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Review Article

METABOLOMICS

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ABSTRACT
To describe the relationship between the genome and phenotype in cells and organisms, increasing efforts are being made in the post-genomic era. It is clear that even a complete understanding of the state of genes, messages and proteins in a living organism does not reveal its phenotype. Therefore, researchers developed the metabolomics study in which metabolomes or the metabolomic complement of functional genomics is studied. Terms such metabolomics, metabolome, metabolites, metabonomics, etc. are mentioned in this text. An overview of target analysis, metabolic fingerprinting and metabolic foot printing is given. The methods for sample preparation and detection are also mentioned in this text. In sample preparation, methods such as quenching, extraction and sample concentration are studied. Also, the various analytical techniques or methodologies used in the metabolomics detection are described. A general description of these techniques i.e. mass spectrometry and NMR spectroscopy is given. The advantages and limitations of these techniques are highlighted.

Key words: Metabolomics, Metabolome, Metabonomics, Metabolic fingerprinting, Mass spectrometry, NMR spectroscopy.

1. INTRODUCTION

Metabolomics refers to the quantitative and comprehensive measurement of the metabolic response of living cells and systems to genetic modifications or pathophysiological stimuli. It can also be defined as a system which focuses on high-throughput characterisation of the metabolome in order to obtain a molecular profile. Metabolomics is a highly suitable system to investigate the genotype interaction in the biological systems and is referred to as phenotype or metabotype. It also involves the analysis and scientific study of metabolites in a biological system. Metabolomics is a study of unique chemical fingerprints that are left behind by specific cellular processes, metabolite profiles of small molecules and the biological endpoint of a condition. In the metabolomics profiling of a cell, metabolite quantification of the cellular system is carried out and an instantaneous snapshot of the physiology of that particular cell is obtained[1-6].

2. METABOLOME

A metabolome is a quantitative complement of all the low molecular weight proteins (usually less than 3000 m/z) and metabolites (like metabolic intermediates, secondary metabolites, hormones and other signalling molecules) present in biological cells in a particular physiological or developmental state. The metabolome is context-dependent, dynamic and changes from second to second[7-9].

3. METABOLITE

The intermediates and products of metabolism are usually referred to as metabolites. Metabolites perform various functions like signalling, stimulation and inhibitions of enzymes, defence, catalytic activity and interaction with other organisms.Metabolites are the end products of cellular processes and are labile species by nature and chemically very diverse. Metabolites are further classified as- primary metabolites and secondary metabolites.

Primary metabolites- These are involved directly in the growth, developmental and reproductive processes. Examples include alcohol, amino acids, antioxidants, organic acids, polyols, vitamins, etc.

Secondary metabolites- These metabolites are not directly involved in the growth and development processes but have an ecological function. Examples are antibiotics and pigments (like resins and terpenes).

Metabolites represent the ultimate reflection of the response of biological systems to changes- both genetic as well as environmental. They, however, are not organism specific[2, 7, 10-12].

4. METABONOMICS

Metabonomics is another terminology used in the study of metabolomics. The quantitative measurement of dynamic multiparametric metabolic response of living systems to genetic modifications or pathophysiological stimuli is referred to as metabonomics. Metabonomics is often confused with metabolomics. The main difference between the two terms is that metabonomics is based on NMR spectroscopy and metabolomics is associated with mass spectrometry[13].

5. ANALYTICAL TECHNOLOGIES

There are various analytical technologies which are employed in the analysis of metabolomes in metabolomics studies. Analysis of metabolomes generally involves the following:

5.1 Sample Preparation:

This involves three steps, namely, quenching, extraction and sample concentration

