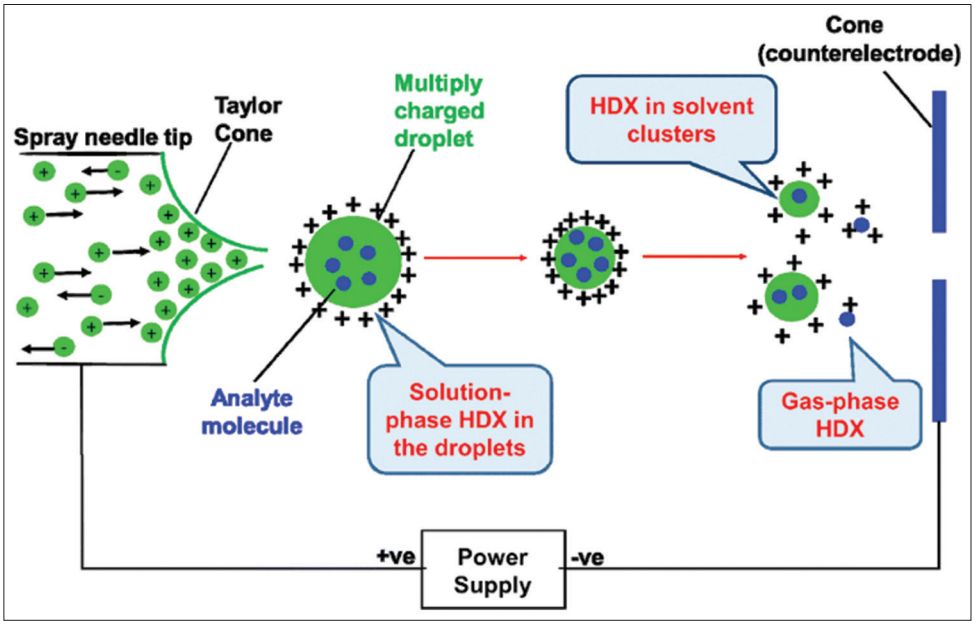

Fig. 4: Electrospray ionization ionizing analytes for detection in liquid chromatography-mass spectrometry workflow

to the clinical diagnosis, food safety to environmental surveillance, and forensics. Microfluidic chips and capillary-based chromatography are often combined into the miniaturized LC-MS systems (Fig. 5) when the sample and reagent consumption are significantly reduced, and the separation efficiency is increased [49]. For example, capillary electrophoresis-MS (CE-MS) miniaturized devices have been deployed for the determination of metabolites in the single cell volume (single or multi-cell samples) or cerebrospinal fluid for the generation of novel insights and for the purpose of personalized therapy.

Sample translocation and laboratory analysis time and cost have been minimized in an environmental science application of portable LC-MS-based instruments to *in situ* analysis of field-spot samples of pesticides, pharmaceuticals, and other contaminants in drinking water [50]. In addition, portable LC-MS systems have also been shown to be used for the detection of contaminants (mycotoxins in the case at hand) in food safety applications and can therefore be used to make on-the-spot real-time processing/farm-based decisions. Likewise, these technologies have also been applied in a clinical diagnostic setting, e.g., field LC-MS portable arrays have been employed for point-of-care testing of infectious and metabolic diseases that capitalized on their potential to analyze biological matrices (e.g., blood and urine) with high speed. Portable LC-MS has been made significantly easier for forensics – drug testing at the point of use, explosive residue analysis,

