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Metabolic Fingerprinting: Quality Control of Phytopharmaceuticals

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ABSTRACT

Metabolomic fingerprinting techniques have received a lot of attention recently and used as a powerful tool for the analysis and quality assessment of the herbal derived medicines. It is increasingly being used in the quality control and standardization of herbal material and phytopharmaceuticals. The most common techniques that are used in metabolomics consist of NMR, GC-MS, and LC-MS in combination with multivariate statistical analyses. In the areas of drug discovery from the natural product for discovering lead compound and quality control of phytopharmaceuticals, Metabolomic fingerprinting or profiling is continuously being applied. The present review has made attention to different aspects of applications of fingerprinting and the state of the art in plant metabolomics.

INTRODUCTION

Medicinal plants are known to be used for the treatment, cure of human & animal ailments for a long time. According to WHO and other reports[1-2], around 80% of the population globally relies on plant-derived natural products and they are widely used and played an important role in maintaining health. Commercialization of these herbs derived products demands safety, efficacy, and quality. Adulteration is a major source for affecting the quality and efficacy of these products. This is mostly because of various factors such as distinguishable morphological features, common names of raw material, and substitution of raw material due to an economic issue. Hence, proper authentication and identification process is necessary to prevent the adulteration. Most of the regulatory agencies suggest macroscopic, microscopic and chemical evaluations like TLC, HPTLC, and HPLC. However, these routine methods have limitations. The intrinsic, as well as extrinsic factors, may affect the chemical composition of herbal drugs. To overcome authentication issues, metabolomic fingerprinting methods are used to prevent intentional and inadvertent adulteration or substitution of targeted nature-derived products. *Metabolomics* is the identification and quantification of all metabolites in a biological system in all types of organisms and plants. Metabolomics is now becoming a well-known technique for the quality control and standardization of plant-derived natural products and phytopharmaceuticals. This system works on a targeted approach (microscopic view) for the specific set of compounds and called metabolic fingerprinting or metabolic profiling. The other approach is untargeted (macroscopic view) which aims at identifying and quantifying all the metabolites present in specific organism[3]. *Metabolic profiling is defined as a* quantitative analysis of a set of metabolites in a selected biochemical pathway or a specific class of compounds. This includes target analysis, the analysis of a very limited number of metabolites, e.g. single analyte as precursors or products of biochemical reactions. And *Metabolic fingerprinting is* an unbiased, global screening approach to classifying samples based on metabolite patterns or 'fingerprints' that change in response to disease, environmental or genetic perturbations with the ultimate goal to identify discriminating metabolites[4-5].

Most simply, metabolomics is the systemic study of unique chemical marker that a specific cellular process leaves behind and studying molecule metabolite profile[6]. The metabolome is the collection of all metabolites in a biological organism, which are the end products of its gene expression. This metabolome expresses the whole process of the physiology of that cell

and gives a more complete picture of a living organism, which mRNA gene expression data and proteomic analyses do not express. Chromatographic and spectroscopic methods generally used to study the metabolome. As metabolites separation carried out by chromatography and then being quantified and identified with various detectors.

***History of Metabolomics*:** The term metabolome was introduced in 1998 for yeast genome[7] along with the term genome, proteome. And when the metabolomics was introduced i.e. 2001, it was not a new process or new concept, but it was more a consequence of the development of the techniques which form the basis of metabolomics. The basics of metabolomics already had been performed before as a TLC profile of medicinal plant extract or GC chromatograms of essential oils. The wider application of two-dimensional techniques for more accurately and precisely the identification of metabolites in the crude sample was increased. In the mid-1980s unmodified biological fluids being studied by using increased resolution spectra[8] i.e. NMR. Also in the early 1990s metabolic fingerprinting of plant material came into use[9]. Along with these, the development of chromatographic techniques such as capillary column GC and small particle HPLC columns was in boom and wider applied. Advances in chemometrics allowed the evaluation of large data sets and distilling significant changes in specific parameters.

***The technique in Metabolomics*:** the most diverse techniques, like UV, IR, MS, and NMR or chromatographic like GC or HPLC, or hyphenated techniques, like GC-MS or LC-NMR are used. Some of the techniques are shown in Fig:1 The areas of metabolomics research where issues of quantification and identification are important, nowadays, NMR and mass spectrometry are the principle detection techniques to be used, having specific advantages and disadvantages. Within mass spectrometry many different techniques can be distinguished, varying in the method of introduction of the sample (direct, GC, LC), in the method of ionization (electron impact, MALDI, electrospray) and in the method of detection (time of flight, FT-ICR).

