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Review article

Current practice of liquid chromatography–mass spectrometry in metabolomics and metabonomics

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ABSTRACT

Based on publication and citation numbers liquid chromatography (LC–MS) has become the major analytical technology in the field of global metabolite profiling. This dominance reflects significant investments from both the research community and instrument manufacturers. Here an overview of the approaches taken for LC–MS-based metabolomics research is given, describing critical steps in the realisation of such studies: study design and its needs, specific technological problems to be addressed and major obstacles in data treatment and biomarker identification. The current state of the art for LC–MS-based analysis in metabonomics/metabolomics is described including recent developments in liquid chromatography, mass spectrometry and data treatment as these are applied in metabolomics underlining the challenges, limitations and prospects for metabolomics research. Examples of the application of metabolite profiling in the life sciences focusing on disease biomarker discovery are highlighted. In addition, new developments and future prospects are described.

Contents

1. Introduction

The application of unbiased global, or holistic, analysis of biological samples (e.g. animal/human biofluids, tissue and cell extracts, in vitro incubation media etc., or plant/food extracts), known variously as metabolomics or metabonomics, are increasing exponentially as the potential of this approach to discover new biomarkers is becoming more widely appreciated. Analytical strategies for metabolomics research aim to characterise the whole metabolite complement of the samples under study and then relate their concentrations to features or properties of the sample [1]. Metabonomics is defined more in terms of seeking differences between the metabolic profiles of test and control groups. In practice these terms are often used interchangeably [2,3]. Metabonomics/metabolomics typically employs multidisciplinary research and collaborative efforts by scientists from

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different fields of expertise including advanced analytical chemistry and statistical analysis, biochemistry, medicine/life sciences, nutritional, agricultural or environmental sciences.

The metabolite coverage provided by these metabonomics/metabolomics studies is, to an extent, still determined by the analytical methodology applied. This means that, with the present state of the art no one method can describe the whole metabolome [4–6].

These holistic methods of profiling represent a hypothesis-free research strategy for the detection of potential biomarkers and should subsequently link them with other metabolites into biochemical networks. However, the methodology risks the identification of artefacts as markers due to e.g., mistakes in sample processing or minor instrumental variations, and this possibility should be eliminated as far as possible by meticulous study design, sample handling and robust analytical methodology. The results should thus be concrete and properly validated after which expert knowledge of the highlighted biochemical pathways along with proficiency in statistical analysis are a necessary prerequisite for success.

Metabolomics may be combined with genomic or proteomic results towards a systems biology approach which integrates the information at different levels/systems to provide biochemical insight in the organism being studied [7–9]. The major application areas for metabolomics are currently to be found in biomarker discovery for medical and life sciences, plant/food and environmental sciences [10,11]. In the medical field metabolomics/metabonomics finds application in the search for early biomarkers of disease (diagnostic markers) [12] for drug efficacy prediction (pharmacometabonomics) [13,14] biomarkers of disease progression (prognostic markers) or drug toxicity (safety assessment) [15]. In plant/food sciences metabolomics can assist in taxonomic studies or in the assessment of quality or origin of (for example) fruits or wine, or olive oil [16,17]. Microbial metabolomics represent an upcoming field. It is now recognised that mammals represent a super-organism where guest-host interactions with e.g. gut microflora may be of primary role in various un-expected phenomena such as the toxicity or metabolic fate of a pharmaceutical [13,18].

Compared with genomics, transcriptomics and proteomics the field of metabolomics may appear new. The truth is that this type of untargeted metabolic profiling has been practiced for decades (e.g. see [19]). However the widespread use of metabolomics and the field's impressive growth in resources is a more recent trend.

Liquid phase separations, particularly High Performance Liquid Chromatography (HPLC) and Ultra High Performance Liquid Chromatography U(H)PLC, have become an indispensable tool for the determination of small molecules in a very wide range of applications and, when combined with mass spectrometry (MS), are becoming the key technology in the quest for extended metabolome coverage and biomarker discovery (for some recent reviews see Refs. [5,20,21]).

2. Analytical technologies

Holistic analysis requires access to information-rich spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy and MS (the latter often combined with a high resolution separation technique) [5,20,21]. Even so, from a separation science perspective, the task of analysing all the low molecular mass molecules contained in a biological sample is overwhelming given their different physicochemical characteristics and concentrations ranges. Obviously volatile molecules, such as those e.g. present in breath, are better analysed using GC [10,11,22] however, the majority of metabolites are polar, involatile, molecules which require derivatization prior to GC analysis. HPLC, and the higher efficiency variants such as U(H)PLC, offer the most versatile tools for the analysis of a multitude of molecules which belong to different groups, have different molecular properties and coexist in the same sample in varying concentrations. Utilisation of MS in direct infusion mode has been proposed for screening purposes and can be very helpful in quantification in complex mixtures, in which case very high mass accuracy is a prerequisite. However, if a chromatographic separation is not applied then the consequence is that an unknown number of molecules, of unknown properties and concentrations are subjected simultaneously to the ionisation process and poor ionisation efficiency will be observed for numerous analytes. As a result direct infusion MS can suffer from extensive ion suppression and the inability to separate isobaric and isomeric substances [20].

CE-MS has been used mostly in so-called targeted metabolomics as a powerful tool for the development of multi-analyte methods, and is particularly well suited for polar/ionisable molecules such as aminoacids. The use of CE in metabolomic profiling is expanding [23–25]. CE however, currently does not reach the robustness, throughput and repeatability achieved by HPLC, hence its utility in holistic analysis is still rather limited. In the following sections we describe the strategies, the needs and the potential of various LC modes in global metabolite profiling.

3. LC-based global metabolic profiling

3.1. Study design

In untargeted approaches towards metabolic profiling, research starts from a hypothesis-free viewpoint aiming to obtain and analyse an appropriate sample-set using an unbiased, generally qualitative, holistic analytical methodology. Large data sets are generated and multivariate statistical analysis is subsequently employed to reduce data complexity and to help reveal underlying trends from which it is hoped that hypotheses can be generated. In this approach several data mining tools are employed and the findings drive the analysis; when a potential biomarker is found, efforts are taken to identify the molecule [20,21]. So-called targeted metabolomics takes a different route. Based on existing knowledge, a large number of metabolites are recognised as analytical targets and a method is developed in order to quantify them in the samples of interest. Following (semi-) quantitative determination statistical analysis is used to visualise trends in the data, to classify the samples and find which of the analysed metabolites contribute to group's separation.

The development of targeted metabolomics using LC–MS/MS can be paralleled with the development of multi-analyte methods. LC–MS/MS in multiple reaction monitoring mode (MRM) on triple quadrupoles provides high sensitivity and specificity and repeatable quantitative determinations. Potential analytical pitfalls can be monitored and corrected e.g. matrix effects can be evaluated, and isotopically labelled (e.g., ^{2}H, ^{13}C, ^{15}N) internal standards can be used to correct for analyte loss during sample preparation. In most multi analyte methods however, the analyst does not expect to find all the analytes in the sample; i.e. in environmental analysis one typically expects to find none of the analytes in the majority of the samples. In contrast in metabolomics one can expect to find all the analytes to be present, albeit in varying concentrations often spanning several orders of magnitude. As a result in untargeted metabolomics analysis validation is not as straightforward. The utilisation of internal single standard(s) cannot have the same utility so some researchers propose the use of a number of internal standards [26,27], while others advocate alternative strategies such as the use of quality control (QC) samples [28–39].