APPLICATIONS OF MODERN MASS SPECTROMETRY

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Applications of Modern Mass Spectrometry

(Volume 2)

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PREFACE

Mass spectrometry is a unique analytical tool that offers unmatched sensitivity and selectivity levels for a wide range of analyses. The most recent applications of mass spectrometry are mostly oriented toward biochemical problems, such as proteomes, metabolomes, high-throughput drug discovery, and metabolism. Other analytical applications are routinely applied in pollution control, food control, forensic science, natural products or process monitoring, and many others.

The present volume of *"Application of Mass Spectrometry"* provides a useful insight into some of these developments. The present 2nd volume of this book series comprises 6 comprehensive reviews written by the leading practitioners of mass spectrometry. These articles present diverse applications of mass spectrometry in fields such as proteomics, peptidomics, drug development and discovery, toxicology, and environmental analysis. Moreover, the use of advanced ionization techniques, i.e., ion mobility, particularly in the analyses of macromolecules, is also discussed in this volume.

Mehmet Atakay *et al*. have discussed the use of an advanced mass spectrometry approach, ion mobility spectrometry (IM-MS), in the field of macromolecule analysis, such as proteomics, glycoproteomics, and polymer characterization. Sarah Otun *et al*. have described the use of different mass spectrometric approaches and tools in the understanding of structural proteomics. Neva Alasağ *et al*. have elaborated on the use of liquid chromatography-mass spectrometry (LC-MS) as a powerful analytical technique for separating and quantifying endogenous neuropeptides in the central nervous system (CNS) and organisms. Louis P. Sandjo *et al*. have focused on the separation and detection of toxic plant-based metabolites in tropical medicinal and edible plants. Shweta Sharma has reviewed the use of mass spectrometry in various stages of the drug discovery and development process, including target identification, hit identification, lead optimization, and drug metabolism and pharmacokinetic studies. Imalka Munaweera *et al*. highlighted the qualitative and quantitative detection of a diverse range of organic contaminants in environmental samples utilizing advanced mass spectrometry.

We are grateful to all the authors for their excellent scholarly contributions and for the timely submissions of their review articles. We would also like to express our gratitude to Mrs. Fariya Zulfiqar (Manager Publications) and Mr. Mahmood Alam (Director Publications) of Bentham Science Publishers for the timely completion of the volume in hand. We sincerely hope that the efforts of the authors and the production team will help readers better understand and appreciate the versatility and robustness of mass spectrometry and motivate them to conduct good-quality research and development work in this exciting area.

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CHAPTER 1

Ion Mobility-Mass Spectrometry for Macromolecule Analysis

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Abstract: The need for conformational information is increasing by the time in studies on macromolecules. For example, proteins may have various functions and properties depending on their folding states that make their conformational analyses very important. Mass spectrometry is one of the most effective analytical techniques that separate ions in the gas phase by their mass-to-charge ratio. It provides useful data on molecular characterization in many areas of research with high precision, accuracy, and sensitivity. Although mass spectrometry is a very powerful analytical technique, it cannot distinguish different species having identical mass-to-charge ratio. The analytical technique combining mass spectrometry with ion mobility spectrometry (IM-MS), which provides information about the three-dimensional structure of an ion, solves this problem by separating them according to their collision cross sections (CCS) in the gas phase. This analytical method also provides the advantages of higher precision and better resolution in the rapid analysis of different types of complex samples. The separation of isomers with the same molecular weight, increasing the dynamic range and distinguishing ions from chemical noise are the most important features that this technique contributes to mass spectrometry. As improvements have been made in IM-MS technology, the number and quality of publications in the areas where this technique is used increases rapidly. In this chapter, the use of IM-MS techniques in the fields such as proteomics, glycoproteomics and polymer characterization are explained by presenting their various applications in the literature.

Keywords: Conformational Characterization, Glycoproteomics, Ion Mobility-Mass Spectrometry, Proteomics, Polymer Characterization.

INTRODUCTION

The status and use of ion mobility-mass spectrometry (IM-MS) in various research areas have been increasing rapidly in recent years [1]. The interest in this analytical method is increasing in parallel with the improvements in the parameters such as high sensitivity, resolution power and low amount of sample

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in the analysis with the developing technology. The basics of ion mobility spectrometry are similar to the principles of mass spectrometric techniques. Thus, these two techniques can be easily used together in a combined system. Modern IM-MS techniques having high ion mobility resolution, sensitivity and applicability for a wide variety of samples started to be developed in the 1990s. These developments coincide with the period when ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) were first used in mass spectrometric analysis [2 - 4]. In 2006, a company manufacturing mass spectrometers combined ion mobility and mass spectrometry techniques in an instrument and launched it on the market. Since then, the use of ion mobility technology has become increasingly common in various research areas for differentiation, identification, and structural analysis of species [5]. Today, commercially available IM-MS instruments having different ion mobility technology provided by different manufacturers are used in studies [1].

