Innovative sample preparation and handling strategies for automated and high-throughput metabolomics

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Chapter 1

Introduction

The rise of metabolomics

Metabolism is commonly known as the process of food intake, energy uptake, and excretion. However, in reality metabolism is much broader and encompasses a plethora of biochemical reactions, as diverse as muscle soreness and recovery, inflammation, infections and disease processes. Small molecules with a mass lower than 1500 Da involved in these chemical processes as intermediate and end products, are called metabolites. Collectively, the full set of metabolites present in an organism is named the metabolome, and contains a wealth of information that directly reflects an organism's physiological state. For this reason, understanding of the metabolome and metabolic processes is essential in, for example, developing preventive and therapeutic treatments. Both qualitative and quantitative information on the metabolome can be gathered using metabolomics, i.e., the comprehensive study of metabolites. Metabolic processes are regulated by internal factors, such as the genome – the unique genetic code of each organism – and the proteome – the set of proteins that are expressed and translated from the genome, but also largely influenced by external factors, such as lifestyle, diet, exercise, but also pollutants, infectious agents, etc.[1] The metabolome, where internal and external factors come together, is thus highly dynamic and highly individual, thus providing a direct chemical readout of the physiological state of an organism. Combining this data with other -omics approaches, in so-called called "multi-omics" or "cross-omics" strategies, gives an insight in the complex interplay between the biological layers and helps to understand health and disease on a detailed molecular level[2]. In this context, metabolomics is expected to play a pivotal role in the implementation of personalized medicine[1,3,4].

Even though the term "metabolome" has been coined in 1998 for the first time[5,6] and popularized by, notably, Fiehn and co-workers in early 2000's[7], the first rudimentary forms of metabolomics date back to ancient times, illustrated by the well-known example of diabetes mellitus diagnosis from urine by taste or using ants[8,9]. The presence of high level of glucose in urine in case of diabetes mellitus causes the sweetness in taste, which was used as a biomarker for diagnostic purpose. This diagnostic approach was useful as a start, but it was not until the early 20th century that more systematic research into the mechanisms of glucose conversion, which can arguably be seen as an early metabolomics approach, finally led to the discovery of insulin as treatment for diabetes mellitus[9,10]. This early example showcases the power of metabolites as markers of disease, which can be used to support clinical decisions, but also, in principle, guide the development of novel treatments. Since the early 2000's, metabolomics has emerged as an important field of research and been implemented in a variety of disciplines, from healthcare research (e.g., unravelling disease mechanisms, drug development and discovery)[11] to food industry and biotech (e.g., food metabolomics and food additives to reduce methane emission of cattle)[12,13].

Currently, the Human Metabolome Database (HMDB) reports more than 200,000 known and identified metabolites[14] (including plant, exogenous, xenobiotic, and predicted metabolites), which illustrates the vastness and incredible complexity of metabolomics. Many of these are predicted intermediate steps in biochemical pathways, discovered via extensive modelling, and not seldomly impossible to measure due to extremely fast reaction rates. Nevertheless, the stunning rise in the number of metabolite entries with each update (Fig. 1) proves the growing efforts and interest in the field of metabolomics, and better understanding of the metabolome. This also highlights the remarkable technological improvements and implementation of dedicated data analysis pipelines in the last decade which fostered the analysis, characterization and annotation of a growing number of metabolites previously not reported.

