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NMR-based metabolomics: a holistic approach for monitoring complex biological systems

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Abstract

Metabolomics is a novel approach with a huge potential to assess and reveal in a complex biological system altered metabolism produced in response to environmental stressors, diseases or exposure to toxicants. The high applicability of this approach is due to its ability to qualitatively and quantitatively characterize the chemical profile of all the low molecular weight metabolites (metabolome) present in cells, tissues, organs, and biological fluids as end products of the cellular regulatory pathways. Thus, providing a snapshot of the phenotype of a biological system, metabolomics offers useful contributions to a comprehensive insight into the functional status of human, animal, plant, and microbe organisms. One of the main analytical platforms employed in metabolomics is the Nuclear Magnetic Resonance (NMR) spectroscopy that, when linked with pattern recognition techniques and data mining tools, can provide an overview of the metabolic status of a complex biological system and comprehensive insights into important metabolic processes. Over the last decade, the number of scientific publications in the metabolomics area has increased exponentially. The literature analysis revealed metabolomics applications in a wide range of fields including medical, animal, plant, environmental and food sciences.

Key Words: Biological systems;Metabolomics;^{1}H Nuclear Magnetic Resonance Spectroscopy; Chemometrics; Research fields

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Introduction

The conventional toxicological methods for stress evaluation, due to their one-dimensional nature, cannot provide the “full picture” of biological effects on organisms upon exposure to stressful conditions. Therefore, in order to overcome such limitations, a current approach is to employ “-omics” techniques that allow for the simultaneous evaluation of a broad number of biomolecules. One of the most recent additions to the -omicsfamily is metabolomics, which involves the study of endogenous, low molecular weight metabolites (<1000 Da), whose production and levels vary with the physiological, developmental, or pathological state of cells, tissues, organs or whole organisms (1). The metabolome describes the composition ofmetabolites at the time of sampling, and includes

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compounds such as lipids, sugars, and amino acids that can provide important clues about the health of individualsand a functional measure of cellular status at that moment in time (1,2).

One of the greatest advantages of metabolomics is that metabolome isthe first to respond to stressors, where in some cases no changes in thetranscriptome and proteome occur (2). Hence, metabolomics may provide the most functional information of the -omics technologies (3), as transcript and protein changes do not necessarily lead to a biochemical change in the organism (4). Furthermore, the metabolome (coined by 5in a fashion analogous to genome and proteome) represents the final "-omic" level in a biological system, and metabolites represent functional entities (6). Metabolites have a clear function in the life of biological systems and there are far fewer metabolites than genes or gene products to be studied (6). However, all -omics technologies provide valuable information and their integration promises to provide the most complete understanding of biological systems.

Metabolomics investigations can bedesigned as targeted studies looking for specific metabolite changes, although this requiressome prior knowledge on the metabolic action of whatever toxicant is being tested. Alternatively the global metabolome is analysed, althoughconstrained by the efficiency and sensitivity of the techniques used to extract and detect metabolites. This method is a relatively non-targeted approach where there is little, if any,prior selection of which metabolic components to measure. Thus, a similar study design canbe used in both a screening mode and mechanistic exploration (7). Among the analytical technologies applied in metabolomics, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy allows a wide range of metabolites to be analyzed simultaneously, offering valuable biochemical and quantitative information on the physiological disturbances induced by endogenous and exogenous factors (8), as well as in discovering metabolic biomarkers in response to environmental stressors or diseases (9-22).

NMR-based metabolomics

NMR spectroscopy is a quantitative technique that can report on hundreds of compounds in a single measurement. It provides information on metabolites comprising nuclei such as ^{1}H, ^{13}C, ^{31}P, which can exist at different energy states in a strong magnetic field because they possess nuclear spin. Lines that can be seen in the spectra are due to transitions between these energy levels.