5.2 Detection (usually done by mass spectrometry or NMR spectroscopy) [14]

In a metabolomics experiment, all the metabolites in a cellular system are quantified. Analysis of metabolites is not simple as there is no simple, automated, analytical technique that can measure 100s to 1000s of metabolites in a quantitative, reproducible and robust manner. Also, methods are not available for amplification of metabolites, and metabolites being chemically labile species, sensitivity is a major issue. Using different analytical techniques, identification and quantification of intracellular as well as extracellular metabolites with molecular mass less than 1000Da is done. However, within the metabolome, there is a large variation in the chemical and structural properties. The metabolome consists of extremely diverse chemical compounds ranging from ionic, inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids, and natural products which are complex in nature. Due to this complexity, it is virtually impossible to detect the complete metabolome. Hence, metabolomes are studied with efficient sample preparations, selective extractions and a combination of different analytical techniques to give a reproducible profile of the sample compound. There are different approaches for the analysis of metabolomes. They are: 1) target analysis and 2) metabolite profiling. Metabolic profiling is further divided into a) metabolic fingerprinting and b) metabolic footprinting.

5.2.1 Metabolome Target Analysis

This approach is restricted to metabolites of, for example a particular enzyme system that would be directly affected by anabiotic or biotic disturbance. It is restricted to the quantitative analysis of a class of compounds which are related to a specific pathway or an intersecting pathway. The analytical procedures employed must include identification and absolute quantification of the selected metabolites in the sample. This technique is very useful for studying the primary effect of a genetic alteration.

5.2.2 Metabolite Profiling

These studies are usually focused on a specific group of metabolites or metabolites associated with a specific pathway. This approach is generally used to trace the fate of a drug or a metabolite. It involves the rapid analysis of a large number of different metabolites. The analysis is generally not quantitative in nature. The main objective is to identify a specific metabolite profile that characterizes a given sample. This approach is sub-divided into:
(a) metabolic fingerprinting and (b) metabolic foot printing.

Metabolic fingerprinting

Metabolic fingerprinting involves the scanning of a large number of intracellular metabolites detected by a selected analytical technique or by a combination of different techniques.

Metabolic foot printing

This is a more recent approach which is technically similar to fingerprinting but is used to measure extracellular metabolites. Metabolic footprinting is a completely non-invasive approach and is also called as footprint or exometabolome. This method is used to detect metabolites in a culture medium. The metabolites secreted by the cells into the medium and the components of the medium which are transformed by the organism by biochemical processes are the compounds which are detected by this method.
Both these approaches of metabolite profiling i.e. metabolic fingerprinting and metabolic footprinting can be used to distinguish between different physiological states of wild-type strains, and between single-gene deletion mutants from those areas of metabolism which are not so closely related. Important physiological information can be extracted using these approaches. The disadvantage is that it requires the identification of the individual metabolites analysed, making the process laborious, tedious and difficult.

6. SAMPLE PREPERATION

In the analysis of metabolomes, sample preparation is of utmost importance. The sample preparation in metabolome analysis is done by three methods namely: 1) Quenching, 2) Extraction and 3) Sample concentration. A detailed description of these three methods is given below.

