Review

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Metabolomics: a review of liquid chromatography mass spectrometry-based methods and clinical applications

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Abstract

Introduction: Metabolomics is a rapidly growing field that aims to understand the complex metabolic pathways involved in health and disease. Liquid chromatography mass spectrometry (LC-MS) based untargeted metabolomics has emerged as a powerful tool for investigating the metabolic changes associated with various diseases, and for identifying potential biomarkers for early disease detection and treatment monitoring. This review provides a comprehensive overview of LC-MS based untargeted metabolomics and its clinical applications.

Content: The advantages and challenges of untargeted metabolomics are discussed, encompassing sample preparation, data processing, sample storage, acquisition mode selection, column strategy, and annotation. The latest advancements in LC-MS technology and data analysis techniques are reviewed, which have facilitated the more accurate and sensitive detection of metabolites in biological samples. The clinical applications of untargeted metabolomics are emphasized, including its utilization in disease diagnosis, treatment monitoring, and personalized medicine. Examples are provided of how biomarkers for various diseases, such as cancer, coronary heart disease, and infectious disease, have been identified through the application of untargeted metabolomics.

Summary and outlook: Overall, LC-MS based untargeted metabolomics is a powerful tool for investigating the complex metabolic changes associated with various diseases, and has the potential to transform clinical diagnosis, treatment, and personalized medicine.

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Introduction

Metabolism in living organisms encompasses all chemical reactions, giving rise to a diverse array of molecules known as metabolites. These endogenous compounds include amino acids, lipids, carbohydrates, and organic acids. The metabolome, consisting of metabolites typically <1,500 Da, represents the comprehensive profile influenced by factors like genetics, environment, nutrition, microbiome, disease, toxicities, infection, and inflammation [1]. Since metabolites serve as essential components of biochemical pathways, they have been analyzed for decades because of their regulatory role and importance in diagnosing diseases [2–4]. Historically, the limited number of metabolites could be investigated concurrently due to the different chemical or physical characteristics of metabolites and analytical limitations. However, due to current chromatography technologies, more metabolites could be analyzed simultaneously in biological samples. These developments have led to the emergence of new approaches entitled metabolomics.

Metabolomics is a new member of the 'omics' family. It is generally defined as the qualitative and quantitative analysis of some metabolites involved in biological reactions [1]. It is a dynamic portrait of the metabolic state of living systems. Thousands of metabolites can be evaluated by metabolomic analysis, and thus metabolic processes can be evaluated with a detailed and holistic understanding [5]. This technology facilitates detailed studies of biological samples, clarifying complex metabolic processes. Its applications, including biomarker discovery, understanding pathophysiological processes, advancing personalized medicine, identifying new drug targets, and enhancing disease monitoring and management, underscore its valuable contributions [6]. This analytical approach includes common steps: sample collection, sample preparation, instrumental analysis, annotation, and metabolic pathway analysis [7].

The aim of this review is to provide an overview of the current state of liquid chromatography mass spectrometry (LC-MS) based untargeted metabolomics and its clinical applications. In this context this review encompasses various aspects of untargeted metabolomics including sample preparation, instrumentation, data analysis, and interpretation. The review will also cover the various applications of untargeted metabolomics in clinical research such as disease diagnosis, prognosis, and therapy monitoring.

Approaches in metabolomics

Targeted metabolomics

Mass spectrometry-based metabolomics analyses can be divided into two main headings: targeted or untargeted [8]. In targeted metabolomics analysis, quantitative measurement of previously biochemically identified metabolites is performed using low-resolution tandem mass spectrometers and multiple reaction monitoring mode (MRM) [9–11]. In this approach, a precise quantitation can be achieved with the calibration curves obtained using standard reference materials [12]. Targeted metabolomics focuses on specific metabolites and related pathways, offering high sensitivity and specificity for accurate detection and quantification. This technique provides a concentrated analysis, yielding a deeper understanding of specific metabolic pathways in biological processes. Notably fast and cost-effective, targeted metabolomics requires less sample preparation and data processing compared to untargeted approaches. Its value extends to unraveling the role of metabolites in disease and contributing to the development of targeted therapies [10, 11, 13].

Untargeted metabolomics

Untargeted metabolomic methods are global in scope and aim to simultaneously measure as many metabolites as possible from biological samples without bias. This approach allows identifying many metabolites, previously biochemically defined or not. However, in contrast to targeted metabolomics analysis, it is not possible to achieve precise quantitation of these metabolites [14, 15]. Typically, high-resolution mass spectrometry (HRMS), such as quadrupole time-of-flight (QTOF) [9] and nuclear magnetic resonance spectroscopy (NMR) [16] can be used for untargeted metabolomics. One of the advantages of untargeted analysis is that hundreds of metabolomes can be detected simultaneously; thus, new targets can be identified to guide diagnosis or treatment. In this approach, compound identification (annotation) relies on the use of different libraries, as it is not cost-effective to employ standard reference material for the identification of each metabolite. Additionally, the datasets obtained through this approach are complex, posing significant barriers to data interpretation [17–21]. Despite the introduction of various metabolomics databases and software in the past decade to tackle this issue, further progress is still necessary. Moreover, the identification of unknown features and the subsequent validation phases demand additional time and effort [17].

Analytical platforms of untargeted metabolomics

Different analytical platforms can be used in untargeted metabolomics. They require a complex and detailed optimization process [22–24]. Due to factors such as complex sample structure, the dynamic range of intensities, biological variation, and structural heterogeneity of metabolites, specific analytical platforms with high resolution and performance are needed to obtain reliable results. NMR and LC-MS, gas chromatograph mass spectrometry (GC-MS), ion mobility coupled with mass spectrometry (IM-MS) or capillary electrophoresis (CE) have been used in metabolomics. Due to methodological variances, the number of detectable metabolites varies across analytical platforms. Ideally, researchers should employ three analytical platforms within the same study to maximize the detection of metabolites. However, there are limited studies where all three platforms are used simultaneously due to financial limitations. The advantages and disadvantages of the analytical platforms were given in Table 1.

LC-MS

Liquid chromatography-mass spectrometry is a highly sensitive and specific separation and identification technique using a mobile and stationary phase. LC-MS is one of the most applied chromatographic techniques for analyzing polar and non-polar metabolites. Different tandem and hybrid configurations such as Q-TOF, triple quadrupole (QqQ), Fourier Transform-Ion Cyclotron Resonance mass spectrometry (Qq-FT-ICR), quadrupole-linear ion trap (QqLIT) and Orbitrap can be used in MS/MS systems. Each system has its advantages and disadvantages [10]. Q-TOF or Orbitrap platforms are mainly used for untargeted metabolomics analyses due to their high resolution and acquisition frequency properties compared to other systems [9]. Different ionization

Table 1: The advantages and disadvantages of the analytical platforms.

Analytical platform	Advantages	Disadvantages
LC-MS	– High sensitivity and specificity – Ability to detect and quantify a wide range of metabolites	– Complex sample preparation – High cost of instrumentation and maintenance
GC-MS	– High sensitivity for volatile metabolites – Ability to detect isomers	– Complex sample preparation – Lower sensitivity for non-volatile metabolites
NMR spectroscopy	– Non-invasive – High reproducibility – No sample preparation required	– Lower sensitivity – Limited to detecting small metabolites

techniques, including atmospheric-pressure chemical ionization (APCI), electrospray ionization (ESI) and atmospheric-pressure photoionization (APPI), can be used for ion suppression or enhancement of the molecules in LC-MS based untargeted metabolomics [25]. More detailed information on ionization techniques will be provided in the following sections. LC-MS is the most preferred analytical platform in metabolomic analyzes due to its higher selectivity, sensitivity and identified metabolite number [26, 27]. Compared to NMR, LC-MS can detect metabolites at the picomolar and nanomolar levels [28]. Amino acids and derivatives, fatty acids, glycerolipids, glycerophospholipids, sphingolipids, and other lipid classes, carbohydrates, sugars, nucleotides, nucleosides, can be detected in biological samples by using LC-MS based untargeted metabolomics [29].