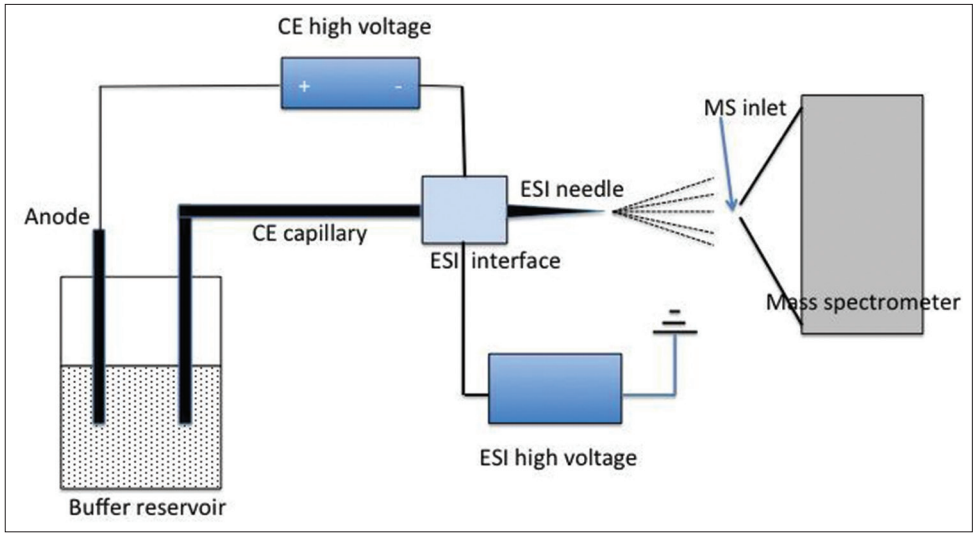

Fig. 5: Depicts capillary electrophoresis-mass spectrometry (MS) enabling high-sensitivity, low-volume portable liquid chromatography-MS analysis

crime scene investigations – with rapid results available to support law enforcement [51].

This real-time data analysis and interpretation have also been achieved by marrying it with artificial intelligence (AI) and machine learning (ML), hence making the systems accessible even to non-experts. Although wireless networking can be exploited to easily wirelessly send the data to stationary databases for analysis, battery-powered and lightweight devices are now ensuring mobility [52]. Portable LC-MS system design is increasingly of interest as an extension of the embedding of environmental considerations into system design, including the use of reusable components and reduction of solvent uptake. At that point in time, these technologies will be advanced enough to affect all sorts of industries by giving real-time high-specificity analytic power that, at the time, will be impossible. LC-MS technologies (micro and hand-held devices) are a means and a platform that gives access to a plus and minus, to novel analytical methods in many applications [53].

LEVERAGING AI AND ML FOR LC-MS DATA INTERPRETATION

The data handling aspect of the integration of AI and ML with LC-MS workflows has been revolutionized in a way that they can now efficiently handle high-dimensional and complex datasets. Traditional LC-MS experiments yield a large volume of spectral and chromatographic data, the manual processing of which is usually very time-consuming, depends on the operator and is subject to variations. Basically, AI and ML-based algorithms have been used to perform almost all the crucial parts of the analysis, such as peak detection, feature extraction, compound identification, and quantification. Thus, they have enormously extended the speed, accuracy, and reproducibility of an analysis [54].

ML techniques such as supervised, unsupervised, and deep learning models have uncovered a remarkable potential to detect very faint patterns in LC-MS datasets which in most cases are ignored by traditional statistical methods. METLIN and Global Natural Products Social Networking are two such resources that facilitate the speedy comparison of the experimental spectra with the curated reference datasets. Thus, annotation confidence in lipidomics, metabolomics, and drug discovery workflows is improved [55]. Besides identification, AI-powered LC-MS platforms have been effectively used in clinical diagnostics for biomarker discovery in infectious diseases, neurological disorders, and oncology. Thus, they are instrumental in personalized medicine strategies [56-59]. Unsupervised learning techniques such as principal component analysis and clustering algorithms help in the accurate classification of experimental conditions and disease states that have been derived from large LC-MS datasets. Reinforcement learning has recently been proposed as a potential solution for the on-the-fly optimization of LC-MS parameters, such as gradient elution profiles and ion source conditions. As a result, adaptive systems that are capable of dynamically enhancing separation efficiency and data quality during acquisition are being developed [60].

ENHANCED SAMPLE PREPARATION TECHNIQUES FOR COMPLEX BIOLOGICAL SAMPLES

Sample preparation is a critical procedure in LC-MS assays, particularly for analyzing complex biological matrices such as blood, plasma, urine, tissue, or cell lysates. However, interfering compounds, in the form of proteins, lipids, salts, and metabolites, are ubiquitous in these matrices, i.e., such agents can help to increase the analytic complexity, decrease the sensitivity, and the quality of the solution. To get trustworthy and high-quality LC-MS analysis, the following recent developments in sample preparation procedures enabled the researcher to efficiently extract, purify, and concentrate the analytes of interest. These advances are driving the applications which require reliable identification and quantification, including proteomics, metabolomics, lipidomics, and clinical diagnostics [61].

