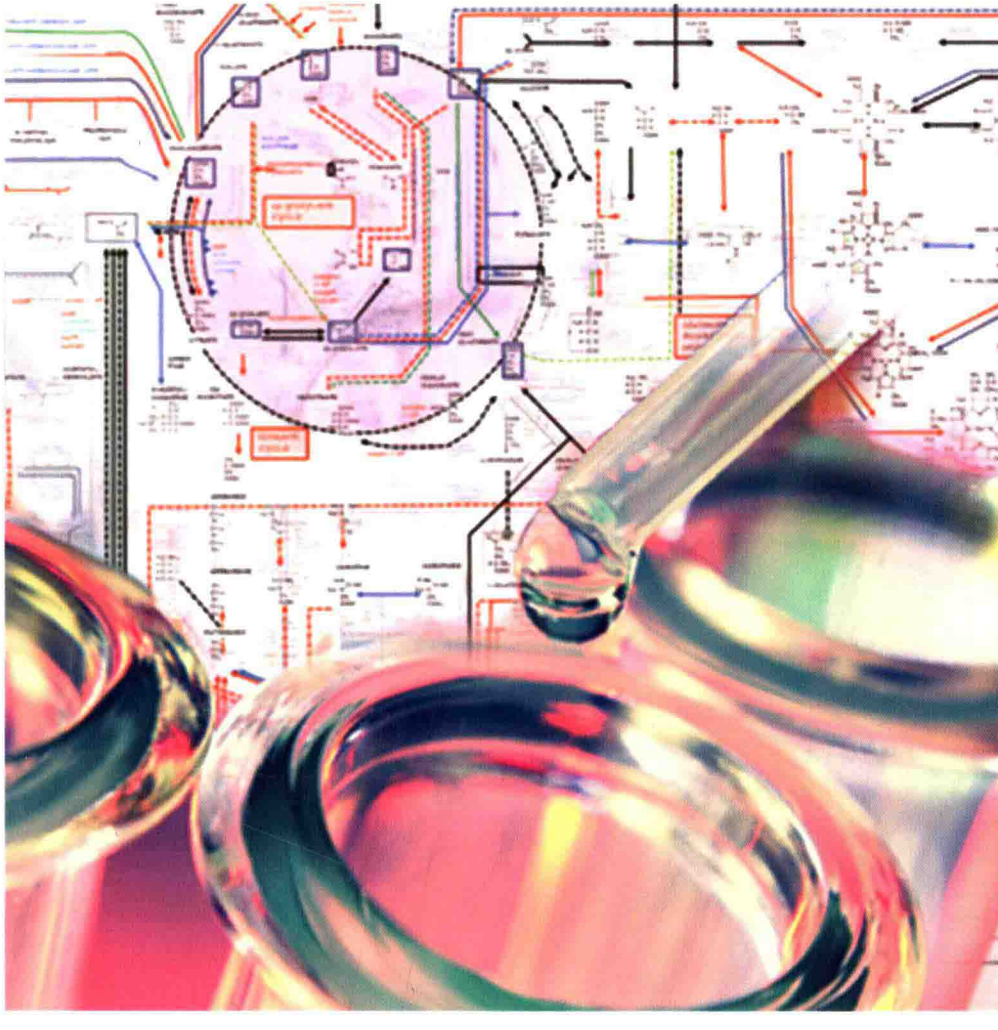
RSC Chromatography Monographs

Edited by Tuulia Hyötyläinen and Susanne Wiedmer

Chromatographic Methods in Metabolomics



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Chromatographic Methods in Metabolomics

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RSC Publishing

RSC Chromatography Monographs No. 18

ISBN: 978-1-84973-607-7
ISSN: 1757-7055

A catalogue record for this book is available from the British Library

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Registered Charity Number 207890

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Preface

Chromatographic techniques, particularly in combination with mass spectrometry, are valuable tools for metabolomics studies. Efficient techniques are required to cover the wide range of metabolites that are present in any biological sample. By global assessment of the metabolic profile, metabolomics can provide an instant snapshot of an organism's metabolic state because the metabolome contains all the biological endpoints of genomic, transcriptomic and proteomic perturbations, in addition to stimuli of environmental factors. The levels of metabolites may be strongly connected to specific conditions, such as apoptotic alterations, hypoxia, oxidative stress and inflammation, hence metabolomics can be used for finding new biomarkers for diagnosis of chronic diseases and for identifying perturbed pathways due to disease or treatment. Most frequently, metabolomics is employed in a comparative mode, where metabolic profiles of sample sets from a normal state and a perturbed state are compared to find changes related to the perturbation, such as genetic knockout, administration of a drug or change in diet or lifestyle.

