Metabolomics: a radical approach to molecular study

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Abstract

Metabolomics is an emerging field that focuses on drug discovery, discovery of biomarkers and provides a new approach to inter relationship between physiological processes and parameters influencing them. It has integrated capacity to shift organic chemistry based characterization to a biochemical context with insight into cellular phenomena. Analytical and computational technologies used in metabolomics allow the characterization of molecules by providing data that can lead to annotation and ultimately to identification. Owing to the complexity of metabolome and diverse properties of metabolites, no single analytical platform can be applied to detect all metabolites in a biological sample. The combined use of modern instrumental analytical approaches such as gas chromatography (GC), HPLC, UPLC, CE coupled to MS and NMR spectroscopy has unraveled the ideal outcomes in separation, detection, characterization and quantification of metabolites and metabolic pathways.

Keywords: Metabolome; GC; HPLC; UPLC; CE; NMR spectroscopy

1.0 Introduction

Metabolomics is the large-scale study of small molecules commonly known as metabolites within cells, bio-fluids, tissues or organisms. It provides an insight into the cell status and describes an actual health condition of organisms. It also provides a unique opportunity to study the influence of genetic variation, disease, applied treatment or diet on endogenous metabolic state of organisms. The beginning of metabolomics traces back all the way to 2000-1500 B.C. when traditional Chinese doctors began using ants in order to evaluate the urine of patients to determine if the urine contained the high glucose of diabetics. At this time others tasted the urine for sweetness in order to check for the same thing. Urine was also a factor in determining diabetes in Ancient Egypt where it was determined by frequent urination. This earliest use of body fluids to determine a biological condition can be considered the first early uses of metabolomics. Santorio Sanctorius is considered to be the founding father of metabolic studies. In 1905, J.J. Thomson developed first mass spectrometer and in same year Otto Knut Olof Folin reported the urine analysis for urea, ammonia, creatnine, uric acid. The second step in start of metabolomic study was Felix Botch of Stanford and Edward Purcell of Harvard published the first NMR in the same issue of Physical Review in 1946 is marked as start of modern metabolomic studies (Shin et al., 2014. A wide range of living organisms have been investigated using metabolomics approach from simple microbes to complex biological systems such as mammals with attempts to profile all metabolites within a cell or biological system. It is important to remember that complete organisms, organs, individual cells and their organelles each possess their own specific metabolome. This naturally changes with age and is gender dependent to some extent. Moreover, the metabolome can be influenced by external factors such as climatic conditions, time of day, season and nutritional habits. It is particularly interesting to employ a hypothesis-free approach to identify unusual metabolite patterns which for example may occur in individual diseases. Therefore, Metabolite levels can be regarded as the final response of an organism to environmental factors, genetic modifications, changes in gut microflora and altered kinetic activity of enzymes (Zamboni et al., 2015). The emerging field of metabolomics gained more importance in recent years as its wide applications in the field of drug discovery and drug development. It aims to design new non-invasive, sensitive and specific diagnostic techniques and development of new therapies. One of the targets of metabolomic studies is biomarker discovery. In agricultural or chemical industry, metabolomics may be used to develop herbicides and pesticides with increasing importance being placed on health and safety related aspects of our food, metabolomics can potentially be a valuable tool to monitor and improve the quality of what we eat.

However, there are still certain challenges that researchers in the field of analysis of metabolome have to face which include technical limitations, bioinformatic challenges and integration. Among them, data analysis is the most time consuming stage of metabolomic workflow and requires close collaboration between analysts, clinicians and experts in chemometric analysis. The research requires effective and precise analytical systems as the samples are complex and many compounds must be identified. This may draw attention to the pathological role of previously overlooked metabolites. This new approach to metabolic research necessitates an expanded experimental design. In the classical experimental design a single or a few parameters are recorded and analysed. However, the quantity of data must be minimized to as great an extent as possible in order to guarantee simple and clear analysis. In contrast in the new approach as much data as possible must be generated as a complement to the classical approach. One frequently employed analytical technique is mass spectrometry. NMR (nuclear magnetic resonance) spectroscopy is also used. One advantage of NMR spectroscopy is that it cannot only identify metabolites but also clarify their molecular structures. On the other hand the use of NMR is greatly restricted by the minimum concentration of µmol/L to mmol/L for individual metabolites (Brietling et al., 2006). Metabolomics enables the study of the metabolic composition of an organism or biological system so that all metabolites are described both primary and secondary. Hence metabolomics stands out from any other organic compound analysis in scale and chemical diversity. Metabolomics can therefore provide valuable tools relevant in a wide range of applications including insight into cellular phenomena through systems-biology approaches (Harrigan et al., 2003).