A nucleus of spin I will have $2I$+1 possible orientations. With a magnetic field applied(B_0), the energy levels split and each possesses a magnetic quantum number, m. In a magnetic field a nucleus with spin 1/2 will have two orientations: m=+1/2, a low energy state aligned parallel to B_0; and m=-1/2, a high energy state aligned anti-parallel to B_0. According to the Boltzmann distribution, the

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lower energy level will contain slightly more nuclei than the higher level. However, these nuclei can be excited into the higher level by electromagnetic radiation with the needed frequency of radiation determined by the difference between the energy levels.

By application of a radiofrequency pulse, the magnetisation vector rotates about the direction of B_0sweeping out a constant angle (Larmor precession) at the Larmor frequency (23), what is detected in an NMR experiment (or free induction signal). Fourier transformation of these signals produces a NMR spectrum. Different types of pulses exist and when the transmitter frequency is exactly the same as the Larmor frequency, the pulse is said to be exactly on resonance (23). Different protons in a molecule resonate at slightly different frequencies, and frequencies at which NMR absorptions (lines) occur scale linearly with the magnetic field strength. As this frequency shift and the fundamental resonant frequency are directly proportional to the strength of the magnetic field, the shift can be converted into a dimensionless value (chemical shift), which is reported relative to a reference resonance frequency (commonly sodium-3-trimethylsilyl-2,2,3,3-d4-propionate – TMSP, or 2,2-Dimethyl-2-silapentane-5-sulfonate – DSS). The difference between the frequency of the signal and that of the reference is divided by the frequency of the reference signal to give the chemical shift, expressed in parts per million (ppm) (23). The chemical shift is used for structural information, as well as the size and shape of peaks in the spectrum. In a complex mixture, the integrated areas under the peaks reveal the concentration of a particular compound.

Workflow in a NMR-based metabolomics experiment

The basic procedure for conducting a NMR spectroscopy-based metabolomics study is depicted in Figure 1. The first step of the experimental design involves the selection of a model organism (i.e. bacteria, invertebrates, fish, insects, plants or humans), a type of external stressor (i.e. exposure to contaminants, changes in environmental parameters or disease), and the mode and duration of exposure. Therefore, based on the aims of the experiments, it is necessary to select the target tissue/organ or biofluid to be investigated and then proceed with a proper extraction of metabolites. After that, samples can be analyzed using the NMR spectroscopy to obtain metabolic profiles in order to identify and quantify a variety of metabolites. NMR data will be then statistically processed by applying both multivariate (i.e. Principal Component Analysis, PCA or Partial Least Squares (PLS)-regression) and univariate statistics (i.e. Student's *t*-test). The final step will be the biological interpretation of data obtained in order to make a connection between the external stressor and the metabolic response of the model organism, by discovering metabolite biomarkers

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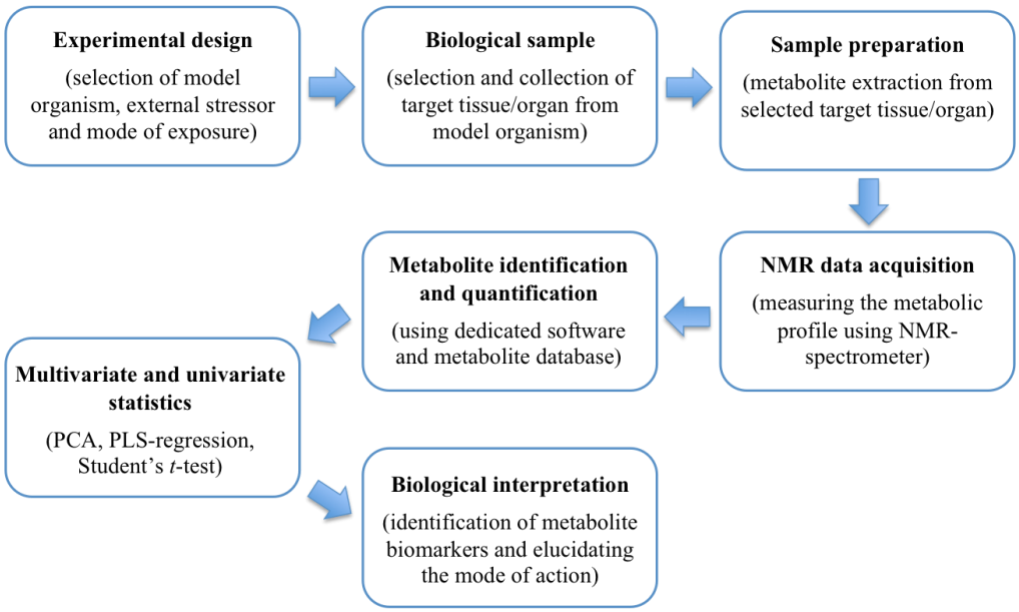
Figure 1. Typical workflow in a NMR-based metabolomics experiment.