One of the main factors behind the advancement of metabolomics has been the development of analytical technologies and in particular advances in mass spectrometry. However, the discovery of metabolic biomarkers remains a significant challenge because of the high number of metabolites, the wide concentration range (over eight orders of magnitude) and the chemical diversity of metabolites. It is not possible to cover the whole metabolome with a single analytical technique, but a set of multiple methods are needed. Moreover, the selection of the optimal method is not always easy. Maybe even more challenging is the mining of the relevant information among the huge amount of data produced by the novel analytical techniques. Here, efficient tools for data processing and modelling are needed.

Metabolomics is currently used within the clinical and pharmacological disciplines not only in biomarker discovery but also widely in drug discovery,

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drug toxicology and personalized pharmacology. Also, metabolomics is an important tool for metabolic engineering in biotechnology and food technology. In clinical disciplines, metabolic biomarkers can be used to diagnose, to select and optimize therapy (type and/or dose), to evaluate the effect of chosen therapy and to monitor disease progression. In the drug development process, on the other hand, biomarkers are being increasingly used in the early clinical development of drug candidates. Biomarkers can be helpful in the identification and validation of novel therapeutic targets, *i.e.*, in the evaluation of both the action mechanism and possible toxicity of the drug target, and also in predicting or monitoring the responsiveness of the patient to the treatment. Metabolomics also has the potential for a significant biotechnological impact in metabolic engineering: as the goal of metabolic engineering is to manipulate metabolite production, metabolomics offers tools for detailed understanding of the underlying mechanisms. Metabolic engineering can also be utilized in food technology.

Two types of approaches are typically used in metabolomics, namely targeted selective analysis and more comprehensive, non-targeted profiling methods. The third analytical approach, diagnostics applications, is then quite different. In the target analysis, only a limited number of preselected metabolites are analysed with a carefully planned analytical protocol. Although this approach allows very sensitive and robust determination of the selected metabolites, it gives relatively limited information on the global metabolome. The non-targeted analyses aim to cover as many metabolites as possible in a single analysis; however, the methods are typically only semiquantitative, hence it is not possible to optimize the method for all compounds. The diagnostic approaches are then focused on one or a few (validated) biomarkers, using a very rugged and robust technology, or even on-site measurement with a small, simple instrument. Different types of instrumentation are needed for the three types of metabolomics, *i.e.*, the targeted, non-targeted and diagnostic metabolomics.

One of the challenges in metabolomics is the lack of standardization of sampling, sample storage and sample pretreatment methods, and also quality control of the analytical methodologies. All of these steps can cause unnecessary methodological variation in the results, which then can hinder the identification of the potentially relevant, biologically significant markers. Often the differences in sampling and sample handling before any actual analysis can cause significant changes in the composition of the metabolites, especially in the levels of labile metabolites. In the whole analytical workflow, strict quality control and correction of batch-to-batch variations are also essential. Another key challenge is the identification of previously unknown metabolites. Typically it is possible to detect 600–1000 metabolites in a single profiling analysis, but usually only 10–30% of these can be identified based on spectral libraries. Future development requires the establishment of spectral databases of metabolites and associated biochemical identities, and also cross-validation of metabolites obtained by NMR or mass spectrometry.

Metabolomics has the potential to become an increasingly effective tool for clinical studies, in drug development, and in food and plant biotechnology. A broader coverage of the human metabolome can also be utilized in stratifying populations based on their metabolic phenotype or metabotype, and to use these data for developing personalized medicine, *i.e.*, to tailor a dose/type of drug or other therapy to an individual (group). This would aid the drug development by decreasing adverse drug reactions and improving disease outcomes. However, further development of more robust and sensitive analytical methodologies and advances in bioinformatics are still needed.

The aim of this book is to give the reader an overview of the chromatographic and electromigration techniques utilized in metabolomics and practical guidelines for the selection of the proper method. Microchip technologies in metabolomics are discussed with special focus on microfabrication methods utilized in microchip-based separations and microfluidic enzyme assays. In addition, critical parameters in sampling, sample preparation and data processing and analyses are discussed.

