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Review Article

Analytical Techniques Used in Metabolomics: A Review

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ABSTRACT

Metabolomics is a branch of chemistry in which endogenous metabolites present in a biological sample are accessed and efforts are made for their identification as well as quantification. The emerging scope and vast plant metabolite profiling has resulted in the development and advancement of modern plant metabolomics and it came out to be an extremely significant tool for attaining a thorough knowledge related to plant biology and physiology. To successfully identify and quantify the plant metabolites, there is a need of compatible as well as reliable analytical techniques. In the recent years, modern analytical techniques like NMR, GC-MS/MS, CE-MS/MS, LC-MS/MS etc. have proved to be quite efficient in the field of metabolomics for characterization and quantification purpose. The present review provides an insight to the utility and applications of the commonly used analytical techniques along with their advantages and disadvantages in the field of metabolomics.

Keywords: Metabolomics, Metabolic fingerprinting, Analytical techniques, Nuclear Magnetic Resonance (NMR), Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-tandem Mass Spectrometer (LC-MS)

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ABBREVIATIONS

GC-MS: Gas Chromatography-Mass Spectrometry; UPLC-MS: Ultra Performance Liquid Chromatography-tandem Mass Spectrometer; CE-MS: Capillary Electrophoresis-Mass Spectrometry; NMR: Nuclear Magnetic Resonance; DI-MS: Direct Infusion Mass Spectrometry; GC × GC-MS: Two dimensional Gas Chromatography-Mass Spectrometry; MSI: MS-Imaging; MRI: Magnetic Resonance Imaging; MRS: Magnetic Resonance Spectroscopy; EI-MS: Electron Ionization Mass Spectrometry; EI: Electron Ionization; eV: electron Volt; CI: Chemical Ionization; APPI: Atmospheric Pressure Photo Ionization; APCI: Atmospheric Pressure Chemical Ionization; MALDI: Matrix Assisted Laser Desorption Ionization; SOP: Standard Operating Protocol; GC: Gas Chromatography; LC: Liquid Chromatography; MS: Mass Spectrometry; RPLC: Reverse Phase Liquid Chromatography; HILIC: Hydrophilic Interaction Liquid Chromatography; µM: micro molar; nM: nano molar; Ppm: Parts per million; SPME: Solid Phase Microextraction; SPE: Solid Phase Extraction; au: astronomical unit; ToF: Time of Flight; ESI: Electron Spray Ionization; NPLC: Normal Phase Liquid Chromatography; ICR: Ion Cyclotron Resonance; FT: Fourier Transform; NPLC-APCI-MS: Normal Phase Liquid Chromatography-Atmospheric Pressure Chemical Ionization-Mass Spectrometry; HPLC: High Performance Liquid Chromatography; GC-Q-MS: Single Quadruple Gas Chromatography-Mass Spectrometry; GC-QqQ-MS: Triple Quadruple Gas Chromatography-Mass Spectrometry; LC-ESI-MS: Liquid Chromatography Electrospray Ionization-Mass Spectrometry

INTRODUCTION

Herbal medicine is one of the biggest gifts of nature to mankind. As per a survey conducted by World Health Organization (WHO), most of the African and Asian countries i.e., 80% of the population use herbal medicines for the purpose of health care (Commisso M, *et al*., 2013). Many researchers use it as a source of inspiration when looking for new drugs because of their ability to generate a variety of chemical structures (Xie T, *et al*., 2015). A number of diseases can be cured by medicines of natural origin. Due to the dubiety of ingredients, most of the mechanisms of action are still not known to us. As to understand Herbal medicines, it is very important to have an in-depth knowledge of their chemical composition. The diversification of the chemical composition of drugs of natural origin is well known to us. This comprises of fatty acids, volatile oils, alkaloids, glycosides, tannins, carbohydrates, amino acids etc. The characteristics of each chemical constituent are different from the other one in many aspects. The dynamic nature of the chemical constituents present in herbal drugs poses a significant challenge to current analytical techniques, and a combination of effective analytical tools is required to identify and classify the variety of chemical compounds in the sample matrix (Wang XJ, *et al*., 2019).