Ion mobility spectrometry is still widely used alone in defense, security and environmental analysis applications [6 - 8]. Over the past two decades, very rapid and significant advances have been made in the development of systems in which ion mobility and mass spectrometry are combined. These advances are particularly concerned with the ability to trap, transmit and focus ions between regions under different vacuum values using electrodynamic fields. Considering the very important analytical advantages provided by IM-MS instruments, ion mobility technique has become a preferable option especially in studies carried out in the "omics" fields [9]. IM-MS technique, which has an important place in various research fields, has become an analytical system that is also sought after and interested in several applications performed on the industrial scale.

Ion mobility spectrometers simply measure the time spent by ions as they travel through an electrical field with the help of a buffer gas. Ion mobility (K), collision cross section (Ω), the Boltzmann constant (k_b), neutral number density (N), mass of ion (m_1), mass of buffer gas (m_N), charge of ion (z), and electronic charge (e, 1.602 x 10^{-19} C) is defined by the Mason-Champ equation [10, 11].

$$K = \frac{\sqrt{18\pi}}{16} \frac{ze}{\sqrt{k_B T}} \sqrt{\left(\frac{1}{m_1} + \frac{1}{m_N}\right)} \frac{1}{N} \cdot \frac{1}{\Omega} \quad \textbf{(1)}$$

The collision cross section (CCS) value of an ion can provide detailed information about its size and shape. When two different molecules having identical mass-to-charge ratios are analyzed using IM-MS, they can be separated from each other

according to their mobility in the ion mobility cell depending on their shape and size characteristics. The arrival time data obtained from the acquisition with an IM-MS device should be converted to the CCS values of the analyzed species according to the performed calibration calculations using standard molecules with known CCS values. There are free software and databases that can be helpful in such CCS calibration and estimation processes. A list of common CCS databases and software used in CCS calibration, prediction, and estimation with their URLs are given in Table **1**.

The collision cross section of an ion depending on its mobility and the type of buffer gas used can also be calculated by appropriate computational methods. Thus, the theoretical CCS values can be compared with the experimental values obtained from IM-MS analyses. The theoretical average CCS values are determined by taking into account the collisions of ions with the buffer gas in the ion mobility cell by the computational simulation of analysis conditions [12]. In structural biology studies, the conformations of biomolecules or their complexes are investigated by comparing the experimental CCS values with data obtained from theoretical calculation [13].

Table 1. List of common CCS databases and software.

CCS Database / Software	Description	URL	Refs.
MOBCAL	calculating CCS values implementing the Projection Approximation, Exact Hard Spheres Scattering model, and MD Calculations	https://nano.lab.indiana.edu/software/	[14, 15]
GlycoMob	CCS database for glycomics	http://www.glycomob.org/	[16]
ISiCLE	simulating CCS values, NMR chemical shifts conformers	https://github.com/pnnl/isicle	[17, 18]
MetCCS	predicting CCS values of metabolites	http://www.metabolomics-shanghai.org/MetCCS/	[19]
LipidCCS	predicting of CCS values for lipids	http://www.metabolomics-shanghai.org/LipidCCS/	[20]
Bush Lab Collision Cross Section database	presenting CCS of small molecules, peptides, denatured proteins, native-like proteins, and native-like protein complexes	http://depts.washington.edu/bushlab/ccsdatabase/	[21 - 28]
Clemmer Group Cross Section Database	presenting CCS of peptides, proteins, and oligonucleotides	https://clemlab.sitehost.iu.edu/Research/Cross Section%20Database/cs_database.php	[29 - 38]
Unified CCS Compendium	interactive repository of experimentally acquired CCS values of molecular standards and classes	https://mcleanresearchgroup.shinyapps.io/ CCS-Compendium/	[39]
CCSbase	presenting CCS values of lipids, water-soluble metabolites, small molecules, drugs, *etc.*	https://ccsbase.net/	[40]
PIXiE	extracting arrival times by drift tube ion mobility spectrometry and calculating the associated CCSs	https://github.com/PNNL-Comp-Mass-Spec/PIXiE	[41]
IMPACT	calculating CCSs of proteins in structural biology and proteomics applications	https://process.innovation.ox.ac.uk/software	[42]
EM∩IM	estimation of CCS from electron microscopy density maps	http://emnim.chem.ox.ac.uk/	[43]
PNNL Collision Cross Section Database	CCS database for metabolites	https://metabolomics.pnnl.gov/	[44]

CHAPTER 2

Recent Advancements in the Detection of Organic Contaminants in Wastewater Using Advanced Mass Spectrometry

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Abstract: With the increase of industrialization and urbanization, pollution of clean water has become a critical issue in the contemporary world. Despite organic pollutants such as pharmaceuticals, pesticides, industrial chemicals, poly- and per-fluoroalkyl substances (PFASs) and hormones, contaminants originating from the industrial effluents, urban run-offs, agricultural run-offs and domestic sewage have become a greater threat to the aquatic eco-systems. The availability of some of these highly potent contaminants at low concentrations and the simultaneous analysis of multiple samples have been identified as the major concerns in current analytical methods in water pollution analysis. In this regard, modern mass spectrometric methods have emerged as suitable techniques for the analysis of smallest concentrations even at a level of nanograms or femtograms while allowing the detection of hundreds of analytes in a single analysis within a short duration of time.