Microextraction techniques

The evolution of microextraction techniques – liquid-phase microextraction (LPME) and solid-phase microextraction (SPME) – are one of the most significant advances. Those approaches show high recovery and selectivity and low sample amounts. An example of SPME is that it does not require sophisticated pre-treatment, since it enables the *in situ* analyte extraction from intricate matrices by a fiber that is functionalized with an extractant phase. This technique for small-molecule plasma and saliva metabolite determination has been applied to clinical diagnosis and environmental sensing. Analytes also may be rapidly and effectively extracted by LPME technologies, e.g., dispersive liquid–liquid microextraction, in particular in the metabonomic investigations of fatty acids and low-molecular-weight metabolites [62].

Protein precipitation

Despite the fact that protein precipitation remains a popular method for the isolation of proteins from biological material, developments have enhanced its efficiency. Examples are just two of the advances, including the use of automated methods and new precipitation agents that not only decrease the amount of analyte co-precipitated but also can be mobilized for subsequent analysis. For example, the optimized, optimized, optimized protein precipitation methods allow correct

Table 1: Various novel instruments and advancements in LC-MS techniques that are transforming biomolecular research

S. no	Technology	Purpose	Method	Application	Limitations	References
1	Orbitar MS	High resolution and mass accuracy	LC-MS with Orbitrap	Proteomics and metabolomic profiling in biomarker	High instrument cost, slower scan speed at ultra-high resolution, complex data processing	Geiger *et al.*, Mol Cell Proteomics, 2010. [18]
2	Time-of-Flight (TOF) mass spectrometer	Ultra-fast acquisition rates	LC-MS with TOF	Metabolite identification in complex biological matrices	Lower mass accuracy compared to Orbitrap, sensitivity trade-off at high speed	Bhagwat *et al.*, TrAC Trends Anal Chem, 2011. [19]
3	Ion mobility spectrometry (IMS)	Enhanced separation of biomolecules	LC-MS coupled with IMS	Lipidomics studies to resolve isomeric lipid species	Increased system complexity, added data interpretation burden	Köfeler *et al.*, J Lipid Res, 2021 [20].
4	MALDI-LC-MS	Spatial biomolecular distribution analysis	Matrix- assisted laser desorption/ ionization	Tissue-based spatial omics studies	Limited quantitative accuracy, matrix interference effects	Zhang *et al.*, npj Imaging, 2024 [21].
5	Quadrupole-orbitrap hybrid system	Improved sensitivity and dynamic range	LC-MS/MS with hybrid analyzer	Single-cell proteomics for cellular heterogeneity analysis	High acquisition time, costly instrumentation	Bekker-Jensen *et al.*, Mol Cell Proteomics, 2020 [22].
6	Nano-LC-MS	Nanoscale analysis of biomolecules	Nano-scale liquid chromatography	Single-cell metabolomics and low-abundance analyte detection	Low robustness, susceptibility to clogging, reduced throughput	Zhang *et al.*, Curr OpinBiotechnol, 2023 [23]
7	High-field asymmetric waveform IMS	Advanced ion filtering	LC-MS integrated with FAIMS	Separation of co-eluting compounds in proteomics	Ion transmission losses, additional method optimization required	Hale *et al.*, Anal Chem, 2020 [24].
8	Tandem mass tag-LC-MS	High-throughput quantification	Multiplexed LC-MS/MS	Disease progression analysis in proteomic studies	Ratio compression, batch effects	Thompson *et al.*, Anal Chem, 2003 [25].
9	Ambient ionization LC-MS	Real-time sample analysis	On-site ionization without sample preparation	Field detection of metabolites in clinical and environmental samples	Reduced sensitivity and reproducibility, matrix effects	Ferreira *et al.*, Clin Chem, 2016 [26].
10	Fourier MS	Ultra-high resolution for large biomolecules	LC-MS with FT-MS	Structural analysis of proteins and protein complexes	Extremely high cost, slow scan speed, specialized infrastructure	Kong *et al.*, Acta BiochimBiophys Sin, 2007 [27].
11	Miniaturized portable LC-MS	Compact, portable analysis systems	Miniaturized LC-MS instruments	On-site environmental sample analysis and clinical diagnostics	Limited resolution and sensitivity compared to benchtop systems	Vargas Medina *et al.*, TrAC Trends Anal Chem, 2020 [28].
12	AI-integrated LC-MS	Automated data processing	Machine learning-enabled LC-MS	Interpretation of large proteomic and metabolomic datasets	Model transparency, risk of algorithm bias, need for curated training data	Zhang *et al.*, Trends Anal Chem, 2024 [29]
13	Ultra-high-performance liquid chromatography-MS	Improved chromatographic separation	Ultra-high-pressure liquid chromatography	Analysis of complex biological samples for biomarker	High backpressure, increased system wear	Zhao and Li, TrAC Trends Anal Chem, 2014 [30].
14	Multi-dimensional LC-MS	Comprehensive biomolecule profiling	Advanced chromatographic separation techniques	Profiling metabolites and lipids	Long analysis times, complex method development	van den Hurk *et al.*, TrAC Trends Anal Chem, 2023 [31].
15	Electro-spray ionization (ESI)	Enhanced ionization efficiency	LC-MS with ESI	Sensitive detection of small biomolecules in metabolomics	Ion suppression, sensitivity to matrix composition	Ho *et al.*, Clin Biochem Rev, 2003 [32].
16	Desorption electrospray ionization	Real-time tissue analysis	Ambient ionization without matrix preparation	Imaging MS for tissue-based biomolecular studies	Lower spatial resolution compared to MALDI, limited depth profiling	Nielen *et al.*, TrACTrends Anal Chem, 2011[33].
17	Ion trap LC-MS	Improved structural analysis	LC-MS with ion trap analyzers	Elucidation of post-translational modifications in proteomics	Limited mass accuracy, space-charge effects	Bueno *et al.*, Anal Chem, 2007 [34].
18	Hybrid (Q-TOF)	High sensitivity and resolution	LC-MS/MS with Q-TOF analyzers	Lipidomics and metabolomics in drug development	Resolution lower than Orbitrap, calibration drift	Ens and Standing, Methods Enzymol, 2005 [35].
19	Nano-ESI LC-MS	Ultra-low sample volume detection	Nano- electrospray ionization in LC-MS	Single-cell analysis and rare biomolecule detection	Poor spray stability, limited robustness	Gaspari and Cuda, Methods Mol Biol, 2011 [36]
20	Hydrogen-deuterium exchange-LC-MS	Protein folding and interaction dynamics	LC-MS with hydrogen-deuterium exchange	Studying the structural dynamics of proteins	Back-exchange, complex data interpretation	Ozohanics and Ambrus, Life (Basel), 2020 [37].