There has been a surge in the amount of 'omics' information in recent years and it has affected different aspects of life sciences, including drug discovery (Kell DB, 2006). Over the past few years, Metabolomics has evolved from a research subject studied by some specialist researchers to a broad field which is at present used by a large number of laboratories and research centers (Gika HG, *et al*., 2018). Changes in the metabolome are often interpreted as a species' reaction to disease, genetic changes, or other factors. In general, the metabolome is thought to be a good predictor of phenotype. Analyzing metabolites in depth is a significant method for studying a species (Dettmer K and Hammock BD, 2004).

Metabolomics (Sumner LW, *et al*., 2015), targeted metabolomics (Wang X, *et al*., 2012), untargeted metabolomics (Richards SE, *et al*., 2010), metabolic foot printing (Dikicioglu D, *et al*., 2012), metabolic fingerprinting, fluxomics (Kell DB, *et al*., 2005) and lipidomics (Saito K and Matsuda F, 2010), are some of the words coined by metabolomic researchers to describe this sector (Theodoridis G, *et al*., 2011). The emerging scope and vast plant metabolite profiling has resulted in the development and advancement of modern plant metabolomics and it came out to be an extremely significant tool for attaining a thorough knowledge related to plant biology and physiology. It is now finding broad applications that practically cover the whole process related to drug discovery as well as development, from the discovery of the lead compound to the post-approval drug monitoring. In the recent era, the conventional methods have been taken over by advance analytical techniques like GC-MS/MS, UPLC-MS, CE-MS along with advanced NMR spectroscopy. The newly developed techniques are able to effectively carry out the processes like separation, identification, and quantification of the present metabolites and their related pathway. It has emerged out as a multi-disciplinary and skilled research area. With the innovative advancements in tech-

nology, evolution in chemometric methodologies, metabolomic studies have provided us a significant insight into the biological and chemical mechanisms that are responsible for carrying out numerous physiological processes (*Figure 1*).

Metabolomics analyses are divided into two main categories: First are targeted and second are untargeted metabolomics (Theodoridis GA, *et al*., 2012; Gika HG, *et al*., 2014). Targeted metabolomics lay stress on determining a particular type of metabolites. The major purpose of this approach is to characterize and quantify as much metabolites as possible present within a particular group (Begou O, *et al*., 2017; Ramautar R, *et al*., 2006). Targeted methods generally demand high sample purification as compared to untargeted approach. In reality, a targeted approach can be employed study specified group of analogous metabolites, biosynthetic pathway, or a whole set of known metabolites from various groups in order to have an insight into the changes in multiple pathways. Untargeted metabolomics investigations lay stress on the simultaneous qualification and quantification of as much metabolites as possible present in a given sample. This top-down strategy is employed to analyze the overall metabolomic profile of the given species (Alonso A, *et al*., 2015). Metabolites range from small, positively charged molecules to larger hydrophobic molecules. Accordingly, a crucial matter in this type of research is attaining overall metabolite profiling over an ununified chemical landscape (Patti GJ, 2011).

LITERATURE REVIEW

Analytical techniques employed in metabolomics

Until recently, it was seen that the activities made in metabolomic studies were restricted to academic institutions. Under such type of environment there is a limited need for automation. The reason could be small sample size, cheap student labor or diverse protocols (Lindon JC and Nicholson JK, 2008; Brown M, *et al*., 2011; Smith CA, *et al*., 2006). With the increasing requirement for fast and precise quantification, new advancements in metabolomics are noticed with emerging accentuation on automation. Automation tends to make reliability and reproducibility better across research laboratories as well as leads to increase in overall throughput. In order to get the most reliable results and regulatory approval, automation has a significant place. This has been achieved greatly by the emerging automated methods (Lehotay DC, *et al*., 2011; Chace H and R Spitzer A, 2011). Metabolomic analysis generally includes diverse analytical platforms. Gas Chromatography coupled to Mass Spectrometry (GC-MS), Nuclear Magnetic Resonance spectroscopy (NMR), Direct Infusion Mass Spectrometry (DI-MS), two-dimensional GC coupled to MS (GC × GC-MS), Capillary Electrophoresis coupled to MS (CE-MS), Liquid Chromatography coupled to MS (LC-MS), are the main analytical techniques used in metabolomic investigations in the present period (Theodoridis G, *et al*., 2008; Gika H, *et al*., 2019; Begou O, *et al*., 2019). A broad range of metabolites present in natural medicinal compounds can be efficiently analyzed by these techniques. Automation of NMR combined with machine learning can outturn into effective analyses in less than a few minutes per sample (Ravanbakhsh S, *et al*., 2015). These developments can result in notable innovations in the domain of automation. In the metabolomics based on GC-MS too similar advancements have been noticed (Aggio R, *et al*., 2011; Ni Y, *et al*., 2012). In large-scale plant metabolomics, the first approach made was GC coupled to Time-of-Flight (ToF) MS (Fiehn O, *et al*., 2000). Reference standards which are derivatized or isotopically labeled, strict SOPs, automated liquid-handling systems and advance software are typically required for LC and GC systems coupled with MS detectors (Wishart DS, 2016). In the recent years, a new technology named GC×GC-MS is capable of detecting approx. 7 times more metabolites in the same sample as compared to GC-MS (Miyazaki T, *et al*., 2017). For the detection of thermostable volatile compounds with complicated and time taking sample preparation procedures, GC coupled with MS is widely used (Wang XJ, *et al*., 2019) (*Table 1*).