Tuulia Hyötyläinen
Susanne Wiedmer

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CHAPTER 1

Selection of Analytical Methodology for Metabolomics

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1.1 Introduction

Metabolomics analysis covers different strategies depending on the goal of the study (Figure 1.1). The most common strategies are based on the following types of approaches:

1. *target analysis,* which aims at the quantitative analysis of a limited number of metabolites, typically a small group of chemically similar metabolites;
2. non-targeted *global metabolic profiling*, which allows the detection of a broad range of metabolites, usually in a semiquantitative manner;
3. *metabolic fingerprinting*, which is a high-throughput, rapid methodology for the analysis of biological samples that provides fingerprints for sample classification and screening;
4. *metabolic footprinting*, which aims at the analysis of metabolites in extracellular fluids;
5. *diagnostic methods* in the clinical laboratory or with a point-of-care approach: very simple, robust methods for quick analysis of metabolic biomarkers.

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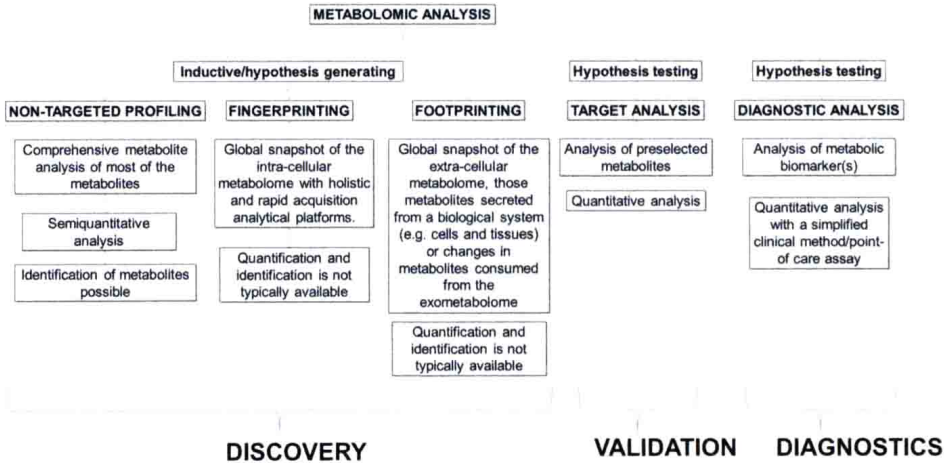

Figure 1.1 Classification of metabolic analyses.

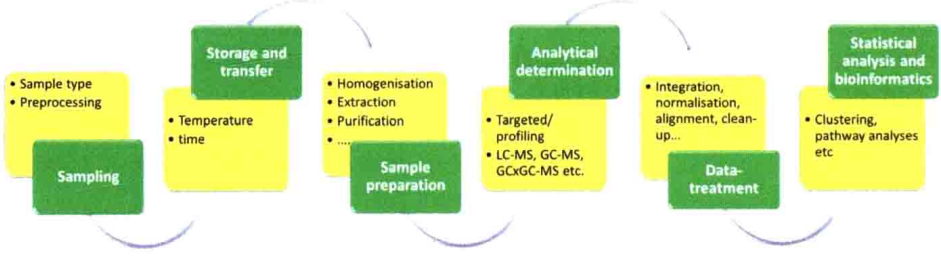

Figure 1.2 Workflow in metabolomics analysis.

The selection of a suitable analytical workflow, from sample pretreatment to analysis and data analysis, is not always straightforward. In the discovery stage, usually global profiling methods are required, and in validation, quantitative targeted methods are needed. In these two approaches, the analytical workflows are very similar. In the diagnostic stage, which may take place in a clinical laboratory or even in point-of-care facilities, the analytical workflow is very different. It also requires a different type of instrumentation, such as microfluidic techniques, which are described in Chapter 7.

The crucial steps of an analytical workflow in the discovery and validation stage are illustrated in Figure 1.2. A well-planned analytical protocol includes a proper design of experiment, optimization of both sample preparation and final analysis, data storage and manipulation, and data processing and validation. Moreover, careful quality control during the whole analytical procedure is essential, especially because many methodologies are global profiling methods for which the analytical steps cannot be optimized to the extent that optimal conditions are obtained for each group of compounds. Thus, even small variations during the analytical procedure can result in large errors in the final results. In addition, the lack of homogeneous standard protocols for the analytical workflow also introduces a barrier for comparing results among laboratories and for reproducing metabolomics experiments.[1]

An ideal analytical system would permit sufficient coverage of the metabolome, allowing simultaneous analysis of a wide concentration range in a high-throughput and rugged fashion. Moreover, it should allow the identification of unknown metabolites in a reliable manner. In practice, no single methodology is sufficient for the analysis of the global metabolic profile and each methodology has its own limitations. At present, the methodologies used in metabolomics for final separation and identification are based mainly on chromatographic methods, such as liquid chromatography (LC) and gas chromatography (GC) combined with mass spectrometric (MS) detection and shot-gun methodologies based on MS alone.[2–7] Capillary electromigration (CE) techniques have also been utilized to some extent.[8–12] In addition, nuclear magnetic resonance (NMR) spectroscopy is used fairly frequently; however, the sensitivity of NMR spectroscopy is often not sufficient ($\sim 10\,\mu mol\,L^{-1}$) for metabolomic studies and will therefore not be covered here.