Preanalytical variations

Sample type

Determining sample type and sampling method is essential for standardizing the preanalytical phase. Different factors such as target metabolites, study objectives, transfer time, hemolysis risk, transfer conditions, targeted system or organ and turnaround time should be considered in determining sample type. Many different sample types have been used in the metabolomics analyses, including serum [30], plasma [31], amniotic fluid, urine [32], cell [33], tissue [32], saliva [34], cerebrospinal fluid [35] and milk [36]. Among them, plasma and serum are the most commonly used biofluids in metabolomics analyses [37]. Although serum and plasma are obtained from whole blood, different tubes are needed and undergo different processes after collection. The liquid component of blood that remains after clotting and removal of blood cells is known as serum. It is obtained by allowing a blood sample to clot in a tube, followed by centrifugation to remove the fibrin, blood cells and any remaining clotting factors [38]. Plasma and serum can be used interchangeably for many metabolites. However, it is important to keep in mind that there may be differences between the two sample types regarding the levels of some metabolites [37]. The most important reasons for the differences between the two sample types are the use of anticoagulants, the difference in the process of the centrifuge and sampling tube. Liu et al. discovered notable variations in 216 metabolites between serum and plasma samples [38]. UPLC-MS analyses have identified potential variations between plasma and serum samples in relation to specific metabolites. For instance, lysophosphatidylethanolamine (18:0) and lysophosphatidic acid (20:0) have been observed to be higher in serum samples compared to plasma. On the other hand, certain phosphatidylcholines such as (16:1/18:2, 20:3/18:0, O-20:0/22:4), lysoPC (16:0), sphingomyelin (18:0/22:0), and linoleic acid were found to be lower in serum samples [39]. Some differences were also detected in amino acids between serum and plasma [40]. Yu and colleagues noted that while the reproducibility of serum and plasma samples was good, the plasma measurements were generally higher than serum [37].

The choice of anticoagulants significantly influences the reproducibility of metabolomics analyses, with different tubes containing individual chemical structures. Common anticoagulants for obtaining plasma include heparin, EDTA, and citrate. In a study comparing the effects of EDTA and citrate in LC-MS-based metabolomics analyses, the type of anticoagulant was found to significantly impact the expressions of various lipids and amino acids. These included glycerophospholipids, acylcarnitine, sphingolipids, diacylglycerols, triacylglycerols, cholesteryl esters, as well as aspartate, histidine, and glutamine [41]. In metabolomic studies, choosing the right anticoagulant is essential because it can greatly impact the quality of the findings. Proper selection and handling of the anticoagulant will help ensure the accuracy and reliability of metabolomic data generated.

Spot urine is a commonly chosen sample for metabolomics analysis due to its ease of collection and non-invasiveness, whereas the collection and storage of a 24-h

urine sample pose challenges, making it less preferred. A key issue in urine-based metabolomics is the substantial concentration variation in metabolome levels among individuals. To address this, before analysis, creatinine and urine osmolality values in the urine sample should be determined, and metabolite intensities must be normalized based on creatinine or osmolality values [42]. Other problems in urine sampling are storage and sample stability. Samples should be stored at 4 °C as soon as possible after collection to decrease contamination and metabolite degradation [43]. Several preservatives such as sodium azide, hydrochloric acid, thymol and boric acid [44, 45] are used in the chemical analyses of urine samples to prevent bacterial overgrowth and metabolite degradation. However, preservatives should be used with caution as they may cause interference.

Given the presence of cells, crystals, or cylinders in urine, it is crucial to remove these structures to prevent issues in analysis and sample extraction. Filtration or centrifugation is commonly employed for this purpose, but care must be taken to prevent the release of metabolites from cells into the urine during these processes. Metabolite stability can be significantly impacted by freeze-thaw cycles; hence, it is recommended to limit cycles to a maximum of two to maintain sample integrity [46]. Roux et al. reported that storage of urine samples at 4 °C inhibited bacterial overgrowth for at least a 72-h period and slowed the chemical degradation process [43].

Tissues have sophisticated compositions containing various morphological cell types and extracellular matrix material. Therefore, obtaining a representative and homogeneous tissue sample requires careful consideration [47]. In animal studies, complete tissue can be harvested; however, limited tissue samples by using biopsy techniques or surgery can be obtained as representative tissue samples in humans. After the harvesting procedure specimen should be rinsed to remove the remaining blood and then frozen as soon as possible to overcome continuing metabolic changes and enzymatic degradation of tissue samples [48, 49].

The cell culture technique is frequently used to establish many disease models, such as cancer and neurological pathologies. It has many advantages, such as working with different cell types, getting results faster than *in-vivo* studies, and obtaining genetically modified lines. Footprint or fingerprint approaches are used in cell culture metabolomics [50]. While the footprint approach aims to detect extracellular metabolites, the fingerprint approach seeks to detect intracellular metabolites [51]. These approaches can be used together or separately. Cell culture metabolomics poses challenges such as the impact of growth medium, standardizing cell number and density, and cell quenching difficulties. To address these issues, a novel and powerful tool called single-cell metabolomics has been introduced. This technique allows for the determination of metabolic profiles in individual cells, eliminating metabolites from different tissues or cell groups. This enhances the probability of identifying primary disease-contributing metabolites [52–54].

Sample storage

Maintaining the quality of samples is of utmost importance in metabolomics since the metabolites under analysis are often vulnerable to decay and instability. Various factors such as temperature, pH, exposure to light and air can all play a significant role in affecting the stability of metabolites in biological samples [55]. To mitigate the impact of storage, it is crucial to exercise caution when handling and storing samples. The temperature at which samples are stored is a critical factor to be considered. To maintain the integrity of metabolites, it is generally recommended to store samples at extremely low temperatures, typically −80 °C or below. Such low temperatures can help impede metabolic processes that may otherwise cause deterioration of the samples. Samples can be stored at −80 °C for extended periods, often for years, without significant degradation. Another important factor to consider is the type of storage container [56–61]. It is advisable to store samples in containers that are airtight and chemically inert, as well as resistant to potential leaching or contamination. While glass or plastic vials are commonly utilized for storing samples, Ensuring the compatibility of the material used with the specific type of sample being stored is crucial [62, 63]. The quality of stored samples is influenced not only by temperature and storage containers but also by the duration of storage. Ideally, samples should be analyzed promptly after collection to minimize degradation. However, circumstances may necessitate longer storage times, making regular quality checks crucial to ensure stability for subsequent analysis. Adhering to standardized methods for sample collection, processing, and storage is essential to maintain optimal quality. Guidelines from organizations like the Metabolomics Standards Initiative (MSI) provide valuable benchmarks for proper sample handling in metabolomics [64]. These guidelines provide recommendations for sample collection, processing, and storage, as well as quality control and assurance measures to ensure the reliability and reproducibility of metabolomics data. Generally, multiple aliquots should be composed for each sample types, which are stored at −80 °C to slow or prevent enzymatic changes of metabolites. Multiple freeze/thaw cycles should not be used.

Sample preparation

Determining the appropriate sample volume is an essential part of the extraction step. The sample volume obtained after the extraction process should exceed the minimum sample volume required for analysis. Generally, 1–100 mg of tissue, 10^6, and 10–250 µL of biofluids are acceptable for the extraction phase. On the other hand, these values may vary between devices. Thus, the minimum sample volume must be determined before the extraction step.

The sample preparation should be fast and involve a minimum number of steps. It should also be non-selective for untargeted metabolomics analysis. Multiple sample types and analytic techniques can be used in metabolomics analyses; therefore, each sample type and analytical platform need different sample preparation procedures. Direct injection of samples is not possible due to the use of sensitive column and detector technology in mass spectrometric techniques [65]. Protein, salt, and some lipids in the sample must be removed [66, 67]. In untargeted metabolomics with LC-MS, a common approach for sample preparation is solvent protein precipitation, often using a one-step extraction method with one or more solvents simultaneously. The choice of solvent is crucial, considering the polarity index, which determines the range of extracted metabolites. Solvents, listed in order of increasing polarity index, include water, dichloromethane, acetone, methanol, acetonitrile, acetone, and hexane. Highly polar solvents pose challenges for effective extraction of lipid-derived metabolites. Researchers must carefully select the solvent based on the desired metabolite profile [27].

Dilute and shoot methods are commonly used in urine and targeted metabolomics as they are fast, simple, and require minimal sample preparation. In this approach, a small volume of urine is diluted with a suitable solvent to reduce matrix effects, and then directly injected into the analytical instrument for analysis [68, 69]. This method is advantageous for compounds with high abundance and low variability in urine, where sample dilution does not significantly affect the analytical signal. It is also useful approach when limited sample volume is available, or the metabolites of interest are not retained by the sample preparation sorbent [70]. However, the dilute and shoot method should be used with caution. The high complexity of urine matrix and the large dynamic range of metabolite concentrations can affect the detection of low abundance metabolites.