LC-MS: Liquid chromatography-mass spectrometry, MALDI: Matrix-assisted laser desorption/ionization, FAIMS: Field asymmetric waveform ion mobility spectrometry

quantification of drug metabolites in plasma samples, which can be found in popular pharmacokinetic studies [63].

Solid phase extraction (SPE)

Due to its excellent selectivity and applicability, SPE is still one of the most frequently used methods in sample preparation. Selectivity and recovery have been enhanced due to the development of SPE materials such as magnetic nanoparticles and molecularly imprinted polymers (MIPs). The MIPs for certain molecular structures are also especially useful for the discrimination of the target analytes from some complex mixtures of hormones or pesticides. Magnetic nanoparticles, functionalized with a selected ligand, can be rapidly and efficiently washed out of biomolecules (proteins and nucleic acids) from cell lysates and tissue [64].

Ultra-filtration and size exclusion

Another exciting innovation in the use of more powerful filtration techniques, which is based on the separation of analytes, using size-exclusion and ultrafiltration techniques which depend on separating analytes by molecular size, is still underway. Ultrafiltration is a relatively innocuous technique for the removal of the prevalent proteins (e.g., albumin) which thereby increases the relative ease of removing the more low-abundance biomarkers. This approach, coupled with LC-MS, has provided the means to identify disease-relevant proteins in the cancer and neurological areas [65].

Liquid–liquid extraction (LLE)

Improved LLE has also been enabled by more environmentally friendly solvents and by automated systems. To still improve extraction efficiency and minimize their environmental impact, ionic liquids and deep eutectic solvents have recently also been investigated as alternatives to classical organic solvents. Automated LLE systems improve throughput and repeatability, especially when hundreds of clinical samples are used in extensive metabolomic research [66].