The selection of a suitable technique depends on several parameters, such as the target of the analysis (target *versus* non-target profiling), the type of analytes and matrix, the amount of sample available, and the concentration of the analytes. Different approaches are needed for target-compound analysis and for global profiling. For targeted analyses, a highly selective sample pretreatment is beneficial, whereas a non-selective approach that is not biased towards any certain groups of metabolite is needed for global screening. In target analysis, on the other hand, selective sample preparation steps can be applied and optimized to improve the data quality, leading to highly sensitive and precise quantification of metabolite concentrations. In the metabolite profiling, it is difficult to find the optimal conditions for all types of analytes, so some compromises must to be made in the selection of experimental conditions.

A general guideline for selection of the method is shown in Figure 1.3. A more detailed selection of sample preparation methods is given in Chapter 2. In the selection of the method of analysis, the physicochemical characteristics of the analytes play a major role. For example, (most) lipids are non-polar and non-volatile and therefore both GC- and CE-based methods are poorly suited for their analysis. The natural choice is LC-MS and, of the LC subtypes, reversed-phase (RP) LC. The choice of the method is more difficult for small polar and semipolar compounds, which in principle can be analysed using GC, LC and CE. Each of the three separation methods have their own advantages and limitations, which should be carefully considered in the method selection.

In metabolomics, LC-MS-based methodologies have been used for the analysis of a wide variety of compound classes, including both non-polar and polar compounds such as lipids, amino acids, bile acids and eicosanoids.[6,13] LC-MS has been used both for target analysis and for non-target profiling, with high-resolution MS typically used for non-target profiling and triple-quadrupole MS systems for targeted analysis. The sensitivity in LC-MS is typically high and the identification of novel compounds is possible, although not always easy. Novel fast LC methodologies, utilizing very high pressures, elevated temperatures and novel column materials, allow high-throughput analyses. In ultra-high-performance liquid chromatography (UPLC), columns