Recently, combinational mass spectrometric analysis has become the state of the art in several qualitative and quantitative analyses of organic pollutants in water. The sensitivity of the detection has been enhanced by coupling with various sample extraction methods, chromatographic techniques and different mass analyzers in mass spectrometry. Utilization of modern sample extraction methods coupled with mass analyzers has facilitated the accuracy of the detection of organic pollutants in water samples. Sample extraction methods involve sophisticated solid-phase extraction, solid-phase microextraction, and liquid-liquid extraction methods, whereas mass analyzers include time-of-flight, orbitrap, ion-trap and triple quadrupole, *etc.* The hallmark of these hyphenated techniques is the ability of allowing the screening of targeted analytes, non-targeted analytes and suspect analytes without the need of authentic standards. This chapter will focus on the recent advancement of mass spectrometry in qualitative and quantitative analysis of several organic contaminants in wastewater samples.

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Keywords: Emerging Contaminants, PFAs, Hyphenated Techniques, Industrial Chemicals, Mass Spectrometry, Non-target Analysis, Organic Pollutants, Pesticides, Pharmaceuticals, Recent Advancements, Sample Extraction, Target Analysis, Wastewater Analysis.

INTRODUCTION

Contamination of water resources is one of the most serious issues that must be addressed in order to preserve the ecosystem and ensure its long-term viability. Currently, the use of enormous numbers of different chemicals all over the world, has a huge impact on the ecosystems due to their unavoidable intrusion. Any chemical that could cause adverse effects to the ecological systems or to human health and also frequently detecting in increasing concentration in the environment but yet not being monitored by the established environmental monitoring programs is referred to as an emerging contaminant (EC) [1, 2] and the existence of such compounds in the environment could even be permanent due their constant input from various unregulated sources. Since the acquisition, consumption, and usage of such contaminants are not governed by any legislation, the elimination of these substances in natural sources *via* treatment plants has not been prioritized. In this regard, pharmaceuticals, personal care products, hormones, per-fluorinated compounds, flame retardants, endocrine disruptors, pesticide, plasticizers, impurities from commercial formulations and surfactants, are all considered ECs that could pose a threat to environmental ecosystems [3].

To acquire early identification and exact quantification of every component capable of compromising ecosystems, and global health integrity, a comprehensive examination of environmental contamination necessitates persistent innovation in technology and analytical methodologies [4]. In this sense, mass spectrometry (MS) stands to be the most effective approach, since it has demonstrated constant and massive growth in concept, apparatus, and implementations since its invention [5 - 7]. MS is distinguished by its high sensitivity, high resolution, quantitative capabilities, and robust repeatable fast analysis. High sensitivity allows for the detection of molecules at trace amounts or in limited samples, while high resolution allows for molecular identification by comparing their mass/charge (*m/z*) or fragmentation patterns [8 - 10]. With its quantitative capabilities, the concentrations of analytes in real samples could be reported, while high speed analysis enables rapid process monitoring or high analytical performance [7, 11].

Basic Components of the Mass Spectrometer

Multiple improvements have been made to increase the performance of MS since its inception in analytical chemistry. To ionize, scan, focus, fragment, and identify chemical structures, a variety of methods have been devised. The main components of a mass spectrometer include an ion source, a mass analyzer and a detector. Only ions of compounds or molecules can be detected by a mass spectrometer and differentiation between radicals and neutral molecules cannot be done. Ionization of materials is hence a prerequisite for MS analysis. To ionize the samples, sample molecules are first exposed to an ion source. The most prevalent ionization techniques are electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI) and matrix-assisted laser desorption/ionization (MALDI) [12, 13]. After ionization, the newly formed ions of molecules are sent to the mass analyzer to be sorted as per their *m/z* ratio. Different types of mass analyzers are used alone or in combinations and they have been classified into two major types as 'beam analyzers' and 'trapping analyzers'. In beam analyzers, ions escape the ion source in a beam and pass through the analyzing field to the detector, whereas in trapping analyzers, ions are trapped in the analyzing field after being produced in the analyzer or injected from an external ion source [13]. Quadrupole (Q), quadrupole ion trap (Q_{IT}), time of flight (TOF), Fourier-transform ion-cyclotron resonance (FT-ICR), and orbitrap are the widely used mass analyzers up to date. Q and TOF are examples of beam analyzers while $Q_{IT,}$ FT-ICR and orbitrap are trapping analyzers. In addition, combinations of the previously mentioned mass analyzers, such as triple quadrupole (QQQ), time-of-flight/time-of-flight (TOF/TOF), and quadrupole/time-of-flight (Q/TOF) are used and are termed as Tandem Mass analyzers (MS/MS). These MS/MS are again classified as tandem-in-space or tandem-in-time. Each step of MS/MS requires a separate analyzer in tandem-i--space devices. In tandem-in-space instruments, beam-type analyzers are utilized. Instruments used in trapping are usually tandem-in-time. The different steps of MS/MS are carried out in the same analyzer but at different times [13]. A multitude of factors impact the choice of a mass analyzer, including the intended *m/z* range to be studied, the mass of the analytes, the required resolving power, the ability to interface with the mass spectrometer's ion source, and the required limit of detection [12, 14]. With the exception of FT-ICR, after passing through the above mentioned mass analyzers, the ions are identified and converted into an electrical signal by the detectors, which is generally proportionate to their abundance in the ion beams. Some detectors, known as point ion detectors, can only detect a single ion at a time, but others, known as array detectors, can scan many masses at once [6]. MS detectors include the photo plate detector, Faraday cup detector, electron multiplier detectors such as the Discrete Dynode Electron Multiplier and Continuous Dynode Electron Multiplier, Micro channel Plate

