UNIVERSITÀ DEGLI STUDI DI MILANO

CONCORSO PUBBLICO, PER TITOLI ED ESAMI, A N. 1 UNITÀ DI TECNOLOGO DI SECONDO LIVELLO, CON RAPPORTO DI LAVORO SUBORDINATO A TEMPO DETERMINATO PRESSO L'UNIVERSITÀ DEGLI STUDI DI MILANO - DIPARTIMENTO DI SCIENZE DELLA SALUTE - CODICE 22485

La Commissione giudicatrice della selezione, nominata con Determina Direttoriale n. 14147 del 09/09/2024, composta da:

Prof.ssa Elena Maria Varoni	Presidente
Dott.ssa Elena Anna Lesma	Componente
Dott. Federico Maria Rubino	Componente
Sig.ra Laura Lagana'	Segretaria

comunica i quesiti relativi alla prova orale:

1. Quando si impiega la spettrometria di massa, cosa si intende per “analisi qualitativa”, “analisi semiquantitativa” e “analisi quantitativa”?

Letter

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Exploring the versatility of mass spectrometry: Applications across diverse scientific disciplines

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Abstract

Mass spectrometry (MS) has become a pivotal analytical tool across various scientific disciplines due to its ability to provide detailed molecular information with high sensitivity and specificity. MS plays a crucial role in various fields, including drug discovery and development, proteomics, metabolomics, environmental analysis, and clinical diagnostics and Forensic science. In this article we are discussing the application of MS across the diverse scientific disciplines by focusing on some classical examples from each field of application. As the technology continues to evolve, it promises to unlock new possibilities in scientific research and practical applications, cementing its position as an essential tool in modern analytical science.

Keywords

Mass spectrometry, TOF, Orbitrap, MALDI, proteomics, MSI, impurity identification, HOS, DMPK, metabolite identification

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Introduction

Mass spectrometry (MS) has undergone a remarkable evolution since its inception in the late nineteenth century, transforming from an elementary technique for studying atomic isotopes to a sophisticated analytical tool with wide-ranging applications across scientific disciplines. This development has been driven by continuous technological advancements and the ever-growing demand for more precise, sensitive, and versatile analytical methods.[1] MS continued to evolve rapidly, with significant milestones including the development of various ionization techniques, such as electron ionization and chemical ionization, as well as the introduction of different mass analyzers like quadrupoles quadrupole ion trap analyzers, ion cyclotron resonance (ICR) analyzers, time-of-flight and orbitrap instruments. The latter half of the century saw the emergence of soft ionization methods like electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), which revolutionized the analysis of large biomolecules and expanded the technique's applications in life sciences. The evolution of MS has accelerated, driven by advancements in three key areas: resolution, integration with other techniques, and data analysis. High-resolution mass analyzers, such as Orbitrap and improved time of flight (TOF) instruments, has greatly enhanced mass accuracy and resolving power. Hyphenated techniques such as liquid chromatography (LC)-MS, gas chromatography (GC)-MS, capillary electrophoresis (CE)-MS and imaging mass spectrometry (IMS) significantly enhance the analytical capabilities of MS by improving separation, increasing sensitivity, and providing more comprehensive information about complex samples. While advances in data analysis, including the application of artificial intelligence and machine learning, have greatly improved the interpretation of complex mass spectrometric data.[1] MS has become an indispensable analytical tool in the pharmaceutical, biopharmaceutical, environmental, and forensic science due to its high sensitivity, specificity, and versatility. MS applications across various stages of drug development, from initial discovery to quality control and regulatory compliance. In the pharmaceutical industry, MS is extensively used for the identification and quantification of small molecule drugs. MS aids in the structural elucidation of drug candidates, impurity profiling, and metabolite identification, which are crucial for understanding pharmacokinetics and potential toxicity.[2] Advanced MS techniques, such as ultra-high-resolution MS, provide detailed molecular information, enabling reliable molecular formula assignments and trace analysis in complex mixtures. In the biopharmaceutical sector, the complexity and heterogeneity of biologics, such as monoclonal antibodies and other protein and peptide therapeutics, present significant analytical challenges. MS is employed to characterize critical quality attributes (CQAs) like post-translational modifications (PTMs) and HOS which can impact product efficacy and safety.[3,4] MS-based methods provide a comprehensive

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2. Quali sono, in termini essenziali, le principali fasi, operative e tecniche, per la messa a punto di un metodo analitico quantitativo con strumenti combinati di cromatografia e spettrometria di massa?