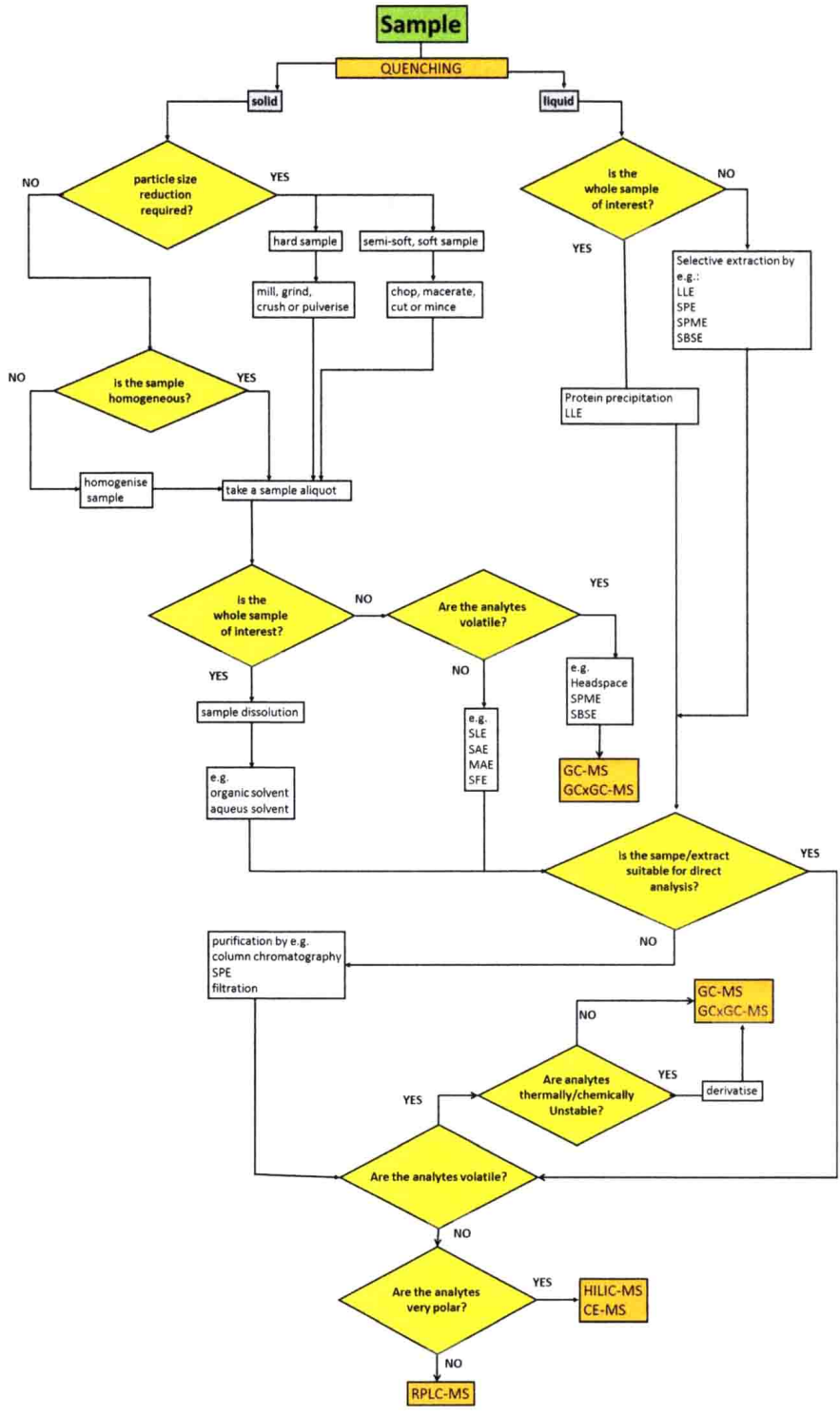

Figure 1.3 General guidelines for the selection of an analytical methodology for metabolomics.

packed with sub-2 μm particle size stationary phase are utilized and the separation efficiency is very high and the analysis time relatively short (5–15 min).

CE is an efficient analytical liquid-phase separation technique by which high-resolution separation of a wide range of analytes is possible, using only minute amounts of samples and running electrolyte solutions. The analytes that can be separated by CE range from single ions to large biomolecules. Owing to the availability of several different CE techniques, charged and uncharged and also polar and non-polar analytes can be separated. A major advantage of the technique is that only minor sample pretreatment is required, in order not to have large particles in the sample clogging the narrow-bore capillaries. The capillaries are always rinsed after each run and provided that no irreversible reactions take place, the retention of analytes is maintained. However, the real Achilles' heel of the method is the poor robustness, which in practice means poor repeatability and reproducibility of migration times.

The main drawback of CE techniques is the poor concentration sensitivity of the method, especially when conventional absorption-based detectors, such as UV-Vis, are employed. Much research has been carried out on enhancing online the concentrations of the injected analytes and various stacking methodologies have been reported. On the other hand, lower limits of detection can be obtained by using online CE-MS and in metabolomics this is more or less the only alternative. The most common ionization method by far is electrospray ionization (ESI). However, CE-ESI-MS puts some demands on the choice of the background electrolyte solution in CE, which in that case must be fairly volatile.

Both targeted and non-targeted CE approaches have been developed over the years, including miniaturized methodologies, which are covered in Chapter 6. The main technological issues when developing CE-MS methodologies have been the development of CE-MS interfaces, which would result in the highest possible coverage of metabolites. Both sheath-liquid and sheathless approaches have been reported. The most commonly used MS analyzer by far in CE-MS online studies is the time-of-flight (TOF) type. Regarding the applicability of CE-MS to metabolomics studies, the most common studies have dealt with non-targeted metabolomics approaches.

GC-based methods, on the other hand, are suitable for the analysis of sufficiently small and volatile compounds (MW $<$ 700), such as fatty acids, small polar compounds, such as carboxylic acids, sugars, amino acids and sterols. The polar metabolites require derivatization before analysis; however, the derivatization can be done in an automatic fashion in modern instruments. The main problem with derivatization is that some compounds produce multiple peaks due to incomplete derivatization. However, the GC-based methods have several advantages over the LC-MS-based approaches which also have been used for the analysis of polar metabolites. One of the main advantages is that the separation efficiency is much higher in GC than in LC methods. An additional advantage of all GC-based methods is the repeatable characteristics of retention, particularly when non-polar stationary phases are used. This makes it possible to use universal retention indices, which are very useful in the identification of unknown compounds, in combination with MS detection.

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