CHAPTER 3

Poisonous Substances in Tropical Medicinal and Edible Plants: Traditional Uses, Toxicology, and Characterization by Hyphenated Mass Spectrometry Techniques

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Abstract: Alkaloids are natural metabolites containing nitrogen atoms, produced for different biological functions by plants, animals, and microorganisms. In most cases, its production is related to the defense mechanism of an organism through allelopathic effects. Because of this allelopathic property, some of these alkaloids are used as pesticides and can somehow be found in food and beverages as exogenous contaminants. Other contaminations by alkaloids come from industrial processing; so, ingestion of contaminated food or drinks can cause poisoning or death. Many of these plants, although composed of toxic substances, are also used as traditional medicines. Therefore, the compilation of these plants, their chemical constituents, and their pharmacological effects remain important. This paper aims to report traditional preparations and the use of edible plants containing toxic components, their toxicological records of a part of these poisonous metabolites, some regulations on their tolerable dose, and appropriate hyphenated techniques related to mass spectrometric for their separation, detection, quantification, and characterization. In addition, a particular emphasis will be placed on the properties of the stationary and mobile phases used for these studies. The fragmentation mechanism pathways based on mass spectrometry data for these substances will be widely described, and the diagnostic peak will be highlighted.

Keywords: Alkaloids, Edible plants, Mass spectrometry, Toxicology, Traditional remedy.

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INTRODUCTION

Many communities in tropical areas have a dynamic relationship with plants in nature including plants-food, plant-remedy, and plant-spiritual thinking. Sometimes, different parts of the same plant can serve simultaneously as food, traditional remedy, or be endowed with spiritual power as believed by certain local communities. In any case, the use of plants as food and as remedy mostly relies on their nutritional value and their chemical constitution. However, the chemical constituents of these plants are not always beneficial for humans and sometimes can present toxic effects. The danger associated with plants used as food and traditional medicines depends mostly on their preparations or manipulation. For instance, the preparation can require cooking or fermentation before consumption or ingestion as a fresh vegetable. So, the thermal exposure and the fermentation step can be crucial factors to avoid intoxication. Therefore, this review will particularly present alkaloids in medicinal and edible plants. Alkaloids are nitrogen-containing natural products whose biosynthetic precursors are proteinogenic (the twenty amino acids found in proteins) and non-proteinogenic amino acids (those which are not incorporated in protein biosynthesis: anthranilic acid, L-dopamic acid, hydroxyproline). Different alkaloid nuclei containing aromatic systems, saturated ring, and acyclic atoms sequences are found among the skeletal structure of these metabolites. Nitrogen-containing metabolites can be separated into five groups, including true alkaloids, protoalkaloids, polyamine alkaloids, amides alkaloids, and pseudo alkaloids. The predominant skeletons in food are pyrrolizidine, tropane, piperidine, quinolizidine, isoquinoline, β-carboline, quinoline, ergot alkaloids, pyrrolidinylpyridine, steroidal alkaloids, pyrrolopurine, and purine [1]. These metabolites are produced by plants, animals, and microorganisms and can present beneficial effects or can be harmful to humans [1 - 3]. Despite the adverse effects associated with alkaloids, some of them are found in trace or are highly concentrated in the daily human diet. Consequently, their existence in beverages, feeds, and foods can cause damages such as hepatotoxicity, cirrhosis, carcinogenicity, mutagenicity, teratogenicity, immunotoxicity, neurotoxicity, and estrogenicity [4, 5]. For this reason, governmental agencies and authorities have set food control strategies and regulations by defining thresholds for toxic alkaloids concentration in food products [6]. The effective concentration of these compounds depends on the amount consumed, and beyond this limit, possible food poisoning can be observed.

Import and conservation of food products always present a high risk of contamination, especially when the contaminant is not traceable and unknown or when the product comes from countries that lack adequate quality of monitoring infrastructure. Therefore, from an economic point of view, the standard of safety

and quality of food products remains a priority for food manufacturers and industries. Quality control, food ingredient, functional ingredients, and so on relied on traditional analytical techniques, such as Nuclear Magnetic Resonance, NMR; Infrared, IR; and Ultraviolet, UV, mass spectrometry (EI, MALDI, ESI, APCI *etc.*) and separation techniques (high-performance liquid chromatography, HPLC; gas chromatography, GC; capillary electrophoresis, CE and supercritical fluid chromatography, SFC). Two of these techniques can be hyphenated, such as liquid chromatographic coupled to mass spectrometry (LC-MS), gas chromatography associated with mass spectrometry (GCMS), and liquid chromatography coupled to nuclear magnetic resonance (LC-NMR). These hyphenated techniques allow simultaneous characterization of the components of a mixture, although the choice of analytical techniques depends entirely on the physical and chemical properties of the sample to be analyzed.