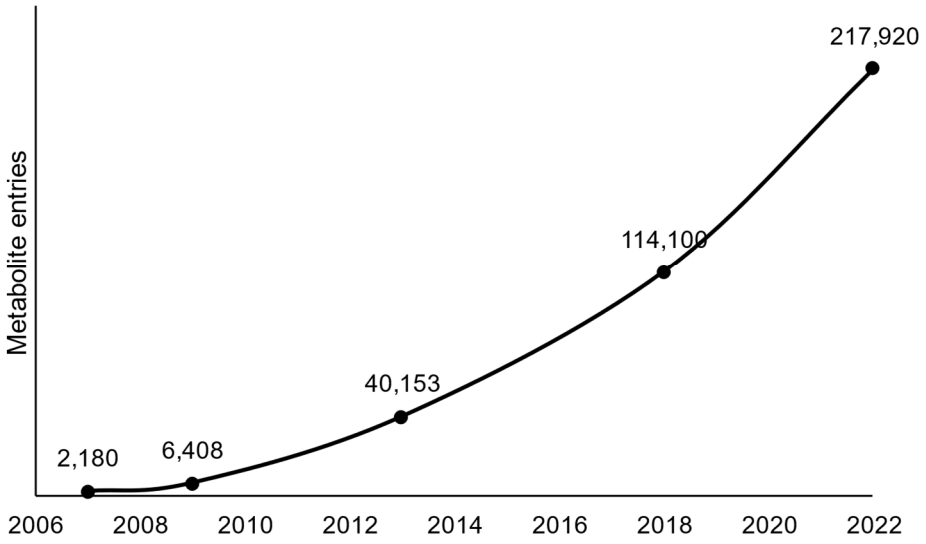

Figure 1. The exponential growth of metabolite entries in the Human Metabolome Database (HMDB) is indicative for the growing interest in the field of metabolomics. Data from Wishart et al.[14,15].

Many metabolites are intermediate steps in the so-called metabolic pathways, a series of linked chemical reactions that result in the production and biotransformation of metabolites. The collection of all these metabolic pathways is called the metabolic network, which is a vast and complex metro-like map combining the reaction paths of various metabolite classes, such as lipids, amino acids, carbohydrates, etc.[16]

Metabolomics for biomarker discovery

Metabolomics is a powerful tool in clinical research and personalized healthcare. Indeed, the metabolic profile of an organism can provide a functional readout of the current health state (phenotype)[17]. Disturbances in the metabolic profile can indicate dysregulation of a pathway, which

can be correlated to disease mechanisms[1,13,18]. However, a metabolic readout ("snapshot") by itself does not always suffice, as the onset and progression of complex diseases are most often multifactorial. Such metabolic snapshots can be used to detect disturbances in the health status, but cannot necessarily identify its cause. Only a combination of metabolomics with other -omics approaches, longitudinal studies and/or mechanistic intervention studies may lead to a more complete picture. For example, Alzheimer's Disease has a genetic predisposition with the ApoE4-allele, among other genes, but also strong environmental risk factors related to lifestyle, diet, exercise and educational level[19,20]. Genomics or metabolomics alone are thus not sufficient to unravel the biomolecular mechanisms associated with Alzheimer's Disease pathology. The integration of -omics approaches is typically referred to as "Systems biology", and is believed to be an essential step towards the clinical implementation of personalized medicine[1,4,21].

All -omics approaches have both shared and specific challenges. In metabolomics, one of the biggest challenges is related to the inter- and intra-individual variability of the metabolome. Indeed, due to the influence of external factors on metabolism, the composition of the metabolome is highly dynamic and highly different between individuals. To tackle this challenge, large-scale cohorts are typically required to ensure a sufficient study power and, in turn, non-biased results and data interpretation[3]. This results in an increased numbers of samples that need to be collected, prepared and measured. Nowadays, it is common to have study designs based on very large clinical cohorts, where thousands of samples are collected to increase the chances of discovering or validating novel biomarker candidates. This is especially true in studies focusing on the discovery of early disease biomarkers using population-based metabolic profiling[1].

Increasing the chance of discovering new biomarker candidates does not only rely on the number of samples, but also on the metabolite coverage. Indeed, the higher the number of metabolites identified and quantified within a metabolic pathway, the more complete the information obtained on biomolecular mechanisms underlying a physiological state. Moreover, many of these metabolites are present at very low concentrations in an organism[22]. This highlights the need for the development of high-end approaches allowing for the analysis of biomass-limited samples[23], samples with low absolute mass of metabolites of interest, which can originate from humans (e.g., CSF, micro dialysates, saliva, sweat), animal models, cell cultures, and - more recently - organ-on-a-chip models[24]. Specifically, biomass-limited samples encompass samples that have workable volumes but extremely low concentrations of target analytes (e.g., endocannabinoids in cerebrospinal fluid[23,25]) or highly-concentrated analytes but low in volume (e.g., mice urine, animal CSF, or microdialysates[26,27]). Therefore, handling and analysing such type of samples is associated with high technological challenges[28]. Despite these challenges, pushing the boundaries of the

number of metabolites that can be detected and accurately quantified in biosamples is essential to enable the access to biological information currently unravelled.