2.0 Metabolomic Techniques

Metabolomics is study of metabolites which correlates biochemistry with analysis. The techniques used for molecular profiling and data analysis of metabolites are chromatography, molecular spectroscopy, mass spectrometry, gas chromatography (GC), high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR), fingerprinting. The Gc, HPLC and NMR helps in compound analysis precisely while fingerprinting is used for data coverage. The samples to be analyzed are crude extracts. NMR, direct injection mass spectrometry (MS) or Fourier transform infrared (FT-IR) spectroscopy are combined with LC/MS, LC/MS/MS and LC/NMR for more precise and accuracy. An overview of each of the approaches is given below (Kasture et al., 2013).

2.1. *Gas Chromatography*

Gas chromatography has gained attention in agriculture. It provides compound separations with high spectrum. It is universal method and very sensitive. In this technique, a small quantity sample can be easily analyzed through gas. The sample is dissolved in a solvent first and then vapourized to separate the analytes. The sample is distributed through two phases: a stationary phase and a mobile phase. A mobile phase involves chemically inert gas such as helium, nitrogen etc. Gas chromatography is one of the unique forms of chromatography that does not need the mobile phase for interacting with the analyte. The stationary phase is either a solid adsorbent or a liquid but with an inert gas. It has been able to detect all organic compounds irrespective of their structure complexity. The non volatile organic compounds are not analyzed directly. They are converted into more volatile and less polar derivatives for data analysis. The drawback of this technique is low sample analysis capacity.

2.2. *High Performance Liquid Chromatography (HPLC)*

HPLC with UV detection is technique used for metabolic profiling of metabolome. It aims at targeted analysis of plant materials based on individual classes. Selection of compounds depends upon type of column, detector and type of solvent used. The technique can be used for known compound as it relies on reference compounds for comparison. The UV spectrum is used for detection of nature of compounds in complex data profiles. The major drawback is it detects the different class of compound present in the sample but not the exact identity of that particular class.

2.3. *Fingerprinting Methods*

Fingerprinting techniques is a rapid method and it analyzes large number of sample in less time. The samples are examined precisely with help of solvent or as intact tissues (magic angle spinning NMR), liquids or semi-solids (NMR and FT-IR), or dried materials (FTIR) for specific chemical information.

2.3.1. *Nuclear Magnetic Resonance (NMR)*

The sample containing H+ metabolites are detected by Proton (1H) NMR and signals are assigned to each compound on basis of reference compounds used for comparison. The 1H NMR spectra however, has a low chemical shift dispersion which makes it crowded and spin-spin couplings make it complicated by multiplying signals. The 13C NMR is more advantageous than 1H NMR with twenty times greater chemical shift dispersion and signal multiplicity in spin- spin interactions are removed by decoupling but inspite of these advantages, it has lower sensitivity which limits its use in complex extract samples (Cajka and Fiehn., 2016).

2.3.2. *Direct Injection MS*

The molecular profiles are obtained by this technique by injecting crude extracts into the high-resolution mass spectrometer. It generates mainly protonated, deprotonated or adduct molecules such as [M+H]+, [M+cation]+ or [M-H]-. These molecules are separated according to their molecular mass in a spectrum. The high resolution helps to analyze the masses precisely and provides a rough estimate of the number of metabolites present in a sample. It also gives a hint to their identities. The only drawback is inability to separate isomers of the same molecular mass.

2.3.2. *FTIR Spectroscopy*

This method is superior over other method for simple sample preparation, the speed with which data can be acquired, and the high degree of reproducibility. Liquid samples spread to make good contact with a flat surface can be measured by the attenuated total reflectance (ATR) method whereas powdered or dried samples are measured by diffuse reflectance. The major drawback is the spectra are less easily interpreted than with the other methods but extremely subtle differences may be picked out using chemo metrics providing a powerful classification tool (Breitling et al., 2006).