Balasaheb et al. investigated the metabolic pathway of Palbociclib (PAB) by UHPLC-Q-TOF/MS/MS and in silico toxicity studies of its metabolites. PAB is a CDK4/6 inhibitor and U.S. Food and Drug Administration (FDA) granted regular approval for the treatment of hormone receptor (HR) positive, metastatic breast cancer in combination with an aromatase inhibitor in postmenopausal women. The in vivo metabolism of the drug was investigated by administering PAB orally to Sprague-Dawley rats and analyzing urine, feces, and plasma samples. Simple protein precipitation (PP) is used to prepare the samples, which are then extracted solidly. The extracted samples were analyzed using UHPLC/Q-TOF/MS/MS. Fourteen metabolites were found in in vivo matrices. The PAB was metabolized using hydroxylation, oxidation, sulfation, N-dealkylation, acetylation, and carbonylation pathways. A few metabolites were also found in in vitro samples. Metabolite identification and characterization were performed by using UHPLC/Q-TOF/MS/MS in combination with TOPKAT and DEREK software.[17]

Johnsi Rani et al. characterize the metabolites of Ambrisentan (AMBR), in *in vitro* and *in vivo* matrices by UHPLC/Q-TOF/MS/MS. In vitro metabolism studies were carried out by incubating AMBR in rat liver microsomes (RLM), as well as rat and human liver S9 fractions. An in vivo study was conducted by collecting urine, feces, and plasma samples at various time points following oral administration of AMBR in suspension form at a dose of 25 mg/kg to 6 male Sprague-Dawley rats. In vivo samples yielded 17 metabolites, including hydroxyl, demethylated, demethoxylated, hydrolytic, decarboxylated, epoxide, and glucuronide metabolites. The majority of the metabolites were found in the feces and urine matrices, with only a few in the plasma matrix. Only 10 metabolites were identified in the in vitro study, which was consistent with the in vivo study. Detailed structural elucidation of all metabolites was done using UHPLC/Q-TOF/MS/MS. The toxicity profile of AMBR and its metabolites were predicted using TOPKAT software.[18]

Chen-Xue Li et al. developed the method for structural characterization of the metabolites of orally ingested hederasaponin B, a natural saponin that is isolated from Acanthopanax senticosus leaves by LC-MS. In this study, ultra-performance liquid chromatography/fusion Lumos Orbitrap MS was used to analyze the metabolites of hederasaponin B in vivo, and potential metabolic pathways were proposed. After oral administration of the parent drug, 47 metabolites were found in rat feces (42), urine (11), and plasma (9) samples. These metabolites were produced by metabolic processes in phases I and II, including deglycosylation, hydroxylation, acetylation, oxidation, gluconalciation, and glycosylation. Deglycosylation is the primary metabolic pathway, accounting for 52.46% of all metabolites in feces sample.[19]

Mass spectrometry imaging. MS imaging (MSI), also known as imaging MS (IMS), is a type of compound-specific imaging. MSI techniques do not require the labeling of chemicals. MSI gives spatial information on parent drugs, drug metabolites, and/or endogenous substances (e.g. biomarker identification) on whole body or tissue slices without the use of (radio) labeling. MSI has been used for over two decades, although its popularity in ADME-related research has recently increased due to the improved sensitivity and resolution in mass spectrometers such as orbitrap and time of flight (TOF) mass spectrometers. In MSI MS's superior selectivity and sensitivity can be used over other spectroscopic techniques in an experiment where mass spectrometric analysis is performed directly from the sample rather than from sample extracts as is done with conventional LC-MS and GC-MS techniques. Mass spectra are systematically acquired from every point on a sample by scanning the sample below the ionization spot (Figure 1). This experiment, which typically runs over one or several hours (depending on the size of the sample and the spatial resolution in the image) results in a collection of thousands of mass spectra where every mass spectrum becomes a pixel in the final image.[9] Laser desorption ionization (LDI) and secondary ion MS (SIMS) are the two most commonly used molecular MSI methods for biological samples. LDI MSI has been demonstrated at laser wavelengths ranging from near infrared to ultraviolet (UV), with pulse durations ranging from nanoseconds to femtoseconds.[20] SIMS has a spatial resolution in the nm range, which is higher than any other MSI technique, but the instruments are also correspondingly more expensive and therefore not as commonly available. Unfortunately, despite recent advancements, SIMS still suffers from substantial ion fragmentation, which contributes to less overall sensitivity when analyzing biological materials. Inorganic analytes can be imaged using laser ablation inductively coupled plasma (LA-ICP) MSI. LA-ICP MSI uses a UV laser to desorb analytes from the sample, which are then ionized in the ICPMS instrument. This allows for a spatial resolution of up to 5 mm, however the analysis only offers information about the distribution of elements in the sample.[21] MALDI-IMS is a nontargeted label-free technique for imaging in which a laser performs a raster scan throughout the whole region of the matrix-treated tissue sample, producing mass spectra for each spot with defined x and y coordinates. The molecular image is created by plotting the ion intensities of any obtained m/z signal as a function of position.[22] MALDI imaging MS was used in a rat to determine the effect of nanoparticle exposure into the biological system through cells and tissues which leads to techniques interaction with the protein molecules resulting in a dynamic NP-protein corona formation. MALDI IMS has been used to analyze the lipids by adding lithium salts to the matrix solution.[23] Analytical probes that can determine molecule composition at the nanoscale are crucial for materials science, biology, and medicine. Mass spectral imaging can visualize the spatial arrangement of many molecular components at a sample's surface.