Metabolomics in industrial screenings

Besides clinical research and healthcare, metabolomics has found its way into industrial applications, for example for identification and prediction of taste metabolites[29] and quality control for food safety[30,31]. However, while the source and composition of these samples may be different from biological samples, such applications largely face the same challenges with respect to dilution, limited volume, and/or presence of interferences in the matrix. Regarding throughput, industrial screenings (e.g., strain selection, and combinatorial chemistry) require rapid analysis and put even higher demands on throughput, with thousands or millions of samples that are measured per year[32,33]. This requires considerable efforts in the development of high-throughput workflows, with special attention for sample preparation, transfer, analysis methods and automation.

The metabolomics workflow

Data acquisition

Nowadays, metabolomics analysis is almost exclusively performed using state-of-the-art mass spectrometry (MS) approaches or nuclear magnetic resonance (NMR)[33,34]. Both techniques have their advantages and disadvantages: MS provides high sensitivity and selectivity, but generally requires more sample preparation, and absolute quantitation is not straightforward. It is destructive to the sample, but uses only very little of it. NMR is inherently quantitative, requires little sample preparation and has a high dynamic range, but with low sensitivity and selectivity. It also requires larger sample volumes, but is non-destructive to the sample. Overall, these two techniques provide complementary data for structural identification and quantification in chemical analysis[33,35,36]. In metabolomics, MS is most frequently combined with a separation technique, such as liquid chromatography, gas chromatography (GC), supercritical fluid chromatography (SFC) or capillary electrophoresis (CE), to increase the selectivity between analytes of interest, reduce matrix effects and increase the sensitivity. While the number of gas chromatography – mass spectrometry (GC-MS) and capillary electrophoresis – mass spectrometry (CE-MS) applications are steadily growing, in the past two decades liquid chromatography – mass spectrometry (LC-MS) has become the gold standard in metabolomics. Out of all the metabolite entries in the HMDB that are supported by measured spectra (about 90% of the entries are predicted metabolites based on computer models

and by the in- and output of pathways), the majority is covered by LC-MS. Nevertheless, all aforementioned techniques, including LC-MS, suffer from selectivity, and the coverage of a single LC-MS platform only spans a small subset of the measurable metabolites[37–39]. As a result, there remains a trade-off between throughput and depth of information, i.e., depth of information may require lengthy analysis or analysis on various platforms at the expense of throughput, and vice versa. Generalized, targeted analyses that focus on a selected subset of metabolites can achieve faster measurements than untargeted strategies that aim to detect and identify as many metabolites as possible[37], resulting in longer measurement times due to lengthy gradient separations or higher number of NMR scans. It should be mentioned that for MS, combined approaches of targeted and untargeted analysis using high-resolution MS is gaining popularity. This combination allows to detect metabolites that are not on the target list whilst still being able to reliably detect the targeted metabolites[40,41].

The rise of metabolomics in various fields brings challenges to both ensure sufficient analytical selectivity and sensitivity, while simultaneously dealing with the increasing numbers of samples that need to be analysed. In this context, every step in the workflow matters. Developments in separation methods and analytical instrumentation over the last decades bring unprecedented coverage and sensitivity, but the processing steps between the sample collection and actual analysis often result in less-than-ideal circumstances, thereby limiting their capacity. This step is certainly where – in this metabolomics workflow – significant efforts should be invested. Indeed, closing this gap is urgently needed to further improve the information per unit of time or costs obtained, which is ultimately expected to bring a major leap forward in the large-scale implementation of metabolomics.