Equipment such as needle tip filters is critical for the success of the extraction phase. Syringe filters are commonly used in untargeted metabolomics sample preparation to remove unwanted particles and impurities from biological samples, including urine, blood, and plasma. Nylon syringe filters with a pore size of 0.22 µm

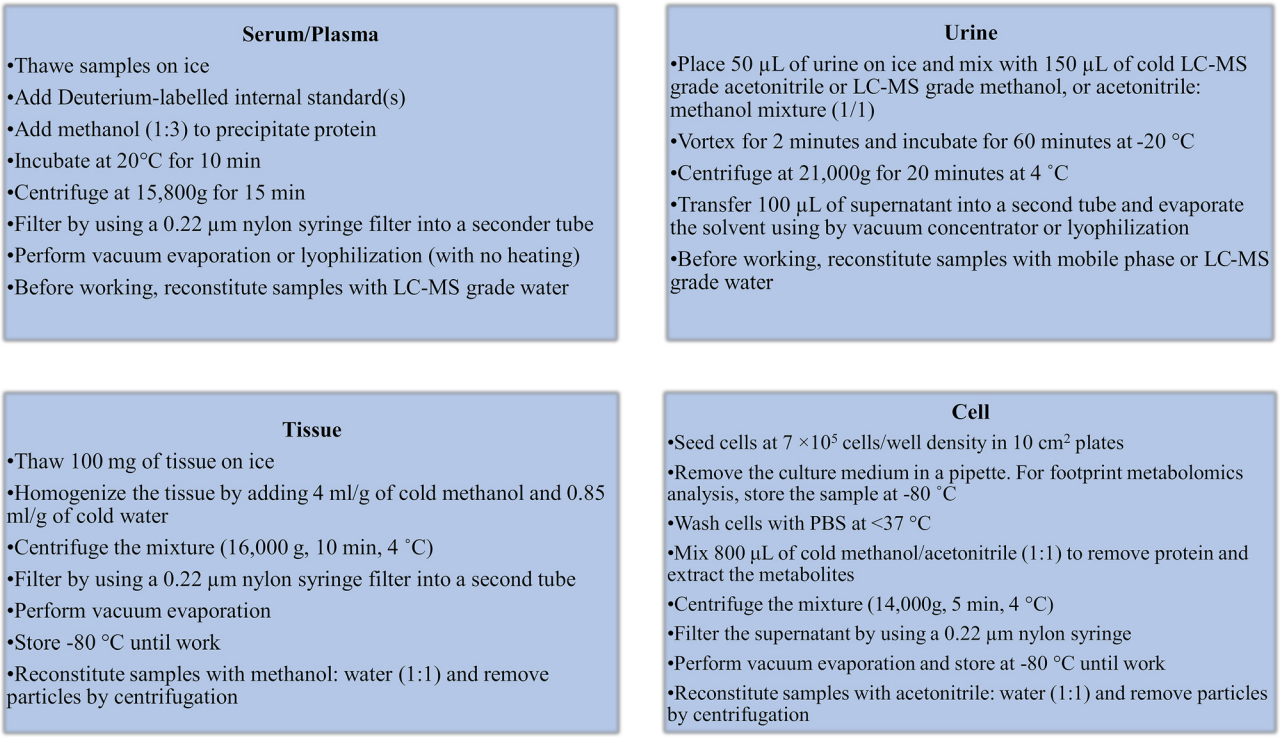

Figure 1: Sample preparation strategies for LC-MS based untargeted metabolomics analysis of diverse sample types.

are commonly used in metabolomics. The use of 0.22 µm nylon syringe filters is especially important for LC-MS-based metabolomics analysis, where the presence of particulates or impurities can interfere with chromatographic separation and reduce the sensitivity and accuracy of the analytical method. All solvents should be evaporated after filtration stage to normalize and optimize the samples. Different techniques can be used, including vacuum concentrators and lyophilization for this purpose [7, 71–73]. Sample extraction procedures for untargeted metabolomics for each sample type were given in Figure 1.

Quality assurance of metabolomics

The validity of measurements in the laboratory is ensured through quality control (QC) procedures, which aim to reduce precision errors and analytic bias. Various internal and external programs can be utilized for this purpose. Quality assurance and control procedures for targeted LC-MS analyses are highly standardized, and guidance on these procedures can be obtained from the Clinical and Laboratory Standards Institute (CLSI) C62-A document [74]. The CLSI C62-A document provides comprehensive information on various aspects related to quality control procedures in LC-MS analyses. This includes guidelines for the selection of appropriate quality control materials, frequency of analysis, and instructions on evaluating corrective actions to be taken in case of failed QC. Additionally, the document provides guidance on ensuring accessibility to QC procedures.

The application of QC procedures in untargeted metabolomics is crucial to identify and rectify potential sources of bias or errors that may impact the results when attempting to identify as many metabolites as possible in a sample. While some alternative approaches to quality control (QC) procedures have been reported for LC-MS based untargeted metabolomics, there is currently no widely accepted or standardized procedure for routine use [75–78]. In untargeted metabolomics, QC procedures can be classified into three main categories: device-related, sample-related, and analysis-related procedures. The device QC procedure involves ensuring the proper functioning of the injector, column, ionization source, and mass spectrometer performance. To carry out these procedures, it is important to obtain detailed information from the producer on the control procedure and frequency of these device parts. Several standard sample and analyze related QC procedures are used in untargeted metabolomics to achieve this goal, including the use of blank, pooled samples, internal standards, replicates, and reference samples.

Blank sample handling is a method used to address background signals, carryover or contaminants that may be present in the instrument or reagents. This is necessary to provide a baseline measurement and ensure accurate results [75]. Another QC procedure commonly used in untargeted metabolomics is the pooled sample. The pooled sample is created by combining equal amounts of the analyzed samples. Adding a pooled sample to the QC procedure in untargeted metabolomics is a useful way to increase the validity and reliability of the results and to enhance confidence in the conclusions drawn from the data. The incorporation of a pooled sample enables researchers to evaluate the performance of their analytical method and detect any potential sources of error or bias that could impact the results [79]. If the results obtained from the pooled sample are significantly different from those obtained from the individual samples, it suggests a problem with the analytical method or sample preparation that needs to be addressed [75, 79, 80].

To correct for potential variations in the sample preparation or measurement process in untargeted metabolomics, internal standards of known amounts are added to the samples. These standards can be deuterated and labeled with stable isotopes. They are particularly useful to normalize the data and account for differences in extraction efficiency, sample preparation, and measurement conditions. Deuterated internal standards have several advantages over other internal standards, and their use can significantly improve the accuracy and reliability of the results [75, 77]. Repeat analysis of a subset of samples, also known as replicates, and the use of reference samples are another useful tools to detect any technical or analytical variation and assess the results' reliability [77, 81].

Column strategies

The choice of column strategy will depend on several factors, including the nature of the sample, the size and complexity of the metabolites, and the study's goals. Some commonly used column strategies in LC-MS based untargeted metabolomics include reverse phase chromatography (RPC) and hydrophilic interaction liquid chromatography (HILIC) [82].

Reverse phase chromatography uses a hydrophobic stationary phase, such as C18, to retain hydrophobic metabolites in the sample. This column strategy is often used to analyze small, polar, and medium polar metabolites, such as amino acids and organic acids [83]. Using RPC in LC-MS based untargeted metabolomics can provide high-resolution separations of hydrophobic metabolites, improve the sensitivity of the analysis, and increase the selectivity of the results.

The HILIC strategy uses a hydrophilic stationary phase such as an amide to retain hydrophilic metabolites in the sample. This column strategy is often used to analyze polar and polarizable metabolites. The retention mechanism of HILIC and RPC is different. HILIC uses hydrogen bonding and dipole-dipole interactions to retain hydrophilic metabolites. It is particularly useful for the analysis of very polar metabolites such as sugars and glycerophospholipids [83–86]. Both HILIC and RPC are valuable column strategies for untargeted metabolomics. A combination of both HILIC and RPC may be necessary to fully characterize the metabolic profile of a complex biological sample [27, 87].

MS acquisition strategies

Acquisition strategies refer to the methods used to obtain mass spectrometric data from a sample. Different acquisition techniques, including full scan MS, selected ion monitoring (SIM), multiple reaction monitoring (MRM), data-dependent acquisition (DDA) and data-independent acquisition (DIA), can be used in LC-MS based metabolomics [88].

Full scan MS is the most basic acquisition strategy and involves acquiring a complete mass spectrum of all the ions present in the sample. In this method, the MS scans over a specified mass range and detects all the ions in the sample. It is often used as a screening method to identify the presence of specific ions [10]. To monitor the intensity of one or more specific ions, SIM mode can be used. In this method, the MS is set to detect only the specified ions, and the intensities of these ions are recorded over time. SIM is often used to increase the sensitivity and specificity of the analysis, as it reduces the background noise and improves the signal-to-noise ratio [89]. MRM acquisition strategies are being used to detect specific transitions between precursor and product ions. In this method, the MS is set to detect only specific precursor-product ion pairs, and the intensities of these pairs are recorded over time. MRM is often used to increase the specificity of the analysis, as it allows for the detection and quantification of specific metabolites in a sample [90–95]. DDA and DIA are the common acquisition strategies used in untargeted metabolomics. To speed up the process of collecting data in untargeted metabolomics, a new mode called DDA has been developed. In this mode, the mass spectrometry (MS) instrument performs a full-scan followed by MS2 analysis on a list of precursor ions chosen from the full-scan spectrum. This allows for both quantitative and structural information to be acquired at the same time, making it easier to identify metabolites. However, DDA has some limitations, such as the potential to miss low abundance metabolites that are not selected for fragmentation and reduced signal intensity for MS1 features. To overcome these limitations, DIA has been recently implemented in metabolomics studies. DIA allows for the generation of MS2 spectra for all precursor ions, which can detect and identify more metabolites, especially those at lower concentrations. In DIA mode, the MS instrument cycles through the precursor ion m/z range with a large precursor ion mass width to fragment multiple precursor ions at once. AIF and MS^{All}/MS^{E} are common DIA methods that enable continuous and unbiased acquisition of MS2 information for all metabolites [10, 96].