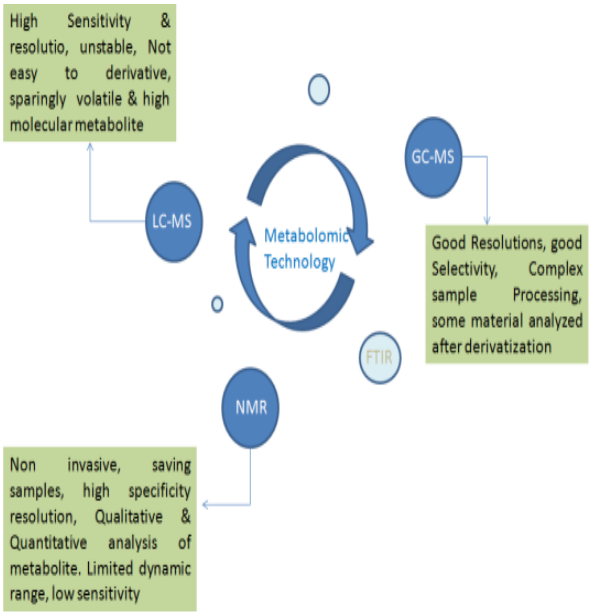

Figure No. 1: Some techniques in Metabolomics.

Metabolomics using Mass Spectroscopy:

The most commonly used mass spectrometry platforms are:

GC-MS: It has the high chromatographic resolving power of gas chromatography is combined with electron impact mass spectrometry. Electron impact ionization provides for each compound a mass spectrum, which through the fragmentations provides much information about its identity. Because Electron Ionization is the most traditional way of ionization, large databases exist and compounds can be rapidly identified. The compounds need to be volatile to be analyzed, it is a major disadvantage of GC-MS. Furthermore, they should be stable during the analysis in which high temperatures are used. By derivatization, many normally not volatile compounds can be converted in volatile adducts, e.g. by silylation, acetylation or methylation.

LC-MS: liquid chromatography is combined with mass spectrometry. In this type of analysis, all types of compounds can be separated by LC and subsequently in-line analyzed by MS, usually electrospray-MS. With modern columns (e.g. UPLC) high resolutions can be obtained. The electrospray mass spectra are, however, less informative than the EI mass spectra. Also, large databases are not yet available. Another problem in electrospray mass spectrometry is the difficulty of ionization of many compounds. It is, however, possible to work in positive or negative ion mode.

FT-ICR-MS: The mass spectrometer has a very high resolution using Fourier transform ion cyclotron resonance MS. Based on the exact masses, this gives a complete resolution of compounds. However, isomers cannot be resolved. For metabolomics, the chromatography step, which might prevent certain compounds to arrive at the mass spectrometer, is often omitted.

CE-MS: Promising results have been obtained using the coupling of capillary electrophoresis coupled to the mass spectrometer.

Metabolic Fingerprinting using NMR

A comprehensive evaluation of all metabolites and to achieve quantitative and qualitative final goal in living organisms, analysis chemistry combined with multivariate data analysis. Different technological platforms, NMR and MS have been successfully used for metabolic fingerprinting analysis. These two techniques have their respective advantages and limitations and are often referred to as being complementary[10-11]. However, as a tool for metabolomics, NMR has some unique advantages over MS-based methods. It can provide a detailed analysis of the biomolecular composition very quickly with relatively simple sample preparation[12–14]. NMR spectroscopy is based on the physical characteristics of compounds, it has very high reproducibility. In metabolomics, all data obtained from the analytical methods should be analyzed by statistical methods to extract all possible information from the data and the detection of metabolites is not the final step. Also, the accuracy and correctness of the data to be analyzed by the statistical methods are inevitably reliant on the robustness of the raw analytical data set. In this aspect, NMR has a unique advantage, the highest reliability in metabolomics. Unlike the retention time in chromatography based techniques, with a few exceptions the chemical shift, coupling constant, and integral of each signal in an NMR spectrum does not change as long as it is measured under the same conditions: the field strength applied, solvent, pH, and the temperature. Despite the low intrinsic sensitivity, the robustness of data and the ability to cover a broad range of metabolites has enabled NMR to be the favored overall “macroscopic” metabolomics and fingerprinting tool. In addition to the advantages of data robustness, the power of NMR in structure elucidation of metabolites cannot be matched by any other method. NMR has a long history in the natural products chemistry field as the tool for structural elucidation. With a proper database, it can generate data that can be kept almost permanently[15]. In this part, the general procedure of NMR metabolomic analysis will be discussed together with multivariate data analysis. The

identification of metabolites is of high importance and this aspect will be highlighted. At the end of this section, specific applications will be discussed using NMR as a metabolic fingerprinting technique.