Sample preparation

Sample preparation represents a key step in the metabolomics workflow to remove matrix interferents that cause background noise or analyte suppression (i.e., ion suppression or enhancement in MS[42,43], peak overlap in NMR44) and pre-concentrate the samples when needed. Typically, the approaches can be differentiated in three categories with increasing complexity, namely: i) dilution and filtration/centrifugation, ii) protein removal, and iii) targeted selective extractions. The first two are generic approaches that excel in simplicity, recovery of analytes, and throughput – especially true for "dilute-and-shoot" approaches[45]. Dilution is simply aimed at reducing the influence of highly-abundant interferents. Protein precipitation removes proteins as major interferents from the matrix and releases analytes that may be bound to proteins. Selective extractions, however, focus on extracting target metabolites from the matrix based on their

physicochemical properties, such as polarity and charge. Selective extractions are more elaborate and are often used in the analysis of complex samples to separate and extract analytes from salts, proteins and other interferents. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the workhorse-techniques in this category. They are often followed up by evaporation and reconstitution, which provides a means of improving the compatibility with the subsequent analytical method. Indeed, the analyte concentration can be increased by reconstituting in a (much) lower volume than the original sample; also, the most-favourable diluent can be used for reconstitution. However, this workflow has its limitations. The evaporation-and-reconstitution step is time-consuming and detrimental for the analysis of (semi-)volatile and/or thermolabile compounds that will evaporate and/or degrade. Moreover, it runs into limitations at small volumes: the reconstitution volume is typically at least several microliters to still be able to redissolve and handle the sample, whereas nano-LC methods only require several to a few hundreds of nanoliters for analysis – a gap spanning several orders of magnitude. It also means there is little preconcentration capacity for low-volume samples that are only several microliters to start with. However, the diluent after precipitation or selective extraction is often high in organic and a poor fit for reversed-phase liquid chromatography (RPLC)-based methods (the most-commonly used chromatographic mode), where ideally the elution strength of the sample diluent is similar to, or slightly weaker than, the mobile phase[46,47]. This evaporative and reconstitution step is therefore needed to meet these requirements. Finally, the classical sample preparation workflows offer limited potential for simultaneous miniaturization and automation for increasing the throughput towards large-scale metabolomics, as will be elaborated in the subsequent sections.

Mind the gap: from sample to analysis

The motivation for miniaturizing the metabolomics workflow is multifold and synergistic. On one hand, miniaturization is needed to be able to work with low-volume and/or biomass-limited samples without further diluting the samples. This is perfectly exemplified by the development of microcoil-NMR for the purpose of analysing small volume samples up to several microliters that would otherwise be impossible with conventional NMR[48,49]. On the other hand, technological advancements in miniaturization of sample preparation, separation and detector technology offer, amongst others, increased sensitivity. Micro- and nano-LC separations suffer from less dilution during separation, and the low flow rates can be beneficial for electrospray ionization, leading to better detection sensitivity[47,50,51]. Also, relatively small injections volumes may lower the effects of the sample diluent on the separation efficiency[46]. Lastly, miniaturization would enable direct

coupling of small-volume sampling like lab-on-a-chip applications directly to the analysis methods[52–54].

Working with small volumes brings two challenges. Firstly, it requires sample preparation methods suited for (sub)microliter sample volumes. Secondly, a flexible means of transferring the sample to the analysis equipment is required; in other words, an interface. Ideally, this interface is agnostic to the further processing of the sample, which is most easily achieved using syringe-based methods. For the conventional selective extractions, i.e., LLE and SPE, a plethora of miniaturized counterparts – called microextractions – has been emerging that are more apt for small sample volumes. Liquid-phase microextractions (LPME) come in the form of amongst others single droplets (single-droplet microextraction, SDME), dispersive methods (dispersive liquid-liquid microextractions, DLLME) and parallel artificial liquid membrane extractions (PALME)[55,56]. Especially single-droplet methods have great potential as a one-step extraction and preconcentration[55,57,58]. Samples can be extracted into (sub-)microliter droplets suspended directly from a syringe. Since the concentration factor is determined by the ratio of the initial and final volume, preconcentration can be achieved even from only a few microliters of initial sample. Other methods, such as DLLME and hollow-fiber liquid-phase microextraction (HF-LPME) pose great difficulty in retrieving the acceptor phase for downstream analysis[59]. Solid-phase microextractions (SPME) extract analytes by retaining them on sorbent material with a small-as-possible bed volume. A wide variety of techniques have been reported already[60]. For instance, a small fiber inside a syringe whereby the eluent can be directly injected in an open MS-interface has been used by Gomez-Rios et al. for the extraction of drugs in urine[61]. Other syringe-based approaches, such as microextraction by packed sorbent (MEPS), provide similarly easy interfacing. While these methods can be powerful for the high-volume, low-concentration type of samples, they are less suited for low-volume samples. The starting volume needed is typically in the order of tens of microliters or higher; likewise, a similar volume is required to elute the analytes from the sorbent material.