Hereby, we will report on toxic alkaloids found in plants and used as food and as medicinal remedies, their pharmacological effect, and how to use the mass spectrometry device for their detection and identification

EXAMPLE OF TROPICAL EDIBLE PLANTS USED IN TRADITIONAL PHARMACOPEIA AS MEDICINE

Plants Rich In Methylxanthine Alkaloids

Coffee Tree

The infusion of leaves and roasted seeds of *Coffea arabica* is used in Haiti to treat anemia, asthenia, and edema. In western India, asthma symptoms are alleviated with the use of an aqueous extract from dried seeds [7]. However, a side effect manifested by excessive bleeding during menstruation was observed in Nepal after consuming *Coffea benghalensis* flowers [8]. Disorders related to coffee intake have been previously demonstrated in several studies [9, 10].

Cocoa Tree

The whole grains of *Theobroma cacao* are used in the traditional Ghanaian pharmacopoeia for the treatment of diabetes, digestive and thoracic problems. In addition, seed powder after fermentation is used to prevent heart disease [11]. Its beans are also used in Mexico as a traditional remedy to cure mouth ulcers and toothache [12].

CHAPTER 4

LC-MS Analysis of Endogenous Neuropeptides from Tissues of Central Nervous System: An Overview

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Abstract: In recent years, various methods and technological advances demonstrated that neurochemical measurements have contributed to significant improvements in our understanding of the relationship between chemistry in the central nervous system (CNS) and the organism. Techniques based on Liquid Chromatography-Mass Spectrometry (LC-MS) are potent approaches for separating and quantifying endogenous neuropeptides in CNS. The separation ability and reliability of LC with sensitivity and selectivity of MS have become a valuable combination for peptide analysis either qualitatively or quantitively. Thus, new peptides have been identified using this technique. When applied to disease models, pathophysiological mechanisms can be identified and used as drug targets or biomarkers. Due to the low concentrations of neuropeptides in the biological samples, they restrict developing analysis methods and the understanding of their biological function. This book chapter focuses on novel developments of LC-MS/MS for endogenous neuropeptides. It has also emphasized the applications that cite preparation techniques used for brain tissue analysis, published in recent years.

Keywords: LC-MS/MS, Endogenous neuropeptide, Sample preparation, Brain tissues, Microdialysis, Central nervous system, Neuropeptide families, Mass spectrometry, Mass analyzers, Ionization sources, Fragmentation techniques, Neuropeptide analysis.

INTRODUCTION

Neuropeptides are the largest class of neuromessengers and have gained significant attention from researchers due to the diversity of their chemical structure, mechanisms of intercellular communication, localization within cells,

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and regulation of their functions in many biological and physiological processes.

Neuropeptide analysis in biological samples has significantly contributed to neuroscience research for identifying new peptides and biomarkers, having a better understanding of the disease, and developing new strategies for treatment. However, there are common challenges related to neuropeptide studies owing to vastly variable molecular weights and structural diversity, present at low endogenous concentrations in biological samples, products of proteolytic processing and posttranslational modifications (PTMs). Despite many difficulties that make neuropeptides study challenging, much advancement has been made on new technological developments, applications, and perspectives in this field [1 - 3].

Radioimmunoassay (RIA) and Enzyme-Linked Immunosorbent Assay (ELISA) methods are widely used for neuropeptide analysis. Although these methods are sensitive, most antisera are not unique to a single peptide and can cross-react with other peptides. Also, only a certain number of neuropeptides can be analyzed with these methods. Using highly selective, sensitive, robust, and accurate liquid chromatography-mass spectrometry (LC-MS), procedures have been carried out to separate and quantify peptides from biological samples. Simultaneous identification and determination of many neuropeptides in a sample are possible using mass spectrometry [4 - 6].

This book chapter demonstrates the use of mass spectrometric analysis of neuropeptide families in mammals to prepare a guide for researchers in this area. It also focuses on preparation and pre-treatment techniques for endogenous neuropeptides.

Neuropeptides

In complex neural networks, the chemical language of nerve cell communication compounds (called neuromessengers), also known as neurotransmitters, plays a crucial role in signal transduction and affects neural activity in the brain. Previously, only a few substances appeared as signal molecules; nowadays, more categories of molecules, including amino acids, biogenic amines, gaseous messengers, lipid derivatives, purines, pyrimidines, appear to have a signaling function in the nervous system [7, 8]. Neuropeptides have only been investigated as neurotransmitters in recent years, and their wide range has made them different from the amino acid and biogenic amine neurotransmitters [9].

The term “neuropeptides” was first launched by David de Wied in 1971. They were firstly found in the nervous system, as indicated by their name. They consist of 3 to 100 amino acid residues, which are much larger and synthesized

differently than classical neurotransmitters. They are released in a smaller amount than small-molecule transmitters. Neuropeptides are thousands of times more active than small-molecule transmitters. Another feature of neuropeptides is that they cause long-lasting effects. Some of these long-term effects include changes in the cell's metabolic function, changes in the activation or deactivation of specific genes in the cell nucleus, and long-term changes in the number of excitatory or inhibitory receptors. These effects can sometimes take days, months, or even years [7, 10].