Research fields using metabolomics

NMR spectroscopy-based metabolomics, when linked with pattern recognition techniques and data mining tools, can detect differences in the profile of metabolites (metabolic biomarkers) in response to environmental stressors, diseases or exposure to toxicants (4,8-22), providing an overview of the metabolic status of a biological system. Metabolite profiling, originally developed for biomedical applications (24), has been increasingly employed in several research areas, including plant (3), microbial (5) and environmental science (8-22).

In ecotoxicology, metabolomics is of particular value for the risk assessment of chemicals in the environment (1). Because metabolomics can provide valuable information on how xenobioticsinfluence physiological functions, this technique has recently been applied to experimental studies of selective exposure on various aquatic organisms, both invertebrates (17,19,22) and fish (12-14,21). Environmental metabolomics has many advantages for elucidating organism–environment interactions and assessing organism health status (4,8). It also allows the identification of specific biomarkers that differentiate pollutant-exposed from unexposed organisms and can be applied for direct evaluation of mixture effects under field conditions (9-16,18,20).

It is widely recognised the value of metabolomics in disclosing the toxicity mechanisms of a variety of environmental contaminants. To date, NMR metabolomics has demonstrated to be well suited for exploring metal(loid) toxicity in a wide range of aquatic species. For instance, it contributed to unveil the signaling pathways of mercury in wild golden grey mullet, pinpointing disturbances in energy and protein metabolism, membrane stabilization/repair processes, oxidative stress and neurotoxicity (12-14). NMR metabolomics has been applied to elucidate the metabolic pathways by

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which polyaromatic hydrocarbons (PAHs) or their mixture manifest their toxicity on aquatic biota, as for instance on marine mussels caged in petrochemical pollutes sites (9-11,16,18). NMR was also able to provide a systematic and holistic view of sub-lethal responses to pesticide exposure, as documented for Japanese medaka larvae treated with a neonicotinoid insecticide evidencing impairments in energy metabolism, as well as altered cholinergic and adrenergic neurotransmission (21). NMR has also produced differential metabolic fingerprints attributable to treatments with different pharmaceuticals, as recently reported in mussels exposed to a synthetic progestin, revealing the occurrence of alterations in energy, amino acids, and glycerophospholipid metabolism (17). In regard to emerging contaminants, to date NMR was successfully applied to evaluate the impact of nanoparticle exposure on biota, as documented for sea urchin embryos with induced interferences in cholinergic and serotoninergic neurotransmission pathways, as well as in biomineralization processes (19). Moreover, although the urgency to gain a comprehensive understanding of the metabolic effects of microplastics (MPs) on biota, information about their underlying mechanisms of toxicity is still limited. In mussels exposed to polystyrene MPs, differential tissue-specific metabolite profiles were recorded, with alteration in osmoregulation, protein and energy metabolism, and neurotransmission (22).Additionally, NMR-based metabolomics has been recently applied in food studies to accurately evaluate the health and safety aspects of food and food processes, as well as the quality status and authenticity of food products (15,20,25).

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