Annotation

Annotation in untargeted metabolomics refers to the process of identifying the chemical structure and name of metabolites in a sample. It aims to match the mass spectral data obtained from the sample with reference spectral data in a database, to identify the metabolites [18, 97]. Several steps are involved in the annotation process, including data preprocessing, feature detection, metabolite identification and confirmation of identification [98]. Data preprocessing involves cleaning and processing the raw data obtained from the mass spectrometer to ensure the data is high quality and free from any artefacts. This step may include removing background noise, baseline correction, and data normalization [99]. The feature detection step involves detecting and extracting peaks from the processed data corresponding to the sample's metabolites. The peaks are then compared to a database of reference spectral data to determine the most likely candidate metabolites [100, 101]. This may involve different algorithms and techniques, such as database searching, spectral matching, and fragment pattern analysis. Identified metabolites should be verified using additional information, such as retention time, mass accuracy, and additional MS/MS data. This step aims to confirm the identification of the metabolites and reduce the risk of false positive identifications [18, 97, 98, 102, 103].

Database searching technique searches the mass spectral data obtained from the sample against a reference database of known metabolites to determine the most likely candidate metabolites. This technique is based on comparing the mass-to-charge ratio (m/z) and the retention time of the metabolites in the sample with those in the reference database. Some commonly used databases include the Human Metabolome Database (HMDB) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [104, 105]. In fragment pattern analysis, the chemical structure of the metabolites is ascertained by examining the fragments generated from the

metabolites during the mass spectrometric analysis. To identify the most likely potential metabolites, the fragments are matched to a database of known fragments [106, 107].

Bioinformatics

Bioinformatics plays a crucial role by providing tools for analyzing, interpreting, and visualizing large and complex data sets. Using bioinformatics can significantly enhance metabolomics studies' speed, accuracy, and reproducibility and provide new insights into the biology and biochemistry of cells and tissues. These are some of the key applications and bioinformatic tools, including commercial and open-source software for analyzing LC-MS based untargeted metabolomics data (Figure 2).

Clinical applications of untargeted metabolomics

The clinical application of untargeted metabolomics has the potential to revolutionize the diagnosis and treatment of many diseases, including cancer, metabolic disorders, and cardiovascular diseases. Some specific examples of the clinical applications of untargeted metabolomics include cancer, infectious disease, diabetes, and cardiovascular disease. Overall, the clinical application of untargeted metabolomics holds great promise for improving disease diagnosis, personalized medicine, drug development, and lifestyle interventions [108, 109].

Cancer and untargeted metabolomics

Cancer is a disease characterized by uncontrolled cell growth and proliferation. One of the hallmarks of cancer is metabolic reprogramming, which refers to changes in how cancer cells use energy and nutrients to support their growth and survival. Metabolic changes in cancer cells can include increased glucose uptake, altered glucose metabolism, changes in amino acid metabolism, fatty acid uptake and synthesis, and changes in mitochondrial function [110]. Understanding the metabolic changes in cancer cells is essential for developing new diagnostic and therapeutic strategies that target these processes. In recent years, increasing interest has been in targeting cancer metabolism to improve cancer treatment outcomes [110–112]. Early diagnosis, tumor classification, response to therapy, and tumor metabolism were some specific examples of the clinical applications of untargeted metabolomics [113]. The metabolic profile of cancer cells is often distinct from normal cells, making it possible to identify metabolic biomarkers that can be used for early cancer detection. Zhu and colleagues discovered that certain metabolic pathways, including inositol phosphate metabolism, primary bile acid biosynthesis, phosphatidylinositol signaling system, and linoleic acid metabolism, play a significant role in the development of colorectal cancer. They suggested that by using GC-MS based metabolomics and pattern recognition techniques, it may be possible to identify cancer-specific changes early on, leading to early diagnosis and the development of new treatments [114]. Research suggests that combining untargeted metabolomics with machine

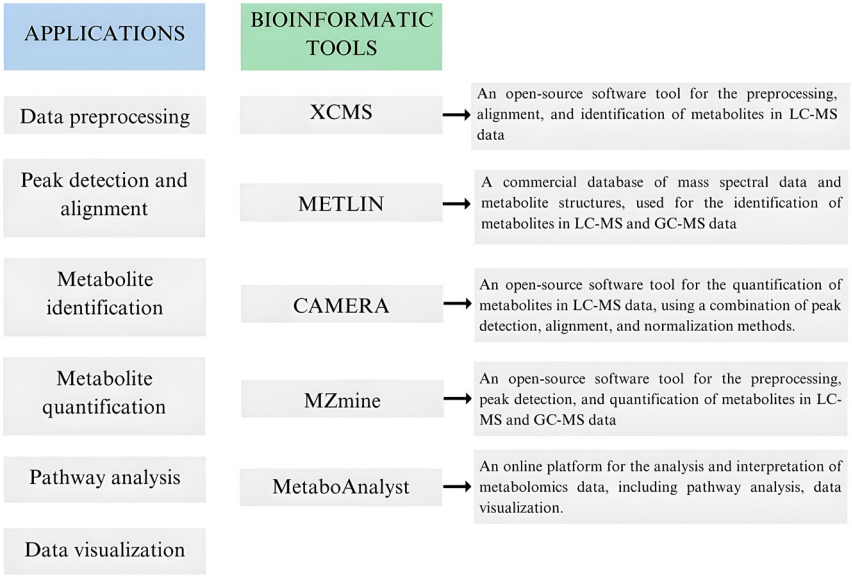

Figure 2: Some commercial and open-source software for analyzing LC-MS based untargeted metabolomics data.

learning approaches in female-specific cancers could offer a substantial advantage in developing early detection methods for these types of cancers [115]. According to Liu and colleagues, urine and plasma samples exhibit distinct metabolic signatures in cases of ovarian tumors. As a result, untargeted metabolomics analyses may have the potential to provide valuable insights into the classification of ovarian cancer. By identifying unique metabolic profiles associated with different subtypes of ovarian cancer, this approach may help improve the accuracy of diagnosis and guide the development of personalized treatment strategies for patients with this disease [116]. Untargeted metabolomics analyses can be used to estimate progression and create a personalized treatment. Yang and colleagues linked alterations in glycerophospholipid metabolism to the development and advancement of esophageal squamous cell carcinoma. Additionally, the authors proposed that targeting glycerophospholipid metabolism could be a potential therapeutic strategy for treating esophageal squamous cell carcinoma. These findings suggest that manipulating the metabolic pathways involved in the synthesis and degradation of glycerophospholipids could be a promising approach to developing new treatments for this type of cancer [117]. Hexadecasphinganine, linoleamide, and N-hydroxy arachidonoyl amine have been identified as potential diagnostic markers for gastric cancer. These molecules may be used as biomarkers to detect the presence of gastric cancer, enabling earlier diagnosis and treatment [118]. Research has indicated that untargeted metabolomics analysis using the LC-MS platform can identify a metabolite panel that has the potential to predict the response of locally advanced rectal cancer to neoadjuvant chemo-radiation therapy. This approach may help to personalize treatment strategies for individual patients by identifying those who are likely to benefit from this therapy and those who may require alternative treatment options. These findings suggest that using metabolomics to guide treatment decisions may improve patient outcomes in locally advanced rectal cancer [119]. The use of receiver operating characteristic (ROC) analysis has identified. These biomarkers include Leukotriene A4, PC (24:1 (15Z)/24:1 (15Z)), TG (17:0/17:0/18:0), hypoxanthine, and cis-ACCP has been identified potential biomarkers for early detection of breast cancer in a LC-QTOF-MS based untargeted metabolomics [120].

Untargeted metabolomics can provide valuable insights into cancer metabolism, diagnosis, the discovery of novel biomarkers, identification of therapeutic targets and personalized medicine, but its application requires careful consideration of the limitations such as complex data analysis, technical variability, and limited coverage of metabolites.