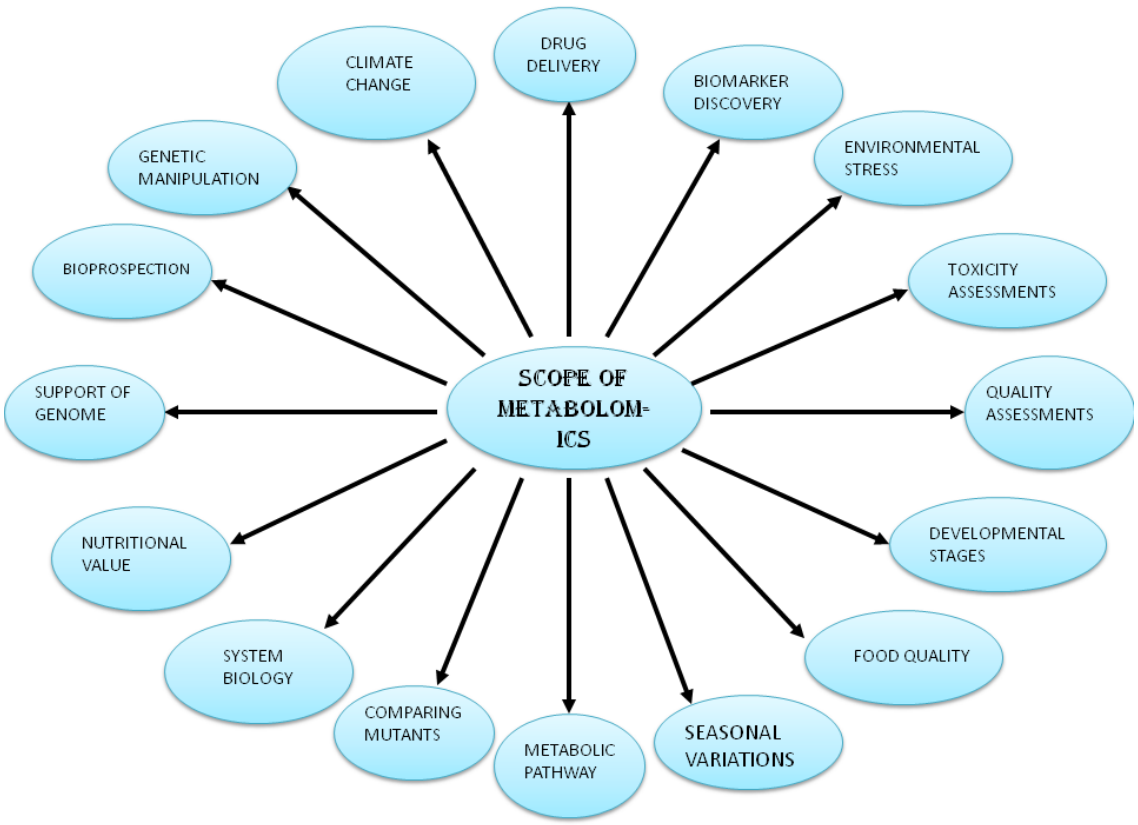

Figure 1: Scope of metabolomics

Table 1: Comparison of different analytical techniques

Properties	Techniques			
	NMR	HPLC-MS	CE-MS	GC-MS
Sensitivity	-	++	+	+++
Reproducibility	High	Lower than NMR	Lower than NMR	Lower than NMR
Resolution	Low	Higher than NMR	Higher than NMR	Higher than NMR
Amount of sample preparation	Low	Medium	Medium	Extensive
Range of metabolites	Polar and Non Polar	RPLC: Non Polar; HILIC: polar	Polar	Volatile polar and non-polar
Identification of metabolites	Easy	Difficult (Database need to be improved)	Difficult (Database need to be improved)	Easy (Spectral libraries)

Note: NMR: Nuclear Magnetic Resonance; HPLC-MS: High Performance Liquid Chromatography-Mass Spectrometry; CE-MS: Capillary Electrophoresis-Mass Spectrometry; GC-MS: Gas Chromatography-Mass Spectrometry; RPLC: Reverse Phase Liquid Chromatography; HILIC: Hydrophilic Interaction Liquid Chromatography

For the purpose of profiling hydrophilic as well as charged metabolites, CE coupled with MS can be considered as an efficient analytical tool (Begou O, *et al*., 2017; Kuehnbaum NL and Britz-McKibbin P, 2013; Soga T, 2007; Ramautar R, *et al*., 2013). Availability of SOPs is important for performing reproducibility studies, but due to their unavailability CE-MS is still taken as a challenge bound tool by scientists (Harada S, *et al*., 2018; Fukai K, *et al*., 2016; Boizard F, *et al*., 2016). While comparing the two techniques NMR and MS, MS is most favoured because of its high resolution and considerable sensitivity (Ren JL, *et al*., 2018). The robust development in MS-Imaging (MSI) tool has added one more dimension to understand metabolomics. All these analytical techniques hold in themselves several pros and cons. When it comes to choose a specific strategy the decision is taken majorly on the basis of the aim of the study being conducted and is mainly arbitration among factors like speed, sensitivity and selectivity (Lei Z, *et al*., 2011).

Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is a technique of utmost importance in the field of metabolomic studies. There are several merits of this technique which includes considerable reproducibility, high throughput, easy identification of metabolite, and non-destructive sample preparation (Markley JL, *et al*., 2017). This technique over a period of time has become the preferred approach for