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LETTER

The Essential Role of Analytical Chemistry in Metabolomics

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In the early 2000s, metabolomics emerged as a cutting-edge research field,[1] focusing on studying metabolites present in biological matrices, such as cells, tissues, biofluids, and whole organisms. Metabolites are small molecules that are synthesized, transformed, or consumed within the complex network of chemical reactions that sustain the biochemical and physiological functions of living organisms (metabolism). From the comparative analyses of these biomolecules in control versus test condition(s), metabolomics offers valuable insight into the biochemical and physiological state of a biological system, implying a closer approximation to its phenotype.

Two experimental approaches are usually employed in metabolomics. The **untargeted metabolomics** approach is based mostly on the **qualitative analysis** of the maximum number of metabolites, including diverse chemical classes and dynamic ranges present in a biological sample. In untargeted metabolomics, studies may rely on the semi-quantitative analysis of differential metabolites when analytical standards are unavailable, focusing on the relative intensities of the instrumental response under different conditions. However, the **targeted metabolomics** approach is defined as the **quantitative analysis** of a selected number of metabolites and/or substrates of metabolic reactions that might be associated with common chemical classes or related to selected metabolic pathways.[2]

Metabolomics is a multidisciplinary area that connects chemistry, biology, statistics, and computational sciences. More specifically, analytical chemistry plays a crucial role in the metabolomics workflow since it encompasses the principles, techniques, and methodologies used to separate, identify, and quantify biomolecules. If we examine the table of contents of a typical textbook on the fundamentals of analytical chemistry,[3] we observe that many of the topics covered are also addressed within the metabolomics workflow.

The first step in the metabolomics workflow is **experimental design**. Here, the type of metabolomics analysis is selected: quantitative (targeted metabolomics) or qualitative (untargeted metabolomics). Other important topics concern **sampling** (sample size calculation, sample collection, metabolic quenching, sample storage) and the selection of the analytical technique. The latter is also relevant for the second step in the workflow, which is **sample preparation**. The best sample preparation technique is selected based on the analytical platform and type of metabolomics analysis chosen. Protein precipitation, liquid-liquid extraction, and solid-phase (micro) extraction are the most common strategies used in metabolomics.

The third step in the metabolomics workflow is **data acquisition**. Here, the **instrumental analysis** is performed. The most common analytical platforms employed are based on **mass spectrometry** (alone or coupled with **separation techniques** such as liquid chromatography, gas chromatography, or capillary electrophoresis) and nuclear magnetic resonance spectroscopy. All these platforms can be used for untargeted metabolomics. In the case of mass spectrometry systems, untargeted metabolomics requires an instrument with high-resolution mass analyzers for further metabolite annotation. Mass spectrometry

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instruments with tandem capabilities are usually employed for targeted metabolomics, and the mass analyzer can be either low- or high-resolution. Additionally, **calibration** and **validation** of the analytical method are required in targeted metabolomics approaches.

The next stage in the metabolomics workflow is **data processing**. Since metabolomics experiments generate large data matrices, data pretreatment steps are necessary, including peak detection and alignment, deconvolution, normalization, data transformation, scaling, and filtering. **Statistical analyses** are then required. Both univariate and multivariate (**chemometrics**) techniques are important to either select the statistically significant features that are altered when comparing two experimental conditions in an untargeted metabolomics strategy or to indicate statistically significant differences in the concentration of the evaluated metabolites in a targeted metabolomics setting.

In an untargeted metabolomics approach, after selecting the relevant features, the next step involves **metabolite annotation**. This step is usually performed with the aid of spectral databanks. However, knowledge of nuclear magnetic resonance spectroscopy or mass spectrometry **spectra interpretation** is very relevant to performing a manual curation from the information acquired through the databanks, which can be incomplete or inaccurate. This is especially true when dealing with data from liquid chromatography coupled to mass spectrometry. In most cases, electrospray ionization is selected, which generates mass spectra with several adducts. This leads to multiple signals for the same compound and unexpected fragment peaks from in-source fragmentation. In addition, there is difficulty in differentiating isomeric and isobaric compounds.

The last step for both targeted and untargeted metabolomics is **biological interpretation**. Here, the connection between the analytical results and the biological problem is established, indicating the altered metabolic pathways that are related to the biological question under study. Alterations in metabolic pathways are caused by disease, treatment, environmental exposure, and genetic modification. The metabolomics results, in turn, reflect the disturbance in the **chemical equilibria** of an organism since metabolic reactions adjust to changes in concentrations of substrates, products, or environmental conditions to maintain homeostasis.

In summary, the discipline of analytical chemistry provides the fundamental tools and methodologies that are the basis of metabolomics. The integration of analytical techniques allows for comprehensive profiling of metabolites, leading to significant insights into several biological processes, including disease mechanisms and potential therapeutic targets.

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