Infectious disease and untargeted metabolomics

Untargeted metabolomics has been applied to studying various infectious diseases caused by viruses, bacteria, and parasites. Untargeted metabolomics can contribute to the study of infectious diseases in the fields of host-pathogen interaction, diagnosis, and monitoring response to therapy. Infections can cause significant changes in the metabolic profile of the host. Studies have identified plasma lipids and alterations in their levels as potential biomarkers for detecting Coronavirus disease 2019 (COVID-19) infection [121]. It has been reported that glucose metabolism and the urea cycle may be targets for treating COVID-19 at various disease stages [122]. Deoxycytidine, ureidopropionate, kynurenine, and multiple short-chain acylcarnitines have been determined as candidate biomarkers for predicting COVID-19 severity [123]. Doğan et al. found downregulations in R-S lactoglutathione and glutamine, besides upregulations in hypoxanthine, inosine, and leukotriene D4 in COVID-19 patients. Considering the identified metabolites, purine, glutamine, LTD4, and glutathione metabolisms were associated with the pathogenesis of COVID-19 [30]. Further research in this area could help improve our understanding of the pathogenesis of COVID-19 and facilitate the development of effective diagnostic and therapeutic strategies. Untargeted metabolomic analyses have also been performed on diseases other than COVID-19, leading to significant new findings. Chen et al. indicated that dysregulation of amino acid metabolism might have a role in regulating inflammation and immunity in patients with sepsis [124]. Untargeted metabolomics analysis revealed that *Mycobacterium tuberculosis* induced tryptophan metabolism in human macrophages. This finding has been associated with a potential therapeutic strategy for pulmonary tuberculosis [125]. In an untargeted serum metabolomics analysis, glycerophospholipid species have been found as potential trichinellosis markers [126]. The study identified dysregulation in the metabolism of amino acids, as well as the biosynthesis of phosphatidylcholines and lysophosphatidylcholines, which have been linked to the development and treatment of hepatitis B virus infection [127]. Untargeted metabolomics provides valuable information about the metabolic changes occurring in the host and pathogen during infectious diseases. This information can be used to develop new diagnostic tools, and therapies, and improve our understanding of the host-pathogen interaction.

Coronary heart disease and untargeted metabolomics

Coronary heart disease (CHD) is a medical condition in which plaque builds up inside the coronary arteries, the blood vessels that supply oxygen-rich blood to the heart muscle. The plaque is made up of cholesterol, fat, calcium, and other substances, and it can narrow or block the arteries, reducing blood flow to the heart. This can lead to chest pain (angina), shortness of breath, heart attack, or other complications. CHD is a common type of heart disease and can be caused by various factors, including high blood pressure, high cholesterol levels, smoking, diabetes, and a family history of heart disease.

A strong relationship exists between metabolic changes and coronary heart disease (CHD). Metabolic changes refer to alterations in the way the body processes nutrients and energy, including glucose, fats, and proteins. Some of the metabolic changes, including insulin resistance, dyslipidemia, obesity, type 2 diabetes, and metabolic syndrome, are associated with an increased risk of CHD. Metabolic changes can contribute to the pathogenesis of CHD through various mechanisms, including damage to the endothelium, inflammation, and plaque formation within the coronary arteries. Management of metabolic changes through lifestyle modifications, medications, and other interventions can help reduce the risk of CHD. There is a growing interest in using metabolomics to understand the pathogenesis of CHD and to identify biomarkers for early detection and personalized treatment.

Several studies have shown that patients with CHD have altered lipid, glucose, and amino acid metabolism, among others. According to Ullah et al., alterations in the metabolism of D-arginine and D-ornithine, glycolysis, oxidation and degradation of branched-chain fatty acids, and sphingolipid metabolism have been linked to coronary heart disease (CHD) [128]. It has been discovered that serum oxyneurine and triglyceride are candidate biomarkers for the early diagnosis of CHD [129]. Nineteen metabolites related to amino acids, lipids, peptides, carbohydrates, nucleotide, and xenobiotics metabolism have been identified as biomarkers in predicting CHD risk [130]. The study observed inverse correlations between creatine, creatinine, and phenylalanine, and positive correlations between mannose, acetaminophen-glucuronide, lactate, and apolipoprotein B, with incidents of cardiovascular disease [131]. Upregulation in metabolites related to the fatty acid oxidation and glucose oxidation pathways, including oleic acid, stearic acid, palmitic acid, linoleic acid, galactose, pyruvic and lactic acids, has been associated with CHD [132]. Branch chain amino acids, L-arginine, linoleic acid, L-serine, L-cysteine, fructose-6-phosphate, glycerol, creatine and 3-phosphoglyceric acid have been identified as candidate biomarkers to explain the pathogenesis of type 2 diabetes mellitus assisted CHD [133].

Biomarkers are measurable indicators that can be used to predict the risk of disease, diagnose the disease, or monitor disease progression. Untargeted metabolomics can help identify biomarkers for CHD. Using untargeted metabolomics, several metabolites have been identified as potential biomarkers for CHD. Furthermore, metabolomics can help identify targets for therapeutic interventions. By identifying specific metabolic pathways dysregulated in CHD, researchers can develop targeted therapies to restore metabolic homeostasis and prevent or treat CHD.

Discussions and future of metabolomics

The future of untargeted metabolomics is promising, and this rapidly growing field of research has the potential to revolutionize healthcare in the coming years. By identifying changes in metabolic profiles that occur before clinical symptoms appear, we can detect diseases at their earliest stages when they are most treatable. This could lead to more effective treatments, improved patient outcomes, and reduced healthcare costs. Another exciting application of untargeted metabolomics is in the field of nutritional research. By identifying the metabolic pathways influenced by specific nutrients and dietary patterns, researchers can develop targeted interventions to prevent and treat disease. This could lead to the development of personalized nutrition plans tailored to the specific needs of everyone. The future of untargeted metabolomics is bright, and we can expect to see this field of research transform healthcare in the coming years. The development of new technologies and methods, coupled with increased collaboration between researchers, clinicians, and industry partners, will help unlock the full potential of metabolomics and improve health outcomes for patients worldwide.