2.4. *LC/MS, LC/MS/MS and LC/NMR*

They are potentially powerful solutions to the problems of structure determination. LC/MS can be used to detect compounds that are not well characterized by other methods. The electro spray ionization (ESI) technique has made polar molecules accessible to direct analysis by MS as well as being compatible with HPLC separations. The structural information is helpful in the identification of new or unusual metabolites or in the characterization of known metabolites in cases where ambiguity exists. The lower sensitivity of LC/NMR is most often used for structural characterization of unknowns rather than for comparative analysis of numerous samples. However, NMR

is a very general detection method and can provide unique structural information so with improvements in sensitivity the use of LC/NMR is likely to grow.

2.5. *Multivariate Analysis*

Multivariate analysis is useful method for samples with complex composition. Due to large member of spectra, visualization is difficult in these samples. It compresses data into more easily managed form. It also provides insight about sample to sample co-relation. Principle component analysis (PCA) is a well-known and effective method of data compression. PCA transforms the original data into a set of 'scores' for each sample, measured with respect to the principal component axes. Scatter plots of the scores wuth respect to PC loadings provide an excellent means of visualizing and summarizing the data and often reveal patterns that cannot be discerned in the original data (Gika et al., 2016).

3.0 Applications of Metabolomics

Metabolomics is one functional level tool used to investigate the complex interactions of metabolites with other metabolites (metabolism). It provides regulatory role of metabolites with interaction to gene, transcripts and protein. It has been used in assessing responses to environmental stress, comparing mutants, drug discovery, toxicology and nutrition studying global effects of genetic manipulation, cancer, comparing different growth stages, diabetes and natural product discovery.

3.1. *Metabolomics in ADMET*

The absorption, distribution, metabolism, excretion and toxicology of drugs (ADMET) are one of the most critical areas for drug testing and drug development. It is one of the most time consuming process. The drug discovery process is concerned with identifying active lead molecules while ADMET is a new approach to identify the leads which are potentially hazardous, thereby preventing them from progressing too far down the drug development pipeline. ADMET is carried out both in preclinical and clinical trial phases of drug development. In pre-clinical studies, ADMET normally requires testing on large numbers of animals and performing detailed histological and pathological analyses. These are supplemented with clinical chemistry studies of blood, cerebrospinal fluid (CSF), urine and faeces. The major drawback is the invasive, manually intensive nature of most ADMET studies which makes them expensive, prone to error and time consuming (Angelo D Alessandro and Lello., 2012).

3.2. *Metabolic fingerprinting*

Metabolic fingerprinting technique is more precise technique which analyzes metabolite patterns in different experimental groups. The aim of this technique is to analyze entire detectable metabolome than analysis of a pre defined set of metabolites. In mass spectrometry-based investigation, metabolite fingerprints are based on variable components with m/z values and detected ions intensities. In case, the metabolites undergo separation of extract sample, the retention time is also taken into consideration. They form the base of molecular profiling and help in sample classification with more multivariate analyzed data. The chemical structure of the detected metabolites in sample extracts typically remains unknown. (Dai et al., 2010).

3.3. *In toxicity assessment*

Metabolic profiling (especially of urine or blood plasma samples) can be used to detect the physiological changes caused by toxic insult of a chemical (or mixture of chemicals). The observed changes can be related to any specific syndromes or specific lesion in liver or kidney. This study is of relevance to pharmaceutical companies wanting to test the toxicity of potential drug candidates, it helps to eliminate compound before it reaches clinical trials on the grounds of adverse toxicity. Thereby, saves the enormous expense of the trials.

3.4. *Functional genomics*

Metabolomics can be an excellent tool for determining the phenotype caused by a genetic manipulation due to gene deletion or insertion. The phenotypic changes in a genetically-modified plant intended for human or animal consumption can be detected easily. It also predicts the function of unknown genes by comparison with the metabolic perturbations caused by deletion/insertion of known genes. Such advances are most likely to come from model organisms such as *Saccharomyces cerevisiae* and *Arabidopsis thaliana* (Zhincheng et al., 2013).

3.5. *Nutrigenomics*

Nutrigenomics is a generalised term which links genomics, transcriptomics, proteomics and metabolomics to human nutrition. The main exogenous factors influencing the metabolic profile are diet and drugs. It reflects the balance of all factors/ forces that play an important role in individual metabolism.

3.6. *Environmental Metabolomics*

This study aims at the interactions of organisms with their environment. The interactions help in assessing organism function and health at the molecular level. As such, metabolomics is finding an increasing number of applications in the environmental sciences, ranging from understanding organism responses to abiotic pressures, to investigating the responses of organisms to other biota. These interactions can be studied from individuals to populations, which can be related to the traditional fields of ecophysiology and ecology (Shin et al., 2014).