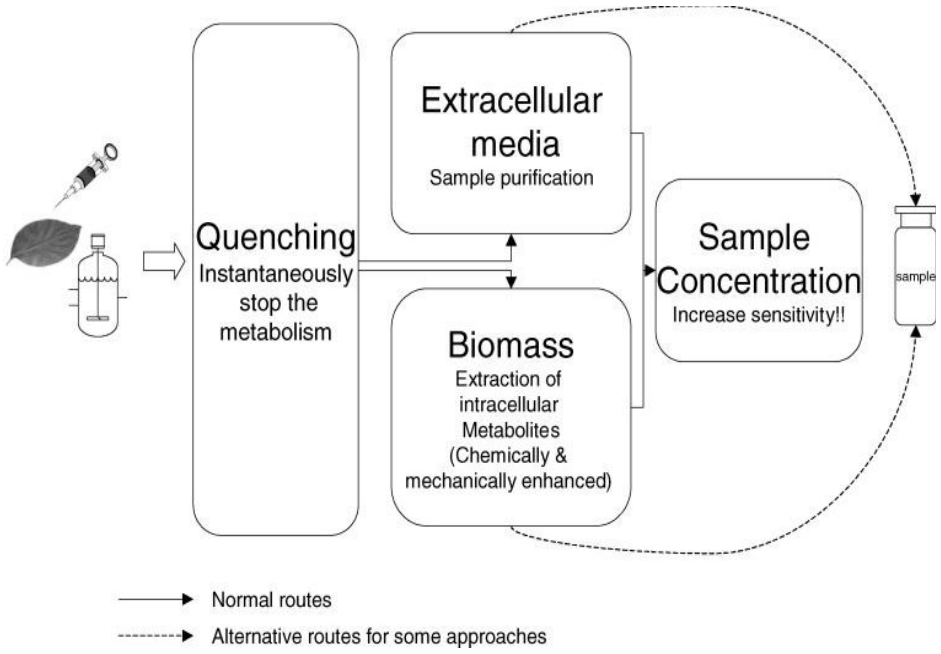

Fig. 1: Steps involved in sample preparation

6.1 Quenching

Rapid quenching of all the biochemical reactions is the first step in metabolome analysis as metabolite levels reflect the final response of a biological system to genetic as well as environmental changes. It is essential to stop all the metabolic reactions rapidly before metabolome measurement is started. A fast inactivation of metabolism is achieved by making rapid changes in temperature and pH. This process of rapidly inactivating the metabolic reactions is termed as quenching. In unicellular organisms or bio fluids, quenching is done by spraying the biomass into very cold (< 40°C) 60% buffered methanol solution. In a microbial cell culture, quenching is limited by the high dilution ratio between the biomass and extracellular medium. Microbial cells can also be quenched by perchloric acid. Fungi are filamentous in nature and hence are different morphologically and physiologically from other unicellular organisms like yeast. Hence, in case of fungi, quenching is done by liquid nitrogen or cold methanol solutions. On the other hand, for animal and plant tissues, liquid N_2 is used to snap freeze the sample, followed by mechanical disruption, which is done to release metabolites. The main quenching methods employed for animal and plant tissues include freeze clamping, immediate freezing in liquid nitrogen, acidic treatments with perchloric or nitric acid. Freezing in liquid nitrogen is considered the most reasonable method to stop quenching in plant cells. For the analysis of extracellular metabolites, it is essential to quench the cellular activity. There are two general strategies employed in the process of quenching:

(a) Combined quenching and extraction is done when there is partial extraction of intracellular metabolite, due to cell wall disruption, during quenching.

(b) Quenching followed by biomass separation from the extracellular medium, washing and extraction of the intracellular metabolites. Due to separation of biomass from the extracellular medium, the interference of extracellular compounds in the extraction of intracellular metabolites is eliminated[7, 14].

6.2 Extraction

A good method for the extraction of a maximum number of metabolites quantitatively is essential for analysing intracellular metabolites. The solvent employed in the extraction process should extract a maximum number of metabolites and at the same time, should be able to prevent any further physical or chemical alterations of the molecule. The whole extraction process should ensure a minimum loss of metabolites. Also, the solvent employed for extraction should be compatible with the subsequent analytical procedures. Usually in the extraction process, more than one solvent is used. For extraction of polar metabolites, polar solvents like methanol, methanol-water mixtures or ethanol are used. On the other hand, for extraction of lipophilic components, non-polar solvents like chloroform, ethyl acetate or hexane are employed. Another popular method of extraction is extraction at elevated temperatures with boiling solvents. This method is simple, accurate, fast and reliable. However, the limitation of this

method is that it is not suitable for the analysis of many intracellular metabolites as most of them are unstable at that elevated temperature (heat labile). The most common methods for extraction of intracellular metabolites include: a) Acidic extraction by use of perchloric acid ($HClO_4$) followed by freeze thawing and neutralization with potassium hydroxide (KOH). This method is mainly used for the extraction of acid stable compounds, nucleotides and water soluble metabolites. b) Alkaline extraction with sodium hydroxide, followed by heating at 80°C. Alkaline extraction is done for compounds which are stable in the presence of alkali. c) Extraction using ethanol which is carried out by boiling the sample in ethanol at 80°C.