Neuropeptides contain many different endogenous peptides, which act as neurotransmitters or neuromodulators in the central and peripheral nervous systems and neurohormones in the endocrine system. They influence the regulation of many biological and physiological functions, including sleep, pain, fear, reproduction, metabolism, depression, learning, homeostatic mechanisms, water retention, *etc* [1, 3, 4, 11 - 19]. According to the research of PubMed, some endogenous neuropeptides and their physiological functions are given, as shown in Table **1**.

Table 1. Some Neuropeptides and Physiological Functions.

Neuropeptides	**Physiological functions**	**Refs.**
Pyrogultamylated arginine- phenylalanineamide peptide (QRFP- 26)	Food intake	[20]
Cortistatin	Inflammation, pain, stress	[21]
Neuropeptide B (NPB)	Feeding, energy metabolism, hormone secretion	[22]
PACAP	Reflex	[23]
Corticotropin-releasing factor (CRF)	Stress	[24]
Orexin B	Passive avoidance learning	[25]
VIP	Feeding behavior, metabolic hormone release, body mass composition and energy balance	[26]
Nociceptin (N/OFQ)	Emotional memory, aversive learning	[27]
Dynorphin-A	Hibernation, climatic modulation	[28]

CHAPTER 5

Advances in Structural Proteomics using Mass Spectrometry

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Abstract: Structural proteomic techniques have recently evolved because of advances in mass spectrometry (MS). Several MS techniques, such as, Hydrogen-deuterium exchange, oxidative footprinting or radical probe mass spectrometry, chemical crosslinking, affinity purification, and ion mobility separation, can now be used to analyse protein interaction networks, conformational changes, protein structures, and other downstream applications. This article examines proteomic MS techniques' progression from convectional to advanced techniques, tandem MS techniques, MS of multiprotein complexes, and emerging MS techniques for structural proteomics. Also, the applications that were gleaned from these techniques were reviewed. Lastly, the future of this rapidly emerging field was highlighted.

Keywords: Affinity purification, Chemical crosslinking, Hydrogen-deuterium exchange, Ion mobility, Mass spectrometry, Oxidative footprinting, Radical probe mass spectrometry (RP-MS), Structural proteomics.

INTRODUCTION

The characterisation of protein structures, protein assemblies and systematic evaluation of protein-protein interactions are instances of structural proteomics, that is, the application of protein chemistry and modern mass spectrometry techniques to these and other proteomic analyses [1]. Several MS-based techniques (such as, hydrogen-deuterium exchange, oxidative footprinting, chemical crosslinking, and affinity purification, to name a few) can now determine the atomic-level structure of the complex, including its shape, cavity size, protein subunit distance, inter-subunit angles, protein connectivity, monomer topology, subcomplex topology, protein-protein interface structure, and more [2, 3]. Nonetheless, the vast diversity, transient nature, and low relative quantities of biomolecules in biological samples make it difficult to determine the essential

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structural characteristics of these protein complexes [3]. Consequently, designing more sensitive MS techniques capable of capturing the dynamic behaviour of protein complexes is crucial to resolving this problem. Nevertheless, there have been actual barriers to implementing such a thorough approach.

Furthermore, the sensitivity, speed, and precision have reinforced MS's status as a crucial structural proteomics tool, enabling researchers to understand several cellular processes' interconnectivity better. The structure and dynamics of multiprotein complexes at any concentration and in any solution using mass spectrometry can be investigated [2]. MS has been combined with other techniques, such as hydrogen-deuterium exchange (HDX), chemical crosslinking (CXL), oxidative footprinting (OFP), limited proteolysis (LP), affinity purification (AP), and ion mobility (IM) separation, to reveal the three-dimensional structure of multiprotein complexes [4].

Structural proteomics uses two primary kinds of MS techniques: 1). those that derive structural information from measurements of protein ions in the gas phase, and 2). those that offer spatial constraints from measurements of proteins in solution [3]. The structural integrity of complex proteins must be preserved during the transition to the gas phase for downstream applications [5]. To do this, MS instruments have been constructed or modified to raise their ion guide pressures, include low-frequency quadrupole mass analysers, and use their more considerable acceleration potentials [3, 5]. Although each MS technique may have a wide variety of physical issues, combining them can yield additional knowledge that can be utilised to solve previously unsolvable structural biology problems. MS may be used, for instance, to discover chemical changes in proteins in a solution intended to affect their structure or dynamics [6].

This article reviews the most recent advancements in the structural characterisation of proteins using MS-based techniques. Although MS measurement of entire protein complexes is a relatively new method for examining protein structure, it is already a solid research tool, and we concentrate on its role in structural proteomics. This review highlights that by combining MS technologies, a range of techniques for understanding protein network composition and three-dimensional structure may be accessible.