evaluating plant metabolites due to its simple and uncomplicated sample preparation protocols and the quick assessment of the NMR spectrum. An important advantage of NMR is that it is not confined to the analysis of tissue or bio fluid extract only. The analysis of whole tissues, solid and semisolid specimens can easily be made through solid-state NMR as well as magic-angle sample spinning (Blondel C, *et al*., 2016; Diserens G, *et al*., 2015; Hong YS, *et al*., 2009; Jang WG, *et al*., 2016), which can help in decluttering the spectra under special conditions. Furthermore, the presence of different nuclei present in the same sample can be studied separately as well as simultaneously by the scientists and hence diverse classes of metabolites can be observed. Moreover, multidimensional NMR methods can also be used to study the relation between distinct nuclei (Emwas AH, *et al*., 2019). Considerable sensitivity and short relaxation times of proton nuclei provided by ^{1}H NMR spectroscopy makes it the preferred method, also it leads to faster analysis in comparison to ^{13}C nucleus which is less sensitive. But due to smaller chemical shifts and scalar coupling. Nevertheless, it is difficult to analyze the spectra of ^{1}H NMR as there exists overlapping of signals which make it quite complicated. One of the reason for this complication is smaller chemical shift values of protons (~12 ppm) and scalar coupling (Dais P and Hatzakis E, 2013) NMR can also be used for imaging of metabolites in case of animate samples Magnetic Resonance Spectroscopy (MRS) and Magnetic Resonance Imaging (MRI) (Calvo N, *et al*., 2015; Lin AQ, *et al*., 2014; Lin G, *et al*., 2017; Simões RV, *et al*., 2008; Yoon H, *et al*., 2016). Compared to LC-MS and GC-MS based approaches, they cannot be employed for the analysis of living samples due to their inherent destructive nature. As an outcome, NMR is an excellent candidate for the metabolite profiling in living cells in real time (Jeong S, *et al*., 2017; Motta A, *et al*., 2010). Moreover, researchers can study the chemistry of compounds in greater depth with NMR than with other techniques. It helps scientists to study the intact molecules, allowing them to see the ^{1}H atoms as well as other atoms (^{13}C, ^{15}N, ^{31}P) (Batool F, *et al*., 2017; Elbaz AM, *et al*., 2015; Jameel AGA, *et al*., 2018; Rehman ZU, *et al*., 2017; Ye T, *et al*., 2009).