6.3 Sample Concentration

It is essential to remove the extraction solvent partially or completely from the sample to be analyzed, as after extraction the intracellular and extracellular metabolites, are often present in dilute solutions. This is because of the usage of a large volume of the extraction solvent.

However, sample concentration can be achieved by vacuum using commercially available devices such as Rotavapor. However, there are some limitations associated with the usage of the Rotavapor for sample concentration, such as: the procedure is time consuming, number of samples that can be concentrated simultaneously is limited and many metabolites are not stable at the temperature which is required for evaporation of the solvent.

Another method commonly used for sample concentration is freeze drying or lyophilisation. This method is used to remove water from aqueous samples for thermo labile compounds in order to avoid thermal degradation. In this method, the advantages of deep freezing and dehydration are combined. The metabolites, dried from a frozen solution, are stabilized by a non-aggressive technology thus avoiding thermal degradation. The metabolites thus obtained show good stability allowing indefinite storage when kept in a cool, dry and neutral atmosphere, and in the dark for light sensitive products.

Other methods for sample concentration are the solid phase extraction (SPE) and solid-phase micro-extraction (SPME). The analytes are concentrated from a dilute sample by passing the sample through an SPE cartridge or by incubating the sample under an SPME fibre. The metabolites of interest are hence trapped, and are subsequently eluted into a small volume of eluting solvent (SPE) or by heating the fibre into a gas chromatography GC injectionport (SPME). However, the application of these methods is limited as different trapping materials are needed to trap different classes of metabolites and all classes of metabolites cannot be concentrated effectively by SPE or SPME methods[14].

7. DETECTION

The two major analytical technologies used to analyse and detect the metabolomics data are mass spectrometry and NMR spectroscopy.

7.1 Mass spectrometry

The metabolites are identified as well as quantified by mass spectrometry. The main advantage of this method is that it is sensitive and analytes in the femtomolar as well as attomolar range can be detected. Hundreds of individual species of a single sample can be detected when this technique is coupled with gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE).

Gas chromatography coupled with mass spectrometry (GC-MS): This method has high separation efficiency and hence many complex biological mixtures can be resolved. When coupled with mass spectrometry, this method permits reliable identification of compounds. This method is used for separation of volatile and non-volatile metabolites after derivatization. The advantage of GC-MS is that it is a low cost method and easy to operate. The limitation is that the sample has to be volatile in order to be eluted in gas chromatography.

Liquid chromatography coupled with mass spectrometry (LC-MS): Molecular identification of polar, less polar and neutral metabolites can be achieved by coupling mass spectrometry with liquid chromatography. The major difficulty encountered in coupling liquid chromatography with mass spectrometry is the incompatibility caused by the flow rate of the liquid into the high vacuum of the mass spectrometer, ionization of non-volatile and thermally labile analytes and the incompatibility caused by non-volatile mobile phase which is used in chromatographic separation.

Capillary electrophoresis coupled with mass spectrometry (CE-MS): Fast and efficient detection of a wide variety of charged as well as uncharged species is achieved when capillary electrophoresis is coupled with mass spectroscopy. The advantages of this method are its high speed, selectivity and sensitivity[2, 7, 14-15].

7.2 NMR spectroscopy

This is the only technique of detection which does not depend on the separation of analytes and thus the sample can be recovered for further analysis. The major strength of NMR spectrometry is its high analytical reproducibility and the simplicity of sample preparation. Also, all types of small molecule metabolites can be measured simultaneously. Hence, NMR is a universal detector. The weakness however, is that, it is relatively less sensitive as compared to the mass spectrometry based techniques[2, 14-15].