Microfluidics

Sample preparation with microfluidic devices is another novel approach. Laboratory-on-a-chip systems that combine cell lysis, analyte extraction, and purification, in one step, are presented here. Through single-cell proteomics, microfluidics are used to capture and microscale analyze cell-to-cell differing proteins for biological diversity and heterogeneity [67].

Dual-phase extraction

Improved recovery of other lipid species including neutral and polar lipids by way of dual-phase extraction and other refined sample preparation procedures has been a major advance in the lipidomics community. This method has been very helpful in the study of lipid metabolism and its significance in diseases such as diabetes and cardiovascular diseases [68].

UTILIZING LC-MS IN SINGLE-CELL AND SPATIAL OMICS STUDIES

LC-MS is a leading-edge analytical platform that has revolutionized single-cell and spatial omics studies in detail. It helps analyze cellular heterogeneity and the spatial arrangement of biomolecules in tissues. Typically, bulk analytical methods mask biologically significant changes by averaging the signals from heterogeneous cell populations. On the other hand, LC-MS provides all the necessary requirements to identify proteins, metabolites, and lipids up to single-cell resolution and in a defined tissue microenvironment, i.e., it has the required sensitivity, specificity, and molecular breadth [69]. Innovations such as nanoscale LC-MS instrumentation, microfluidic sample handling, and CE coupled with MS, have made it possible to detect biomolecules that are in very low quantities and that originate from single cells in a most reliable manner. These developments have opened a wide avenue for research and have driven significant progress in diverse fields such as cancer biology, immunology, neurology, and developmental biology, where cellular diversity and spatial context not only affect function but also disease progression. For instance, single-cell proteomics through LC-MS has helped differentiate tumor cells from the rest of the stroma in heterogeneous cancer tissues, which in turn leads to the discovery of disease-specific biomarkers and therapeutic targets and their subsequent identification [70]. Likewise, single-cell metabolomics have contributed to the identification of metabolic reprogramming that accompanies immune cell activation and exhaustion, thus giving significant insights that are applicable in the field of immunotherapy.

Further, LC-MS spatial omics utility has grown through its association with laser capture microdissection, allowing for molecular profiling of tissues at the regional level. This strategy has been a major component in the understanding of the origins of different kinds of metabolic changes in diseases, such as the formation of amyloid plaques in Alzheimer's disease [71,72] and metabolic dysregulation in diabetic nephropathy that is localized to specific regions of the body [73-75]. Besides that, isotope-labeling techniques like tandem mass tag labeling have boosted multiplexed spatial proteomics [76,77], thereby enabling quantitative protein expression comparisons between different tissue regions [78-80]. The increasing complexity of single-cell and spatial LC-MS datasets has prompted the use of AI and ML-based analytical methods for data interpretation. ML algorithms have been successfully employed to correlate molecular distributions with histopathological features, mainly in cancer, thus enabling prognostic biomarker discovery and patient stratification [81,82]. While there are still challenges in throughput, spatial resolution, and sample preparation, improvements in ultra-high-performance LC-MS, nESI, automation, and microfluidics are gradually making sensitivity, reproducibility, and scalability better [83,84]. Essentially, LC-MS-based single-cell and spatial omics technologies are redefining molecular systems' hidden levels and the basis of diseases that were previously inaccessible [85].

FUTURE ASPECTS OF LC-MS AND ITS ADVANCEMENT IN BIOMOLECULAR RESEARCH

The future path of LC-MS is less and less characterized by small improvements in sensitivity or resolution and more and more by disruptive innovations that change the way biomolecular data are generated, interpreted, and used [86,87]. A major emerging theme is the instrumentalization of LC-MS systems that can adapt and make decisions autonomously instead of relying on static acquisition workflows [88]. Such systems perform on-the-fly data analysis and employ AI to decide on-the-fly changes in the acquisition settings such as chromatographic gradients, collision energies, or ion transmission without interrupting the measurement [89]. By contrast to standard protocols that require a predefined acquisition schemes, adaptive LC-MS intends to exploit the sample-specific information to the utmost, which is especially the case for heterogeneous or low-abundance biomolecules [90].