IDENTIFICATION OF PROTEIN STRUCTURES BY MASS SPECTROMETRY

Protein sequences are abundant, with approximately 141,621,564 entries in the UniprotKB, but protein structures are scarce (158,271 in the UniprotKB as of February 2023, www.pdb.org). Alternative, high-throughput methods for studying protein structure are necessary to reduce this disparity. Despite their frequent

inability to provide complete, high-detail structural information, the value of these higher-throughput methods lies in their ability to offer either a localised, highly detailed view of a small portion of the structure or a global, high-sensitivity view of the structure's dynamical behaviour [7]. Many MS-based methods are utilised to determine protein structure, highlighting the divergence from the conventional focus on identifying and measuring these macromolecules. To assess the stoichiometry of a protein or protein complex, the conventional approach of mass spectrometry entails an examination of the protein or protein complex [8].

Conventional Protein MS Techniques

Mass spectrometry is essential for precise sequencing and characterisation of proteins [9]. Whole proteins may be ionised using electrospray ionisation (ESI) or matrix-assisted laser desorption/ionisation (MALDI). Both approaches characterise proteins in ways compatible with modern mass spectrometers' capabilities and mass range. The initial stages involve ionising proteins in one of two ways and passing them into a mass analyser in their natural form. In protein research, this method is known as "top-down" analysis (Fig. **1c**). For low-throughput single-protein research, the top-down technique is often utilised [10]. Proteases such as trypsin and pepsin are used in electrophoretically separated proteins to enzymatically degrade the separated proteins into smaller peptides in solution or gel. Protein analysis from the "bottom-up" (Fig. **1a**) has also been used to investigate the distribution and position of post-translational changes, such as phosphorylation, on proteins [11]. Another possible paradigm gaining support is the "middle-down" paradigm (Fig. **1b**). This research focuses on proteolytic peptides larger than the average tryptic peptide [12]. Both ESI and MALDI employ these models, and they are further discussed.

Electrospray Ionisation (ESI)

Electrospray ionisation (ESI) is used in mass spectrometry to generate ions by delivering a high voltage to a liquid to create an aerosol (Fig. **2a**) [13]. It works against the inherent propensity of macromolecules to break apart when ionised, making it an extremely successful ionisation approach [14]. Unlike traditional ionisation methods, ESI may generate multiple-charged ions, expanding the analyser's mass range and allowing it to handle the kDa-MDa orders of magnitude often seen in proteins and similar polypeptide fragments [15]. Due to the low degree of fragmentation during ESI, it is also known as a "soft ionisation" technique [16]. Although the molecular ion (or, more precisely, a pseudo-molecular ion) is usually always identified, the simple mass spectrum obtained offers very little structural information. This disadvantage may be overcome by employing a tandem mass spectrometer (ESI-MS/MS) in combination with ESI

CHAPTER 6

Recent Trends of Modern Mass Spectrometry: Application towards Drug Discovery and Development Process

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Abstract: Mass spectrometry has evolved significantly in recent years and has become a powerful analytical tool in the field of drug discovery and development. It allows for the identification and characterization of small molecules, peptides, and proteins in complex biological samples with high sensitivity and accuracy. This chapter provides an overview of the recent trends in modern mass spectrometry and its application towards the drug discovery and development process. It discusses the advancements in mass spectrometry technology, such as high-resolution mass spectrometry (HRMS), ambient ionization mass spectrometry (AIMS), data-independent acquisition (DIA) mass spectrometry, tandem mass spectrometry (LC-MS/MS), and how they have enabled the analysis of complex biological samples. The chapter also highlights the use of mass spectrometry in various stages of the drug discovery and development process, including target identification, hit identification, lead optimization, and drug metabolism and pharmacokinetic studies. Additionally, it discusses the challenges and future prospects of mass spectrometry in drug discovery and development. Overall, mass spectrometry has revolutionized the drug discovery and development process and will continue to play a crucial role in the future.

Keywords: Ambient ionization mass spectrometry (AIMS), Drug discovery, Drug development, Data-independent acquisition (DIA) mass spectrometry, High-resolution mass spectrometry (HRMS), Mass spectrometry, Tandem mass spectrometry (LC-MS/MS).

INTRODUCTION

Mass Spectrometry (MS) has long been recognized as a powerful analytical technique with widespread applications in various fields, including chemistry, physics, and biology. In recent years, mass spectrometry has emerged as a key technology in drug discovery and development, playing a crucial role in the iden-

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tification, characterization, and quantification of small molecules and large biomolecules [1, 2]. With the growing complexity of drug molecules and the demand for faster and more efficient drug discovery and development processes, there has been a significant increase in the adoption of modern mass spectrometry techniques [3].

In this chapter, we will discuss the recent trends of modern mass spectrometry in the context of drug discovery and development. We will explore how mass spectrometry has evolved over the years, driven by advancements in instrumentation and methodologies, and how it has been successfully applied in various stages of the drug development process. We will also discuss the recent and important application of mass Spectrometry in the field of drug discovery.

PRINCIPLE

To understand the current state of mass spectrometry in drug discovery and development, it is important to first highlight its fundamental principles. It is an analytical technique that measures the mass-to-charge ratio (m/z) of ions in a sample [4]. It involves the ionization of a sample, typically through techniques such as electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), followed by separation and detection of these ions based on their m/z values. The resulting mass spectrum provides information on the identity, quantity, and structure of the sample components, making it a valuable tool for the identification and quantification of drug molecules [5, 6].