3. Partendo da un campione biologico, descrivere in maniera sequenziale le procedure pre-analitiche e analitiche per ottenere un'analisi della lipidomica

Primary structure determination: The primary structure is the specific order of amino acids linked together by peptide bonds to form the polypeptide chain. It starts at the N-terminus (amino end) and ends at the C-terminus (carboxyl end) of the protein. The primary structure is unique to each protein and determines its higher-order structures and function. Edman degradation and MS is essential for confirming the amino acid sequence of proteins and peptides.[26] Techniques like peptide mapping and tandem MS/MS sequencing provide detailed information about the primary structure of biopharmaceuticals.[27,28] Proteins undergo digestion using numerous complementary proteases to provide appropriate peptides for de novo sequencing using liquid chromatography-tandem MS (LC-MS/MS) in a bottom-up approach. The majority of MS-based antibody sequencing strategies rely on a bottom-up liquid chromatography-tandem MS (LC-MS/MS) approach in which the antibody product is digested into smaller peptides before being analyzed by MS.[29] The peptides can be identified or structurally determined by using top-down (no digestion) and bottom-up (with digestion) approach using LC-MS/MS.[30] Primary structure for mRNA based vaccine or drug was determined by digesting the vaccine by RNase enzyme and oligonucleotides were separated by IP-RP-HPLC and analyzed by UV and MS/MS. Up to 388 oligonucleotides could be determined with this approach.[31]

Intact protein analysis: Top-down MS allows for the analysis of intact proteins and protein complexes, providing information on overall molecular weight, subunit composition, and noncovalent interactions.[28,30] Orbitrap and TOF mass analyzers have seen with improvements in mass resolution, enabling more effective analysis of intact proteins and "top-down" proteomics approaches. Combining different ion analyzers or separators in a single instrument, like the Orbitrap fusion Lumos Tribrid mass spectrometer, to increase overall capabilities in terms of sensitivity, dynamic range, resolution, accuracy, and scan-to-scan reproducibility.[32] Protein identification methods can be divided into two categories: database searches and de novo sequencing procedures. The database search approach is frequently used and becoming more prevalent. It detects proteins by creating theoretical spectra in silico from a given protein sequence database and then comparing experimental spectra to the theoretical ones to discover the closest matches.[30] De novo sequencing refers to determining the primary genetic sequence of an organism without using a reference sequence. It involves assembling individual sequence reads into longer contiguous sequences (contigs) or ordered contigs (scaffolds) in the absence of a reference genome.[33]

Post-translational modification analysis: Recent advances in MS-based proteomics have enabled the identification and quantification of numerous post-translational modification (PTM) sites in a single experiment. MS with new high-performance instrumentation and bioinformatic algorithms can identify and characterize PTMs such as glycosylation, phosphorylation, and deamidation, which are critical for the function and stability of biopharmaceuticals.[34] MS-based PTM analysis requires sufficient peptide fragmentation information (MS2 scans) to accurately identify and localize PTMs. Several fragmentation techniques, including as collision-induced dissociation (CID), higher energy collisional dissociation (HCD), electron capture dissociation (ECD), and electron transfer dissociation (ETD), have been used in the last decade for this purpose.[35]

Higher-order structure analysis: HOS analysis is an important component in determining CQA in biopharmaceuticals and is compelled by regulatory guidelines such as ICH Q6B. Advanced MS techniques, such as hydrogen-deuterium exchange (HDX-MS) and cross-linking MS (XL-MS), reveal secondary, tertiary, and quaternary protein structures. On a sub-millisecond timescale, FPOP labels solvent-accessible amino acid side chains with hydroxyl radicals, resulting in a "snapshot" of protein structure faster than most conformational changes.[36,37]

Disulfide bond mapping: Disulfide bonds are essential for protein structural stability and function. It is critical to understand the interactions between numerous cysteine residues inside a protein. The creation of one disulfide bond causes a 2-Da drop in molecular weight, which most mass spectrometers detect. MS-based techniques have shown to be particularly beneficial in disulfide bond research. Enzymatic digestion under controlled conditions, followed by chromatographic separation by LC and MS detection methods, continues to be the dominant method for cysteine linkage analysis.[38–42] Top-down MS is beneficial for disulfide bond assignment because it avoids the possibility of disulfide rearrangement, which occurs in mild alkaline conditions during trypsin digestion. Using collision-activated dissociation (CAD) and ECD with liner ion trap/Fourier transform ion cyclotron resonance (FTICR), the five-disulfide links in human salivary α-amylase were identified without digestion.[43] However, technological issues such as protein solubility, sensitivity, throughput, and the need for a high-end instrument remain unresolved, preventing top-down MS from being widely used. Top-down analysis can only provide limited information on proteins with interlocked disulfide links. Thus, disulfide linkages were predominantly broken in top-down analysis to boost the protein backbone sequence coverage. For bigger proteins with more cysteines, such as monoclonal antibodies, ETD-based methods, dimethyl labeling coupled with RADAR, and MALDI with ISD have been found to be successful.[30,44,45]

Impurity and degradation product identification: LC-MS approaches are commonly used to monitor chemical modifications such as Met oxidation, Asn deamidation, and Asp isomerization following storage of formulated bulk solutions, lyophilized products and finished drug products under real time, intermediate and accelerated conditions.[46] MS is used to demonstrate stability of the therapeutic protein and peptides in the

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