Further ahead and also less clearly defined, there is the concept of application-controlled multi-dimensional separations where LC is considered just one of many interacting elements in a self-optimizing analytical ecosystem rather than the fixed front-end. Closed-loop combinations of LC, IMS, and high-resolution MS potentially open the way toward on-the-fly selection of the most structurally informative ions to be studied further. Whereas LC–IMS–MS systems at present mainly provide enhanced isomeric resolution, their potential future use is in releasing analytical power selectively for chemically ambiguous species thus solving the major inefficiency of current untargeted workflows in which most of the depth of analysis remains unused [91]. Miniature LC-MS technologies are likewise moving past simply portable devices to context-aware analytical deployment. Some of the newly developed microfabricated ion sources, chip-based separations, and vacuum-efficient mass analyzers may in fact be the first steps toward the creation of situational LC-MS platforms capable of operating in clinical wards, environmental field sites, or manufacturing environments. Such a shift, however, brings in critical trade-offs, especially those between robustness and analytical depth [92]. Research in the future has to find a way to separate reduced separation path lengths and ion transmission

losses from the demand for sensitivity that is clinically actionable, particularly in the case of trace-level biomarkers. In biomolecular research, single-cell LC-MS is set to move from merely demonstrating concepts to making discoveries based on the formulation of new hypotheses

Without such developments, the field of single-cell LC-MS may continue to be simply descriptive rather than being predictive in biological interpretation. One more significant, although less examined, direction is the use of LC-MS for predictive biomolecular modeling. When integrated with ML frameworks trained on longitudinal datasets, LC-MS can no longer be seen merely as an analytical tool for the past but rather as a system that looks into the future and is able to predict metabolic fluxes, drug responses, or disease trajectories [93]. Such a fundamental change in paradigm would very much alter the current drug discovery processes by allowing for the prioritization of experiments guided *in silico* and not by exhaustive empirical screening [94]. Nevertheless, innovations in LC-MS that are to be realized in the future are hindered by structural constraints [95]. The planned system upgrades which will make them more complex may lead to problems with reproducibility, acceptance by the regulatory authorities, and comparability of results across different laboratories. In addition, AI-driven systems raise issues related to the transparency of algorithms, potential biases in data, and difficulties in interpretation – the problems that, among others, are of paramount importance in clinical and regulatory contexts [96]. Tackling these problems will necessitate the establishment of standards across the community, development of explainable AI frameworks, and benchmarking strategies that are not limited to instrument performance but also include decision-making reliability [97].

COMPARATIVE ADVANTAGES AND LIMITATIONS OF LC-MS TECHNOLOGY IN BIOMOLECULAR RESEARCH

The extensive diversification of LC-MS platforms within the last few years has been a game-changer for biomolecular research. However, it is becoming increasingly difficult to select an optimal system, as this decision mainly depends on trading off between different analytical parameters rather than simply maximizing one single performance metric [98].

SENSITIVITY AND THROUGHPUT

Coupling nano-LC with high-resolution mass analyzers, such as Orbitrap or ion trap, provides outstanding sensitivity, thus allowing the detection of peptides, metabolites, and PTMs in very low-abundance [99]. Such sensitivity is extremely useful, for example, in single-cell proteomics or in the very early stages of biomarker discovery, where the availability of the sample is highly limited. On the downside, these systems are typically less robust and have lower throughput because of long chromatographic gradients, clogging susceptibility, and increased maintenance requirements. Whereas, the UHPLC-Q-TOF or triple quadrupole (QqQ) systems operating at higher flow rates can still offer a higher sample turnover rate and better quantitative reproducibility [100]. Thus, they are more appropriate for large-scale screening, pharmacokinetic studies, and clinical assays, but at the same time, the sensitivity for ultra-low abundance analytes is compromised.

RESOLUTION VERSUS SCAN SPEED

Resolution versus scan speed is another major factor that influences the analytical strategy. To cite an example, Orbitrap and FT-ICR instruments, being high-resolution mass analyzers, provide the highest mass accuracy and resolving power by which one can easily distinguish isobaric species and complex molecular mixtures [101]. Such features are absolutely necessary for untargeted metabolomics, lipidomics, and proteogenomics [102]. Although ultra-high resolution is usually accompanied by reduced scan speeds, the number of data points across narrow chromatographic peaks which are particularly in fast UHPLC separations can be limited. TOF and hybrid Q-TOF instruments allow a feasible compromise by offering moderate to high resolution with the rapid acquisition rates, thus, profiling can be done exhaustively without chromatographic fidelity being sacrificed. Hence, the choice of the platform should depend on what the dominant analytical priority is molecular specificity or temporal resolution [103,104].