However, there are various disadvantages too associated with NMR, as it lacks sensitivity in contrast to other commonly applied techniques. NMR is noticed to be 10 to 100 folds less sensitive as compared to LC-MS and GC-MS techniques. This clearly indicates that analysis based on NMR normally reveals details of 50-200 metabolites with concentrations >1 μM, whereas a LC-MS based analysis is capable of revealing details on 1000+ metabolites with concentrations ranging from >10 to 100 nM.

GC-MS analysis

GC-MS is generally referred as a versatile analytical technique (Kaplan O, *et al*., 1990), the reason being the robustness, outstanding ability to separate compounds, highly selective nature, responsiveness and also the reproducible results (Tsugawa H, *et al*., 2011). This technique is among the most systematized techniques in the field of metabolomics, having established analytical protocols for carrying out the metabolite analysis found in medicinal plants such as amino acids, sugars, sterols, hormones, catecholamines, fatty acids, aromatics etc. It has several advantages when compared to LC-MS as it has higher chromatographic resolution in contrast to the liquid phase. Also, the database is quite broad which makes the analysis simpler and faster. Furthermore, GC-MS can analyze a wide variety of compounds using only one type of column, and the Electron Ionization approach which includes fragmenting molecules as per their respective structure, which aids in the detection, hence eliminates the requirement

for tandem mass spectroscopy. Because of the above mentioned points, GC-MS has turned out to be valuable equipment in metabolomics. As it can test small compounds having weights less than 600 au, it can be used for untargeted metabolomic studies related to primary metabolism activity (Fiehn O, 2016).

There are various options to conduct metabolomic studies for the characterization of volatile constituents, ranging from simple headspace injections (Papadimitropoulos ME, *et al*., 2018) to adsorption of volatile compounds on the adsorption matrices, accompanied by thermal desorption in the GC-MS system, usually by Solid-Phase Microextractions (Tikunov Y, *et al*., 2005), through active adsorption techniques (Dixon E, *et al*., 2011), or through passive adsorption techniques in which those materials are used which have comparatively large capacity than SPME fibers (Zhang Z and Li G, 2010). Moreover, GC-MS utilization for the metabolomic studies of primary metabolites is less frequent and limited to plant research only.

Among the various convenient ionization sources employed in the field of metabolomics, Electron Ionization Mass Spectrometry (EI-MS) is the regularly used MS detector employed in metabolomic investigations related to GC. EI, a 'hard' ionisation technique which can produce repeatable molecule fragmentation and well-defined mass spectral fingerprints. At 70 eV, Ionization is carried out across all EI instruments, and mass spectra are usually considered repeatable across instruments with different mass analyzers (Aksenov AA, *et al*., 2014).

However, Chemical Ionization technique is not as much utilized extensively as Electron Ionization with context to metabolomics. The explanation for this may be that Chemical Ionization technique comes under soft ionization technique, which results in fragments which are not that much sensitive and hence are useless for identification purpose. However, since the CI technique cannot produce a large number of molecular ions, it is only useful in targeted methodologies (Beale DJ, *et al*., 2018; Jaeger C, *et al*., 2016).