DRUG DISCOVERY METHOD USING MASS SPECTROMETRY

Mass spectrometry (MS) plays a crucial role in modern drug discovery, particularly in the identification and characterization of potential drug candidates. Here's a detailed overview of how mass spectrometry is utilized in the process [7 - 10] (Fig. **1**).

Sample Preparation and Introduction

The process begins with the preparation of samples containing the compounds of interest, which could be from natural sources, synthetic chemicals, or biological extracts. These samples are then introduced into the mass spectrometer for analysis.

Ionization

In the mass spectrometer, the sample is ionized to create charged particles. This step is critical as it allows the molecules to be analyzed based on their mass-t--charge ratio (m/z). Common ionization techniques include electrospray

ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), each suitable for different types of compounds.

Mass Analysis

Once ionized, the ions are accelerated and separated based on their mass-t--charge ratio in the mass analyzer. There are several types of mass analyzers used in drug discovery MS, including:

Time-of-Flight (TOF)

Measures the time it takes for ions of different masses to reach a detector.

Quadrupole

Uses radiofrequency voltages to selectively transmit ions based on their m/z ratio.

Ion Trap

Captures and releases ions based on their mass-to-charge ratio using electromagnetic fields.

Detection and Data Analysis

The ions reaching the detector are converted into electronic signals, which are then processed by a computer. This data provides information about the mass-t--charge ratios of the ions present in the sample.

Identification

MS can identify unknown compounds by comparing their mass spectra to databases of known compounds.

Characterization

MS can provide detailed structural information about the molecular composition of the compounds, including fragmentation patterns.

Quantification

MS is also used for quantifying the amount of specific compounds present in a sample. This quantitative data is crucial in assessing the potency and efficacy of drug candidates.

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ATTA-UR-RAHMAN, *FRS*

Prof. Atta-ur-Rahman, Ph.D. in Organic Chemistry from Cambridge University (1968) has over 1559 international publications in several fields of organic chemistry (h index 76, citations 38,200) including 86 international patents, 70 chapters in books, 875 research publications, and 391 books (11 authored and 380 edited). He received the following awards: Fellow Royal Society (FRS) London (2006), UNESCO Science Prize (1999), Honorary Life Fellow Kings College, Cambridge University (2007), Academician (Foreign Member) Chinese Academy of Sciences (2015), Highest Civil Award for Foreigners of China (Friendship Award, 2014), High Civil Award Austria ("Grosse Goldene Ehrenzeischen am Bande") (2007), Foreign Fellow Chinese Chemical Society (2013), Sc.D. Cambridge University (UK) (1987), TWAS (Italy) Prize (2009). He was the President of Network of Academies of Sciences of Islamic Countries (NASIC), Vice President TWAS (Italy), Foreign Fellow Korean Academy of Science & Technology, President Pakistan Academy of Sciences (2003-2006) and (2011 – 2014). He was the Federal Minister for Science and Technology of Pakistan (2000 – 2002), Federal Minister of Education (2002) and Chairman Higher Education Commission/ Federal Minister (2002-2008), Coordinator General of COMSTECH (OIC Ministerial Committee) (1996-2012), and the Editor-in-Chief of Current Medicinal Chemistry.

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M. Iqbal Choudhary is a distinguished national professor of organic/bioorganic chemistry and honorary advisor at the International Center for Chemical and Biological Sciences (H. E. J. Research Institute of Chemistry, and Dr. Panjwani Center for Molecular Medicine and Drug Research), and coordinator general of COMSTECH (OIC Ministerial Committee). He is among the most prominent scientists of Pakistan, recognized for his original contributions in the fields of natural products and bioorganic chemistry. He has written and edited 27 books and he is the author of over 1275 research papers and chapters in top international science journals as well as 64 US patents with an H-index: 86 and citations: 43,716. He is the volume editor of many international book series and journals. He has served as a visiting faculty in many prestigious universities of the world. He is the fellow of major science academies of world (TWAS, IWAS, PAS), and received prestigious awards and honors, including Chinese Government Friendship Award (2022), Mustafa (PBUH) award (2021), ECO Excellence Award in Education (2016) and MRC Team Impact Prize, UK (2024).

SYED GHULAM MUSHARRAF

Prof. Dr. Syed Ghulam Musharraf is amongst the most notable young scientists based on his seminal contributions to mass spectrometry and its applications. He obtained post-doctoral training from Austria and USA. On his return, he established a world-class mass spectrometry research laboratory in Pakistan. He has effectively used several mass spectrometric tools for the high-throughput analyses of bioactive compounds and the standardization of botanicals. He is pioneered in biomarker-based metabolomics research in Pakistan. He worked extensively on different cancers, as well as on thalassemia for molecular understanding of disease. He is the author of over 250 research publications (journal cumulative impact factor over 1200, h-index = 38, Google Scholar). Based on his scientific contributions, he has received several international/national awards and honors, including civil award and a D. Sc. Degree.