3.7. *Metabolomics in Organ Transplantation*

Metabolite measurements have been important part of organ transplant monitoring for more than 40 years. Major metabolite measurements have been restricted to creatinine and glucose to analyze injury associated changes. With use of GC-MS and technique like NMR, LCMS, spectral pattern analysis etc to monitor organ function precisely and accurately for minute metabolic changes. The two key aspects for data analysis of organ physiology are organ function and organ injury. Any dysfunction or change from homeostasis is to be analyzed and records are to be kept for reference and comparison purposes. The good diagnostic or prognosis biomarker is an important component of research which can prove to be of much greater importance in near future. More recently with help of NMR chemical shift imaging techniques, few measurements are performed in vivo. These primarily aim to measure inorganic phosphate or phosphate metabolites like ATP, ADP and phosphocreatine (Lewis et al., 2008).

3.8. *Specific and Sensitive Biomarkers of drug-induced hepato toxicity and nephrotoxicity*

The major concern to both the FDA and pharmaceutical companies is the number of increasing cases of serious adverse effects (SAE) in marketed drugs. In drug trials, the potential toxicity of drugs remain masked in preclinical animal studies as researcher's fail to identify the potential toxicity of a new chemical entity (NCE) and lack of sensitivity and specificity of known biological biomarkers to identify the organ injuries especially the main exposed organs like liver and kidneys. The Metabolomics have the capability of providing translational diagnostic and prognostic biomarkers specific for early stages of liver and kidney injury making it easy to eliminate the potential chemical component which can prove to cause toxic or any adverse effect in population. It also saves time and money of pharmaceutical companies that they spend on drug trails and drug withdrawl from the market (Johnson et al., 2016)

3.9. *To discover metabolic patterns associated with diseases*

Metabolomics aims to study the changes in metabolic patterns in humans associated with nutrition. The existing biological markers for type 2 diabetes mellitus have limited value for the assessment of individual risks. All the factors involved in disease progression such as genetics, nutritional habits, age, or sex are considered to draw statistical conclusions. There is a lot of inherent biological variability which pose constraints on validation of the analytical methods. Using diabetes as an example, the economic and scientific needs for accurate diagnostic tools are discussed with respect to the available analytical and computational approaches for cost-effective high throughput methods (Wishart et al., 2016).

3.10. *Metabolomics and alcohol related dysfunctions*

Numerous metabolomics approaches may contribute to alcohol-related research. It studies the alcohol-related metabolic dysfunctions such as alterations in fat metabolism and thiamine deficiency. It has used analytical data to study different metabolic pathways affected by alcohol abuse and support discovery and development of novel medications for the treatment of alcoholism and related conditions (Duc Du et al., 2019).

3.11. *Use of metabolomics in cancer research*

Metabolomics are considered to visualize the cancer cell proliferation and differentiation pattern. As per warberg effect, highly proliferating cells tend to utilize the energy from glycolytic pathway even in presence of oxygen. The study helps us to document the warberg effect in broad variety of tumors and gives an overview about the oxidative stress. It represents one leading cause of genetic instability underpinning carcinogenesis and provides opportunities for therapeutic window to targeted drug therapies (Johnson et al., 2015).

4. Challenges of Metabolomics

The goals of metabolomic studies are quantitative assessment of biochemical differences reflected in the metabolome, differential analysis between and among sample groups and identifying compounds responsible for observed changes. There are still certain limitations for researchers in this emerging field which include (Suhre et al., 2011):

- Complexity and diversity of biological samples.
- Chemical diversity of small molecule metabolites.
- Multiple sources of variability from sample analysis methods, workflow and reagents.
- Lack of analytical standards, particularly for unknown metabolites.
- Incomplete information – the majority of compounds detected by LC/MS are unknown.
- Time and expense of unknown structure elucidation.
- The need for robust, reliable data handling and bioinformatics.
- Throughput issues for preparing and analyzing large numbers of samples and standards.

Conclusion

Metabolomics is a new window for researchers to a sophisticated level of information about biological systems. It aims for development of novel diagnostic tests and therapies and also holds a future in assessing responses to environmental stress, comparing mutants, drug discovery, toxicology and nutrition studying global effects of genetic manipulation, cancer, comparing different growth stages, diabetes and natural product discovery.

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