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References

1. Castelli FA, Rosati G, Moguet C, Fuentes C, Marrugo-Ramírez J, Lefebvre T, et al. Metabolomics for personalized medicine: the input of analytical chemistry from biomarker discovery to point-of-care tests. Anal Bioanal Chem 2022;414:759–89.
2. Horgusluoglu E, Neff R, Song WM, Wang M, Wang Q, Arnold M, et al. Integrative metabolomics-genomics approach reveals key metabolic pathways and regulators of Alzheimer's disease. Alzheimer's Dementia 2022;18:1260–78.
3. Thistlethwaite LR, Li X, Burrage LC, Riehle K, Hacia JG, Braverman N, et al. Clinical diagnosis of metabolic disorders using untargeted metabolomic profiling and disease-specific networks learned from profiling data. Sci Rep 2022;12:6556.
4. Duarte-Delgado NP, Cala MP, Barreto A, Rodríguez LSC. Metabolites and metabolic pathways associated with rheumatoid arthritis and systemic lupus erythematosus. J Transl Autoimmun 2022;5:100150.
5. Alseekh S, Aharoni A, Brotman Y, Contrepois K, D'Auria J, Ewald J, et al. Mass spectrometry-based metabolomics: a guide for annotation, quantification and best reporting practices. Nat Methods 2021;18: 747–56.
6. Zhou J, Zhong L. Applications of liquid chromatography-mass spectrometry based metabolomics in predictive and personalized medicine. Front Mol Biosci 2022;9:1049016.
7. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc 2011;6: 1060–83.
8. Rakusanova S, Fiehn O, Cajka T. Toward building mass spectrometry-based metabolomics and lipidomics atlases for biological and clinical research. TrAC Trends Anal Chem 2022;158:116825.
9. Schwaiger-Haber M, Stancliffe E, Arends V, Thyagarajan B, Sindelar M, Patti GJ. A workflow to perform targeted metabolomics at the untargeted scale on a triple quadrupole mass spectrometer. ACS Meas Sci Au 2021;1:35–45.
10. Defossez E, Bourquin J, von Reuss S, Rasmann S, Glauser G. Eight key rules for successful data-dependent acquisition in mass spectrometry-based metabolomics. Mass Spectrom Rev 2023;42:131–43.
11. Roberts LD, Souza AL, Gerszten RE, Clish CB. Targeted metabolomics. Curr Protoc Mol Biol 2012;98:30.2.1–24.
12. Visconti G, Olesti E, González-Ruiz V, Glauser G, Tonoli D, Lescuyer P, et al. Internal calibration as an emerging approach for endogenous analyte quantification: application to steroids. Talanta 2022;240: 123149.
13. Jia M, Peng Z, Yang K, Su C, Wang Y, Yan C. A high-throughput targeted metabolomics method for the quantification of 104 non-polar metabolites in cholesterol, eicosanoid, and phospholipid metabolism: application in the study of a CCl 4-induced liver injury mouse model. Analyst 2020;145:3575–91.
14. Di Minno A, Gelzo M, Stornaiuolo M, Ruoppolo M, Castaldo G. The evolving landscape of untargeted metabolomics. Nutr Metabol Cardiovasc Dis 2021;31:1645–52.
15. Steuer AE, Brockbals L, Kraemer T. Untargeted metabolomics approaches to improve casework in clinical and forensic toxicology —"where are we standing and where are we heading?" WIREs Forensic Sci 2022;4:e1449.
16. Fang Y, Duan C, Zhang J, Dai Y, Xia Y. NMR-based untargeted metabolomics approach to investigate the systemic lipid metabolism regulation of norisoboldine in collagen-induced arthritis rats. Eur J Pharmacol 2021;912:174608.
17. Hoffmann MA, Nothias L-F, Ludwig M, Fleischauer M, Gentry EC, Witting M, et al. High-confidence structural annotation of metabolites absent from spectral libraries. Nat Biotechnol 2022;40:411–21.
18. Dunn WB, Erban A, Weber RJ, Creek DJ, Brown M, Breitling R, et al. Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. Metabolomics 2013;9:44–66.
19. Salek RM, Arita M, Dayalan S, Ebbels T, Jones AR, Neumann S, et al. Embedding standards in metabolomics: the metabolomics society data standards task group. Metabolomics 2015;11:782–3.
20. Shahaf N, Rogachev I, Heinig U, Meir S, Malitsky S, Battat M, et al. The WEIZMASS spectral library for high-confidence metabolite identification. Nat Commun 2016;7:12423.
21. Folberth J, Begemann K, Jöhren O, Schwaninger M, Othman A. MS2 and LC libraries for untargeted metabolomics: enhancing method development and identification confidence. J Chromatogr B 2020; 1145:122105.
22. Wang JH, Byun J, Pennathur S. Analytical approaches to metabolomics and applications to systems biology. Semin Nephrol 2010;30:500–11.
23. Johnson CH, Gonzalez FJ. Challenges and opportunities of metabolomics. J Cell Physiol 2012;227:2975–81.
24. Pinu FR, Goldansaz SA, Jaine J. Translational metabolomics: current challenges and future opportunities. Metabolites 2019;9:108.
25. Garcia-Ac A, Segura PA, Viglino L, Gagnon C, Sauvé S. Comparison of APPI, APCI and ESI for the LC-MS/MS analysis of bezafibrate, cyclophosphamide, enalapril, methotrexate and orlistat in municipal wastewater. J Mass Spectrom 2011;46:383–90.
26. Miggiels P, Wouters B, van Westen GJ, Dubbelman A-C, Hankemeier T. Novel technologies for metabolomics: more for less. TrAC Trends Anal Chem 2019;120:115323.
27. Cajka T, Fiehn O. Toward merging untargeted and targeted methods in mass spectrometry-based metabolomics and lipidomics. Anal Chem 2016;88:524–45.
28. Knuuttila M, Hämäläinen E, Poutanen M. Applying mass spectrometric methods to study androgen biosynthesis and metabolism in prostate cancer. J Mol Endocrinol 2019;62:R255–67.
29. Gallart-Ayala H, Teav T, Ivanisevic J. Metabolomics meets lipidomics: assessing the small molecule component of metabolism. Bioessays 2020;42:2000052.
30. Doğan HO, Şenol O, Bolat S, Yıldız ŞN, Büyüktuna SA, Sarıismailoğlu R, et al. Understanding the pathophysiological changes via untargeted metabolomics in COVID-19 patients. J Med Virol 2021;93:2340–9.
31. Doğan HO, Şenol O, Karadağ A, Yıldız SN. Metabolomic profiling in ankylosing spondylitis using time-of-flight mass spectrometry. Clin Nutr ESPEN 2022;50:124–32.
32. Babu AF, Csader S, Männistö V, Tauriainen M-M, Pentikäinen H, Savonen K, et al. Effects of exercise on NAFLD using non-targeted metabolomics in adipose tissue, plasma, urine, and stool. Sci Rep 2022;12:6485.

33. Estrada-Pérez AR, Rosales-Hernández MC, García-Vázquez JB, Bakalara N, Fromager B, Correa-Basurto J. Untargeted LC-MS/MS metabolomics study on the MCF-7 cell line in the presence of valproic acid. Int J Mol Sci 2022;23:2645.
34. Saheb Sharif-Askari N, Soares NC, Mohamed HA, Saheb Sharif-Askari F, Alsayed HAH, Al-Hroub H, et al. Saliva metabolomic profile of COVID-19 patients associates with disease severity. Metabolomics 2022;18:1–16.
35. Li K, Schön M, Naviaux JC, Monk JM, Alchus-Laiferová N, Wang L, et al. Cerebrospinal fluid and plasma metabolomics of acute endurance exercise. FASEB J 2022;36:e22408.
36. Thomas S, Gauglitz JM, Tripathi A, Vargas F, Bertrand K, Kim JH, et al. An untargeted metabolomics analysis of exogenous chemicals in human milk and transfer to the infant. Clin Transl Sci 2022;15:2576–82.
37. Yu Z, Kastenmüller G, He Y, Belcredi P, Möller G, Prehn C, et al. Differences between human plasma and serum metabolite profiles. PLoS One 2011;6:e21230.
38. Liu X, Hoene M, Wang X, Yin P, Häring H-U, Xu G, et al. Serum or plasma, what is the difference? Investigations to facilitate the sample material selection decision making process for metabolomics studies and beyond. Anal Chim Acta 2018;1037:293–300.
39. Kaluarachchi M, Boulangé CL, Karaman I, Lindon JC, Ebbels TM, Elliott P, et al. A comparison of human serum and plasma metabolites using untargeted 1 H NMR spectroscopy and UPLC-MS. Metabolomics 2018;14:1–12.
40. Suarez-Diez M, Adam J, Adamski J, Chasapi SA, Luchinat C, Peters A, et al. Plasma and serum metabolite association networks: comparability within and between studies using NMR and MS profiling. J Proteome Res 2017;16:2547–59.
41. Khadka M, Todor A, Maner-Smith KM, Colucci JK, Tran V, Gaul DA, et al. The effect of anticoagulants, temperature, and time on the human plasma metabolome and lipidome from healthy donors as determined by liquid chromatography-mass spectrometry. Biomolecules 2019;9:200.
42. Kim K, Taylor SL, Ganti S, Guo L, Osier MV, Weiss RH. Urine metabolomic analysis identifies potential biomarkers and pathogenic pathways in kidney cancer. OMICS 2011;15:293–303.
43. Roux A, Thévenot EA, Seguin F, Olivier M-F, Junot C. Impact of collection conditions on the metabolite content of human urine samples as analyzed by liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectroscopy. Metabolomics 2015;11:1095–105.
44. Want EJ, Wilson ID, Gika H, Theodoridis G, Plumb RS, Shockcor J, et al. Global metabolic profiling procedures for urine using UPLC–MS. Nat Protoc 2010;5:1005–18.
45. Stevens VL, Hoover E, Wang Y, Zanetti KA. Pre-analytical factors that affect metabolite stability in human urine, plasma, and serum: a review. Metabolites 2019;9:156.
46. Rotter M, Brandmaier S, Prehn C, Adam J, Rabstein S, Gawrych K, et al. Stability of targeted metabolite profiles of urine samples under different storage conditions. Metabolomics 2017;13:1–9.
47. Zhang, X-F., Huang, Y., Gao, G., Cui, X. Current progress in bioprinting. In: Tripathi A, Melo J, editors. Advances in biomaterials for biomedical applications. Advanced structured materials. Singapore: Springer; 2017, vol LXVI. https://doi.org/10.1007/978-981-10-3328-5_6.
48. Smith L, Villaret-Cazadamont J, Claus SP, Canlet C, Guillou H, Cabaton NJ, et al. Important considerations for sample collection in metabolomics studies with a special focus on applications to liver functions. Metabolites 2020;10:104.
49. Fomenko MV, Yanshole LV, Tsentalovich YP. Stability of metabolomic content during sample preparation: blood and brain tissues. Metabolites 2022;12:811.
50. Kim D-H, Jarvis RM, Xu Y, Oliver AW, Allwood JW, Hampson L, et al. Combining metabolic fingerprinting and footprinting to understand the phenotypic response of HPV16 E6 expressing cervical carcinoma cells exposed to the HIV anti-viral drug lopinavir. Analyst 2010;135: 1235–44.
51. Bujak R, Struck-Lewicka W, Markuszewski MJ, Kaliszan R. Metabolomics for laboratory diagnostics. J Pharmaceut Biomed Anal 2015;113:108–20.
52. Hu R, Li Y, Yang Y, Liu M. Mass spectrometry-based strategies for single-cell metabolomics. Mass Spectrom Rev 2023;42:67–94.
53. Lanekoff I, Sharma VV, Marques C. Single-cell metabolomics: where are we and where are we going? Curr Opin Biotechnol 2022;75: 102693.
54. Zenobi R. Single-cell metabolomics: analytical and biological perspectives. Science 2013;342:1243259.
55. Hernandes VV, Barbas C, Dudzik D. A review of blood sample handling and pre-processing for metabolomics studies. Electrophoresis 2017; 38:2232–41.
56. Pinto J, Domingues MRM, Galhano E, Pita C, do Céu Almeida M, Carreira IM, et al. Human plasma stability during handling and storage: impact on NMR metabolomics. Analyst 2014;139:1168–77.
57. Cuhadar S, Koseoglu M, Atay A, Dirican A. The effect of storage time and freeze-thaw cycles on the stability of serum samples. Biochem Med 2013;23:70–7.
58. Breier M, Wahl S, Prehn C, Fugmann M, Ferrari U, Weise M, et al. Targeted metabolomics identifies reliable and stable metabolites in human serum and plasma samples. PLoS One 2014;9:e89728.
59. Mitchell BL, Yasui Y, Li CI, Fitzpatrick AL, Lampe PD. Impact of freeze-thaw cycles and storage time on plasma samples used in mass spectrometry based biomarker discovery projects. Cancer Inf 2005;1: 98–104.
60. Anton G, Wilson R, Yu Z-H, Prehn C, Zukunft S, Adamski J, et al. Pre-analytical sample quality: metabolite ratios as an intrinsic marker for prolonged room temperature exposure of serum samples. PLoS One 2015;10:e0121495.
61. Zivkovic AM, Wiest MM, Nguyen UT, Davis R, Watkins SM, German JB. Effects of sample handling and storage on quantitative lipid analysis in human serum. Metabolomics 2009;5:507–16.
62. Kirwan JA, Brennan L, Broadhurst D, Fiehn O, Cascante M, Dunn WB, et al. Preanalytical processing and biobanking procedures of biological samples for metabolomics research: a white paper, community perspective (for “precision medicine and pharmacometabolomics task group”—the metabolomics society initiative). Clin Chem 2018;64:1158–82.
63. International Society for Biological and Environmental Repositories (ISBER). Collection, storage, retrieval and distribution of biological materials for research. Cell Preserv Technol 2008;6:3–58.
64. Summer L, Amberg A, Barrett D, Beale M, Beger R, Daykin C, et al. Proposed minimum reporting standards for chemical analysis. Metabolomics 2007;3:211–21.
65. Mushtaq MY, Choi YH, Verpoorte R, Wilson EG. Extraction for metabolomics: access to the metabolome. Phytochem Anal 2014;25: 291–306.
66. Lin CY, Wu H, Tjeerdema RS, Viant MR. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. Metabolomics 2007;3:55–67.