Other soft ionization techniques comprises of Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Photo Ionization (APPI). In present era, the coupling of APCI sources is done to mass systems with high resolution like Ion Cyclotron Resonance (ICR), Fourier Transform (FT) orbit rap, Time-of-Flight (ToF) systems. GC is coupled with different mass analyzers differing in their resolutions and accuracies. Among the different mass analyzers used in GC-MS, the commonly used ones are low mass resolution quadruple mass filters including GC single Quadruple (GC-Q-MS) and triple Quadruple instruments (GC-QqQ-MS). The main advantages of quadruple based systems generally cover their considerable responsiveness and better dynamic range, but the disadvantage is that they can be impacted from slower scan rates and lower mass accuracy relative to high mass resolution based systems.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

LC-MS is the coupling between Liquid Chromatography and Mass Spectrometry (Warren CR, 2013) . Usually, a separation procedure is performed prior to the MS analysis in most metabolomic studies. As a flexible and efficient separation process, High Performance Liquid Chromatography (HPLC) which allows separation of chemical entities with variable polarity range using isocratic elution method (the ratio of water-solvent is kept constant throughout the procedure) or gradient elution method (the ratio of water-solvent is kept variant throughout the process). Some of the most widely used organic solvents in Liquid Chromatography are water, acetonitrile, formic acid and methanol. Generally for simple samples, Isocratic elution is preferred. Gradient elution has an advantage of providing quick analysis, narrow peak and somewhat alike resolution in comparison to the former elution technique (Zhou B, *et al*., 2012).

A MS system is generally comprised of these three prime parts: an ion source, a mass analyzer, a detector. Converting sample molecules into ions is basically the role of ion source, while the ions are resolved by the mass analyzer prior to their measurement by the detector. A number of choices are there as ion sources like Chemical Ionization, Electrospray Ionization, Atmospheric Pressure Chemical Ionization, Atmospheric Pressure Photoionization, and MALDI. Because of the diversification in the metabolite's chemical properties of metabolites, sometimes it becomes a requirement to perform the analysis in positive mode as well as -ve mode of ionization to further amplify metabolome coverage. ESI has undoubtedly become the preferred method in metabolomic investigations based on LC-MS. The reason being that it's a soft ionization technique due to which a large number of ions are produced by charge exchange in solution and frequently forms generates complete molecular ions that further assist in identification.

A reduction in complexity of sample and reduced matrix effect while ionization process is generally observed during chromatographic separation. In LC-ESI-MS, Reverse Phase Liquid Chromatography (RPLC) can efficiently separate compounds with semi-polar nature like flavonoids, phenolic acids, alkaloids as well as other glycosylated entities. Columns like aminopropyl columns are used in Hydrophilic Interaction Liquid Chromatography (HILIC) to elute out compounds with polar nature like sugars, amino acids etc. Even though Normal Phase Liquid Chromatography (NPLC) is capable of separating compounds with high polarity, the utilization of non-polar organic mobile phase is observed to be more suited with APCI-MS rather than ESI-MS. For the purpose of investigating the analytical profile of hydrophobic lipids such as sterols, triacylglycerols, and fatty acid esters NPLC-APCI-MS is generally employed. LC-ESI-MS has become the preferred method for the purpose of carrying out metabolomic studies in complex samples (Schellinger AP and Carr PW, 2006).

The merit of combining HPLC system with MS involve improved MS responsiveness and signal reproducibility due to a reduction in complexity of sample, which reduces matrix interference in the whole process of ionization. Furthermore, since background noise is reduced, better separation through chromatography will result in reliable MS results. Recent advances in LC, such as capillary monolithic chromatography and Ultra-Performance LC (UPLC), have made noteworthy improvement in analysis speed as well as peak resolution (Bowen BP and Northen TR, 2010; Guillarme D, *et al*., 2007).

DISCUSSION AND CONCLUSION

Rather than analyzing individual metabolite one at a time, metabolomics can cover the entire metabolome by detecting all potential compounds present in a sample. With the coming of automated techniques, a surge in metabolomics data has been observed. In the recent years, machine learning has also played a huge role in streamlining of the overall process, turning complicated data into simpler one and also reduced the time required for scanning. Coupling of various analytical techniques with MS detector has proved to be a boom in the field of metabolomics. Every analytical technique has merits as well as demerits of its own. The choice of the analytical technique to be employed in a study mainly depends on the type of sample, and also the resolution we want in our study. The aim of this review is to give an insight to the role of various analytical techniques employed in metabolomics along with their benefits and applications. The emerging advancements in automated techniques is an ultimate approach for the detecting numerous metabolites present in a living sample, speeding up the incorporation of metabolomics into systems biology.

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