COST, OPERATIONAL, AND ACCESSIBILITY COMPLEXITY

From the point of view of cost, operational complexity, and accessibility, LC-MS technologies are quite different from one another in terms of the degree of heterogeneity they display [105]. On the one hand, advanced high-resolution platforms demand a large amount of capital for their acquisition, require a specially designed facility, and a highly skilled workforce; thus, they may be inaccessible to laboratories in resource-poor settings [106]. Moreover, data processing and interpretation for untargeted, high-resolution datasets require advanced computational tools and experts in the field [107]. On the other hand, QqQ LC-MS systems, which are the major instruments in regulated bioanalysis, are characterized by low operational complexity, easy-to-follow standardized workflows, and good robustness [108]. While they cannot boast of being discovery-oriented and having the breadth of high-resolution systems, their relative affordability and easy method transfer make them accessible to drug development and clinical diagnostics laboratories for routine work [109].

CONCLUSION

The emergence of LC-MS technology will undoubtedly have a direct impact on the trajectory of biomolecular research. Analysis of the complexity of biomolecules in health and diseases will require even more LC-MS when limited to analytical platforms, computational algorithms, and sample preparation methods. The unparalleled sensitivity, specificity, and versatility make it valuable for investigating novel biological levels, ranging from single-cell analysis over systems biology to precision medicine. Single and spatial omics are two of the most exciting fields in which LC-MS will be able to make an impact. LC-MS will allow scientists to characterize cellular heterogeneity and complex molecular networks at unprecedented resolution – by analyzing protein, metabolite, and lipid levels at single-cell resolution. These skills are important focal points for research in immune responses, cancer microenvironments, and development biology.

Single-cell studies will become more common and accessible to date as the instrumentation side advances and nanoscale LC-MS array and ultra-high-resolution mass analyzer, for instance, enable basic and applied researchers with new insights. Improvements in LC-MS will be viewed as a tool by spatial omics, mapping biomolecules within the morphology of tissues. Spatial resolution and ability to detect higher quantities will be provided by methods, including imaging MS (e.g., MALDI-LC-MS), and protein, lipid, and metabolite molecules will be mapped accurately to tissues. Enhanced tissue-specific mechanisms of diagnostic and therapeutic interventions, such as neurodegeneration and tumor growth, will be obtained from expanded knowledge of our current state of knowledge. A resurgence of this field to change biomolecular research is the combination of LC-MS with AI and ML.

LC-MS can generate large-scale data which can be processed by these kinds of technologies and identify a link/pattern that is not only difficult to discern by humans. AI-enabled LC-MS techniques are exploiting data extraction and interpretation automation and will drive drug discovery, biomarker discovery, and personalized medicine exponentially. These developments will extend the scope and cost-effectiveness of LC-MS to a global level and thereby will make LC-MS accessible to a worldwide network of research laboratories. Furthermore, LC-MS will still be playing a crucial role in multimodal omics. Combined use of LC-MS and genomes, transcriptomics, and epigenomics will make a comprehensive analysis of biological systems achievable. This will demonstrate how many biomolecular layers have to interact to modulate cellular functions as well as disease processes. In fields such as synthetic biology and systems biology, this systems-based approach will be of paramount value. The application of LC-MS

for disease tracking, biomarker discovery, and precision medicine will continue to grow with technology which will change the life sciences and medicine. LC-MS will continue to be at the vanguard of scientific innovation, influencing the direction of research and medicine by pushing the limits of biomolecular analysis.

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AUTHORS CONTRIBUTIONS

Saravanan Ravindran: Contributed to conceptualization, literature synthesis, and initial drafting. Performed formal analysis of included studies and critical evaluation, resource curation, and data validation. Rajaganapathy Kaliyapermal: Supervised the research design and finalized the manuscript. Shaik Meharaj: Reviewed, edited, and refined the intellectual content. All authors approved the final version and agreed to accountability for the work.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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