67. Raterink R-J, Lindenburg PW, Vreeken RJ, Ramautar R, Hankemeier T. Recent developments in sample-pretreatment techniques for mass spectrometry-based metabolomics. TrAC Trends Anal Chem 2014;61: 157–67.
68. Klupczynska A, Plewa S, Sytek N, Sawicki W, Dereziński P, Matysiak J, et al. A study of low-molecular-weight organic acid urinary profiles in prostate cancer by a new liquid chromatography-tandem mass spectrometry method. J Pharmaceut Biomed Anal 2018;159:229–36.
69. Vuckovic D. Current trends and challenges in sample preparation for global metabolomics using liquid chromatography–mass spectrometry. Anal Bioanal Chem 2012;403:1523–48.
70. Fernández-Peralbo M, De Castro ML. Preparation of urine samples prior to targeted or untargeted metabolomics mass-spectrometry analysis. TrAC Trends Anal Chem 2012;41:75–85.
71. Begley P, Francis-McIntyre S, Dunn WB, Broadhurst DI, Halsall A, Tseng A, et al. Development and performance of a gas chromatography-time-of-flight mass spectrometry analysis for large-scale nontargeted metabolomic studies of human serum. Anal Chem 2009;81:7038–46.
72. Diémé B, Lefèvre A, Nadal-Desbarats L, Galineau L, Hounoum BM, Montigny F, et al. Workflow methodology for rat brain metabolome exploration using NMR, LC–MS and GC–MS analytical platforms. J Pharmaceut Biomed Anal 2017;142:270–8.
73. Cheng K, Brunius C, Fristedt R, Landberg R. An LC-QToF MS based method for untargeted metabolomics of human fecal samples. Metabolomics 2020;16:1–8.
74. CLSI document C62-A. Liquid chromatography-mass spectrometry methods. Approved guideline, 1st ed., 3rd ed. Wayne, PA: CLSI; 2014.
75. Dudzik D, Barbas-Bernardos C, García A, Barbas C. Quality assurance procedures for mass spectrometry untargeted metabolomics. a review. J Pharmaceut Biomed Anal 2018;147:149–73.
76. Kirwan JA, Gika H, Beger RD, Bearden D, Dunn WB, Goodacre R, et al. Quality assurance and quality control reporting in untargeted metabolic phenotyping: mQACC recommendations for analytical quality management. Metabolomics 2022;18:70.
77. Lippa KA, Aristizabal-Henao JJ, Beger RD, Bowden JA, Broeckling C, Beecher C, et al. Reference materials for MS-based untargeted metabolomics and lipidomics: a review by the metabolomics quality assurance and quality control consortium (mQACC). Metabolomics 2022;18:24.
78. Lewis MR, Pearce JT, Spagou K, Green M, Dona AC, Yuen AH, et al. Development and application of ultra-performance liquid chromatography-TOF MS for precision large scale urinary metabolic phenotyping. Anal Chem 2016;88:9004–13.
79. Sangster T, Major H, Plumb R, Wilson AJ, Wilson ID. A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. Analyst 2006;131:1075–8.
80. Dunn WB, Wilson ID, Nicholls AW, Broadhurst D. The importance of experimental design and QC samples in large-scale and MS-driven untargeted metabolomic studies of humans. Bioanalysis 2012;4: 2249–64.
81. Kirwan JA, Weber RJ, Broadhurst DI, Viant MR. Direct infusion mass spectrometry metabolomics dataset: a benchmark for data processing and quality control. Sci Data 2014;1:1–13.
82. Frigerio G, Moruzzi C, Mercadante R, Schymanski EL, Fustinoni S. Development and application of an LC-MS/MS untargeted exposomics method with a separated pooled quality control strategy. Molecules 2022;27:2580.
83. Kohler I, Giera M. Recent advances in liquid-phase separations for clinical metabolomics. J Separ Sci 2017;40:93–108.
84. Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique. Anal Bioanal Chem 2012; 402:231–47.
85. Hemström P, Irgum K. Hydrophilic interaction chromatography. J Separ Sci 2006;29:1784–821.
86. Guo Y. Recent progress in the fundamental understanding of hydrophilic interaction chromatography (HILIC). Analyst 2015;140: 6452–66.
87. Ivanisevic J, Zhu Z-J, Plate L, Tautenhahn R, Chen S, O'Brien PJ, et al. Toward 'omic scale metabolite profiling: a dual separation–mass spectrometry approach for coverage of lipid and central carbon metabolism. Anal Chem 2013;85:6876–84.
88. Fenaille F, Saint-Hilaire PB, Rousseau K, Junot C. Data acquisition workflows in liquid chromatography coupled to high resolution mass spectrometry-based metabolomics: where do we stand? J Chromatogr A 2017;1526:1–12.
89. Murray KK, Boyd RK, Eberlin MN, Langley GJ, Li L, Naito Y. Definitions of terms relating to mass spectrometry (IUPAC recommendations 2013). Pure Appl Chem 2013;85:1515–609.
90. Matraszek-Zuchowska I, Wozniak B, Posyniak A. Comparison of the multiple reaction monitoring and enhanced product ion scan modes for confirmation of stilbenes in bovine urine samples using LC–MS/MS QTRAP® system. Chromatographia 2016;79:1003–12.
91. Van Poucke C, Van De Velde M, Van Peteghem C. Combination of liquid chromatography/tandem mass spectrometry and gas chromatography/mass spectrometry for the detection of 21 anabolic steroid residues in bovine urine. J Mass Spectrom 2005;40:731–8.
92. Impens S, Van Loco J, Degroodt J, De Brabander H. A downscaled multi-residue strategy for detection of anabolic steroids in bovine urine using gas chromatography tandem mass spectrometry (GC–MS3). Anal Chim Acta 2007;586:43–8.
93. Seo J, Kim H-Y, Chung BC, Hong J. Simultaneous determination of anabolic steroids and synthetic hormones in meat by freezing-lipid filtration, solid-phase extraction and gas chromatography–mass spectrometry. J Chromatogr A 2005;1067:303–9.
94. Rejtharová M, Rejthar L. Development and validation of an LC-MS/MS method for the determination of six gestates in kidney fats. Food Addit Contam 2013;30:995–9.
95. Cohen Freue GV, Borchers CH. Multiple reaction monitoring (MRM) principles and application to coronary artery disease. Circ Cardiovasc Genet 2012;5:378.
96. Guo J, Huan T. Comparison of full-scan, data-dependent, and data-independent acquisition modes in liquid chromatography–mass spectrometry based untargeted metabolomics. Anal Chem 2020;92: 8072–80.
97. Zhou Z, Luo M, Zhang H, Yin Y, Cai Y, Zhu Z-J. Metabolite annotation from knowns to unknowns through knowledge-guided multi-layer metabolic networking. Nat Commun 2022;13:6656.
98. Nash WJ, Dunn WB. From mass to metabolite in human untargeted metabolomics: recent advances in annotation of metabolites applying liquid chromatography-mass spectrometry data. TrAC Trends Anal Chem 2019;120:115324.
99. Katajamaa M, Orešič M. Data processing for mass spectrometry-based metabolomics. J Chromatogr A 2007;1158:318–28.
100. Yu T, Park Y, Li S, Jones DP. Hybrid feature detection and information accumulation using high-resolution LC–MS metabolomics data. J Proteome Res 2013;12:1419–27.