Like other methods using MS or chromatography, NMR-based metabolomics includes sample preparation, extraction, multivariate data analysis, and identification of metabolites the generally accepted procedure of NMR-based plant metabolomics. Steps involved in sample preparations using MS/NMR analysis for Quality Control of plant-derived medicine are shown in Fig 2. To obtain reproducible and reliable results, the sample preparation steps need much caution. In the metabolomic analysis, environmental conditions (temperature, soil pH), developmental stage, age, type of tissues and harvesting time greatly affect the metabolome obtained even from the same genotype. For these aspects sampling of the targeted plant, materials should be carefully planned to avoid increased biological variation interfering with the final data interpretation. The sample should be frozen immediately to avoid any biochemical change in the material just after harvesting. The next step is grinding and extraction of the material to liberate the metabolites from the cells. Biochemical reactions may occur in the material during the extraction and reflected in changes in the metabolome. Before extraction, drying the material either by heat or freeze-drying, keeping the material at a low temperature, or grinding at low temperatures and/or in the presence of a solvent that denatures enzymes involved in metabolite alteration must be done to avoid the biochemical reactions. Denaturation can also be achieved by brief microwave treatment. For the development of an optimum extraction method, Ultra-Turrax®, mechanical grinding just previously deep-freezed in liquid nitrogen, sonication with a probe head or cup horn and bead-beating before solvent extraction were evaluated for the efficient breakage of the cell using mycobacteria, Mycobacterium Bovis. Of the evaluated methods sonication was found to be superior to others[16]. Metabolomics targets at comprehensive fingerprinting of all metabolites the extraction methods used should cover all possible metabolites in an organism. A two-phase solvent system consisting of chloroform: methanol: water (2:1: 1) has been applied to extract both polar and nonpolar compounds in a single extraction where pH affects the profile of metabolite e.g. alkaloids[17-19]. When dealing with a large number of samples because of the long processing time and the possibility of degradation or losses during processing, this two-phase extraction method was found to be problematic. For better quality NMR spectra, Recently, a simple direct extraction method with deuterated NMR solvents has been developed for sample preparation[20-24]. Single solvent systems have been used for some

studies e.g. Methanol with TFA was found to be a suitable solvent for the extraction of alkaloids[25] while for more polar metabolites perchloric acid was routinely used, to prevents enzymatic degradation of metabolites[26]. However, a combination of CD_3OD and D_2O in different ratio of 30% to 70% was found to be a more preferable solvent, since it can extract more diverse metabolites, depending on the study. To extracts, a wide range of metabolites including amino acids, carbohydrates, fatty acids, organic acids, phenolics, and terpenoids in a single step, mostly a mixture of methanol-d4 and KH2PO4 buffer in D2O (pH 6.0) has been used. To deal with a large number of samples, and to save time as well, a direct extraction method with deuterated NMR solvents can be used. the fluctuation of chemical shifts of signals in the NMR spectra can avoid possibly by having a buffer insolvent. However, when commercial herbal preparations are analyzed a more targeted approach is used focusing on the known major bioactive compounds in the plant material.

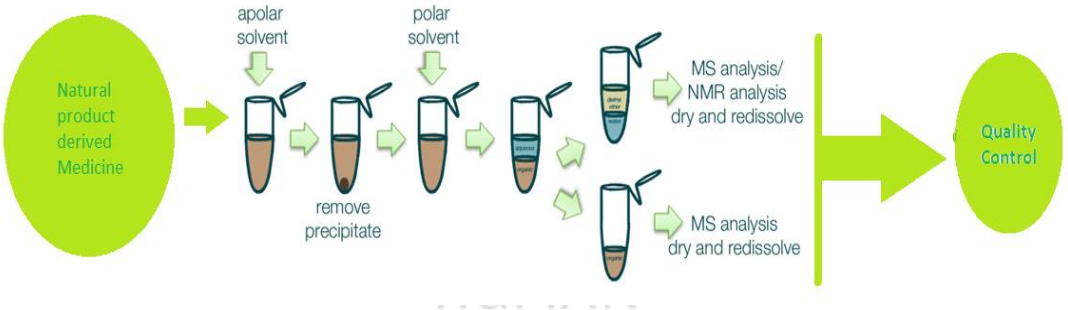

Figure No. 2: Steps involved in sample preparations using MS/NMR analysis for Quality Control of plant-derived medicine.

SUMMARY

In very few years of existence, Metabolomics has gone through great development and much more application are awaiting. Diversity in metabolites in plants as compared to human and animals, make it much more difficult to arrive at a routine system for sample preparation. Optimized sample preparation is required for every experiment. Most diverse techniques like UV, IR, MS & NMR are widely used for quality control of plants derived products. In 1D NMR only a limited number of metabolites can be observed & it increases by using 2D NMR. This NMR spectrum like TOCSY, HSQ & J resolved are useful to reveal hidden signals & important to identify the metabolites. However, the most serious problem is to detect minor components in the presence of a large spectrum. This can be overcome by using LC in combination with MS. Metabolic fingerprinting is ideal for medicinal plant

authentication besides morphological, anatomical and chemical markers. In the future, metabolic fingerprinting can be used to get completely rid of adulterants and spurious materials that have ruined plant-derived products and phytopharmaceuticals.

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