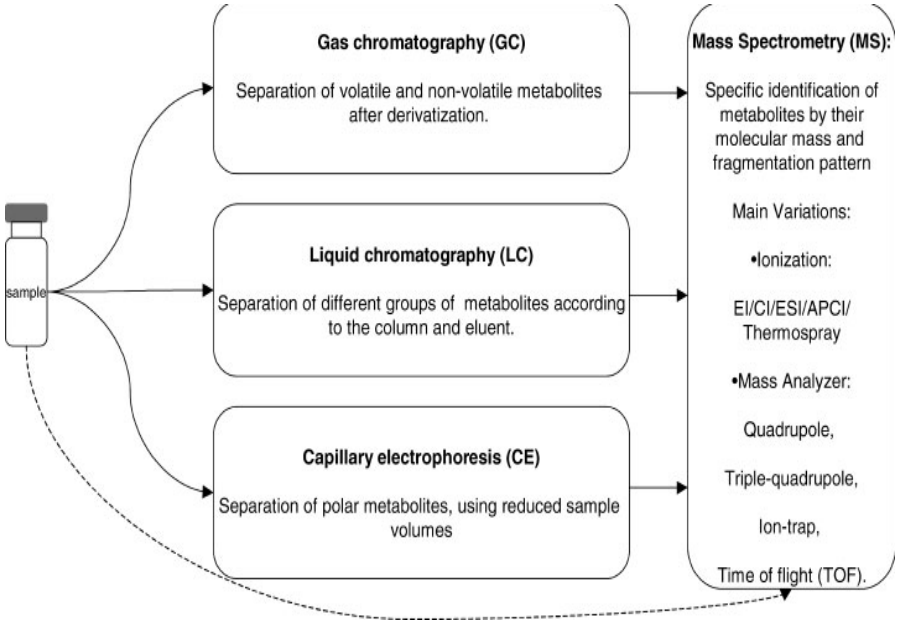

Fig 2: Alternative ways for MS analysis of metabolites

8. APPLICATIONS OF METABOLOMICS

The main applications of metabolomics are as follows:

8.1 Assessment of Toxicity

The physiological changes that occur in the body due to toxins or chemicals can be detected by metabolic profiling of the urine or blood plasma samples.

8.2 Functional Genomics

A phenotype caused by gene manipulation like gene deletion or insertion can be detected using metabolomics. Phenotypic changes in genetically modified plants intended for human consumption can also be detected. Metabolomics can be an excellent tool for studies of atherosclerosis, profiling of ovarian follicular fluid and plasma, identification of biomarkers, etc.

8.3 Nutrigenomics

Nutrigenomics links genomics, transcriptomics, proteomics and metabolomics to human nutrition. Metabolomics is used to determine a biological endpoint or metabolic fingerprints. Metabolomes are influenced by various factors such as age, sex, genetics, body composition and sometimes underlying pathologies. In general a metabolome in a given body fluid is influenced by endogenous factors such as age, sex, body composition and genetics as well as underlying pathologies[13, 16-18].

9. FUTURE SCOPE FOR METABOLOMICS

Genomics, transcriptomics and proteomics could not fully exploit discovery driven sciences. Metabolomics has a unique opportunity to do so. In our simplistic model of gene→ transcript→ protein→ metabolite, there is a wide scope for finding extremely useful disease biomarkers and the correlation between these four biomolecules can be carefully studied. The data collection and analytical technologies for genomics and proteomics is advanced and hence the metabolomics study can be carried out in detail with greater accuracy.An important goal of this technology is to evaluate or monitor the health status of patients by application in clinical studies. MS and NMR are the two main technologies used in metabolomics detection. Of these, MS plays a major clinical role as compared to NMR. MS is applied routinely in clinical and diagnostic laboratories. The ability of MS to directly measure a specific metabolite in a biological sample is employed using methods primarily built upon isotope-dilution-MS. Most clinical MS assays are conducted in reference laboratories. However, this scenario is likely to change over the next few years as mass spectrometers have become a standard laboratory instrument. These studies have the ability to translate discovery into assays that can be applied routinely within clinics. This will have an enormous impact on public

health. Disease-specific biomarkers can be created using this technology. The hope is that metabolomics studies will be be more successful than genomics and proteomics, and can learn from the other omic technologies to not make the mistake of collecting a lot of data and giving very little information[15].

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