101. Pirttilä K, Balgoma D, Rainer J, Pettersson C, Hedeland M, Brunius C. Comprehensive peak characterization (CPC) in untargeted LC–MS analysis. Metabolites 2022;12:137.
102. Huan T, Tang C, Li R, Shi Y, Lin G, Li L. MyCompoundID MS/MS search: metabolite identification using a library of predicted fragment-ion-spectra of 383,830 possible human metabolites. Anal Chem 2015;87: 10619–26.
103. Li Y, Kuhn M, Gavin A-C, Bork P. Identification of metabolites from tandem mass spectra with a machine learning approach utilizing structural features. Bioinformatics 2020;36:1213–8.
104. Misra BB. New software tools, databases, and resources in metabolomics: updates from 2020. Metabolomics 2021;17:49.
105. Scheubert K, Hufsky F, Petras D, Wang M, Nothias L-F, Dührkop K, et al. Significance estimation for large scale metabolomics annotations by spectral matching. Nat Commun 2017;8:1494.
106. Graça G, Cai Y, Lau C-HE, Vorkas PA, Lewis MR, Want EJ, et al. Automated annotation of untargeted all-ion fragmentation LC–MS metabolomics data with MetaboAnnotatoR. Anal Chem 2022;94: 3446–55.
107. Chen L, Lu W, Wang L, Xing X, Chen Z, Teng X, et al. Metabolite discovery through global annotation of untargeted metabolomics data. Nat Methods 2021;18:1377–85.
108. Wishart DS. Emerging applications of metabolomics in drug discovery and precision medicine. Nat Rev Drug Discovery 2016;15: 473–84.
109. Jacob M, Lopata AL, Dasouki M, Abdel Rahman AM. Metabolomics toward personalized medicine. Mass Spectrom Rev 2019;38: 221–38.
110. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. Sci Adv 2016;2:e1600200.
111. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discovery 2011;10:671–84.
112. Kery M, Papandreou I. Emerging strategies to target cancer metabolism and improve radiation therapy outcomes. Br J Radiol 2020;93:20200067.
113. Schmidt DR, Patel R, Kirsch DG, Lewis CA, Vander Heiden MG, Locasale JW. Metabolomics in cancer research and emerging applications in clinical oncology. CA A Cancer J Clin 2021;71:333–58.
114. Zhu G, Wang Y, Wang W, Shang F, Pei B, Zhao Y, et al. Untargeted GC-MS-Based metabolomics for early detection of colorectal cancer. Front Oncol 2021;11:729512.
115. Gupta A, Sagar G, Siddiqui Z, Rao KV, Nayak S, Saquib N, et al. A non-invasive method for concurrent detection of early-stage women-specific cancers. Sci Rep 2022;12:1–12.
116. Liu X, Liu G, Chen L, Liu F, Zhang X, Liu D, et al. Untargeted metabolomic characterization of ovarian tumors. Cancers 2020;12: 3642.
117. Yang T, Hui R, Nouws J, Sauler M, Zeng T, Wu Q. Untargeted metabolomics analysis of esophageal squamous cell cancer progression. J Transl Med 2022;20:127.
118. Yu L, Lai Q, Feng Q, Li Y, Feng J, Xu B. Serum metabolic profiling analysis of chronic gastritis and gastric cancer by untargeted metabolomics. Front Oncol 2021;11:636917.
119. Jia H, Shen X, Guan Y, Xu M, Tu J, Mo M, et al. Predicting the pathological response to neoadjuvant chemoradiation using untargeted metabolomics in locally advanced rectal cancer. Radiother Oncol 2018;128:548–56.
120. Recber T, Nemutlu E, Beksac K, Cennet Ö, Kaynaroğlu V, Aksoy S, et al. Optimization and normalization strategies for long term untargeted HILIC-LC-qTOF-MS based metabolomics analysis: early diagnosis of breast cancer. Microchem J 2022;179:107658.
121. Occelli C, Guigonis J-M, Lindenthal S, Cagnard A, Graslin F, Brglez V, et al. Untargeted plasma metabolomic fingerprinting highlights several biomarkers for the diagnosis and prognosis of coronavirus disease 19. Front Med 2022;9:995069.
122. Jia H, Liu C, Li D, Huang Q, Liu D, Zhang Y, et al. Metabolomic analyses reveal new stage-specific features of COVID-19. Eur Respir J 2022;59: 2100284.
123. Roberts I, Wright Muelas M, Taylor JM, Davison AS, Xu Y, Grixti JM, et al. Untargeted metabolomics of COVID-19 patient serum reveals potential prognostic markers of both severity and outcome. Metabolomics 2022;18:6.
124. Chen Q, Liang X, Wu T, Jiang J, Jiang Y, Zhang S, et al. Integrative analysis of metabolomics and proteomics reveals amino acid metabolism disorder in sepsis. J Transl Med 2022;20:1–15.
125. Xiao G, Zhang S, Zhang L, Liu S, Li G, Ou M, et al. Untargeted metabolomics analysis reveals Mycobacterium tuberculosis strain H37Rv specifically induces tryptophan metabolism in human macrophages. BMC Microbiol 2022;22:249.
126. Chienwichai P, Thiangtrongjit T, Tipthara P, Tarning J, Adisakwattana P, Reamtong O. Untargeted serum metabolomics analysis of Trichinella spiralis-infected mouse. PLoS Neglected Trop Dis 2023;17:e0011119.
127. Yu L, Zeng Z, Tan H, Feng Q, Zhou Q, Hu J, et al. Significant metabolic alterations in patients with hepatitis B virus replication observed via serum untargeted metabolomics shed new light on hepatitis B virus infection. J Drug Target 2022;30:442–9.
128. Ullah E, El-Menyar A, Kunji K, Elsousy R, Mokhtar HR, Ahmad E, et al. Untargeted metabolomics profiling reveals perturbations in arginine-NO metabolism in middle eastern patients with coronary heart disease. Metabolites 2022;12:517.
129. Jiang H, Li L, Chen W, Chen B, Li H, Wang S, et al. Application of metabolomics to identify potential biomarkers for the early diagnosis of coronary heart disease. Front Physiol 2021;12:775135.
130. Wang Z, Zhu C, Nambi V, Morrison AC, Folsom AR, Ballantyne CM, et al. Metabolomic pattern predicts incident coronary heart disease: findings from the Atherosclerosis Risk in Communities Study. Arterioscler Thromb Vasc Biol 2019;39:1475–82.
131. Tzoulaki I, Castagne R, Boulange CL, Karaman I, Chekmeneva E, Evangelou E, et al. Serum metabolic signatures of coronary and carotid atherosclerosis and subsequent cardiovascular disease. Eur Heart J 2019;40:2883–96.
132. Fatima T, Hashmi S, Iqbal A, Siddiqui AJ, Sami SA, Basir N, et al. Untargeted metabolomic analysis of coronary artery disease patients with diastolic dysfunction show disturbed oxidative pathway. Metabolomics 2019;15:1–12.
133. Adela R, Kasarla SS, Saquib N, Gupta SK, Bajpai S, Kumar Y, et al. Untargeted metabolomics reveals altered branch chain amino acids, glucose and fat metabolism contributing to coronary artery disease among Indian diabetic patients. Mol Omics 2023;19:321–9.