Electrokinetic extractions represent another attractive approach for the simultaneous extraction and preconcentration of metabolites. Generally, these methods offer high enrichment factors, are easy to automate and provide a flexible interface[62,63]. For example, single-droplet, three-phase electroextraction results in high enrichment factors by extracting into a droplet of 0.5-1.0 μL, which is ideally suited for coupling to microscale separation approaches[64,65]. Moreover, this process can be performed in an automated fashion that directly integrates with existing workflows[66,67]. However, one major drawback of electrokinetic extractions is that analytes have to be charged to

be extracted. Moreover, the choice of acceptor phase is limited, which may impede the possibilities or limit the potential of further hyphenation.

After extraction and prior to the actual analysis, samples can be preconcentrated to counter any dilution. Online evaporation methods, either with reconstitution[68] or directly coupled to analysis [69,70] can provide better preconcentration towards smaller sample volumes, but are still too slow or elaborate to be realistically applied to large-scale routine analysis.

These examples illustrate the infinite innovation and creativity observed and required with respect to the sample preparation steps. However, often only one aspect is optimized in terms of miniaturization, hyphenation or throughput, and direct integration with standard lab automation equipment is often not considered. Innovative sample preparations methods that tackle all aspects are thus urgently needed to solve the challenges for large-scale metabolomics integration.

Automation: towards Lab 4.0

Workflow automation is inevitable in solving the challenge of combined increased sensitivity and throughput[71,72]. Industrial automation has entered the era of the so-called Industry 4.0[73], following up Industry 3.0 where automation was first introduced, with large-scale automation and interconnectivity of processes to collect and use vast quantities of data. This transformation is also already visible in, amongst others, medical diagnostics laboratories[71,73] and high-throughput screening in combinatorial chemistry for drug delivery[74]. Strikingly, metabolomics workflows are still lagging behind on other -omics fields – not due to lack of ambition, but due to the limited possibilities of suitable and reliable techniques to deal with complex samples. Dedicated sample preparation methods that cater to both the requirements of miniaturization and automation are still lacking. Automated sample preparation methods to increase sample throughput are emerging and evolving[3], such as the RapidFire™ system (Agilent), which offers rapid SPE coupled to direct injection MS, resulting in a throughput of up to 30 s per sample[75]. However, conventional SPE cartridges are not suited for low-volume samples. Alternatively, online fractionation using a series of trapping columns is effective and fast, but has significant void volume and requires relatively high volumes of elution solvents, namely, in the millilitres range[76].

It is often overlooked that existing operating procedures cannot be readily automated. While automation offers new possibilities for sample handling and hyphenation that were previously unimaginable and can provide a "two birds one stone"-solution for both sensitivity and throughput. This discrepancy is adequately illustrated with traditional LLE. A trained analyst relies on the visible interface between the layers and steady hand-eye coordination to extract the organic

layer. However, transferring this exact procedure to an automated platform would require a complex mechanism of a camera and feedback to the mechanism that controls the syringe or pipette position. Instead, an adaptation – or rather a translation of this workflow – is needed. Automated methods can simply rely on the consistency of the pipetted volumes and the needle position, and repeatably aspirate a set volume of acceptor phase at a set depth in the container. More advanced methods can use conductivity measurements to detect the liquid interface and aspirate the acceptor phase much closer to the interface to maximize the recovery[77]. In translating operating procedures, potential improvements enabled by automation should be reviewed in terms of reduced sample and solvent consumption, faster extraction, increased repeatability, and direct integration with analysis equipment. These motives should ultimately lead to a paradigm shift in technological development, workflow architecture, and study design towards the successful implementation of a Lab 4.0-approach in metabolomics.

Scope of this thesis

This thesis focuses on two important aspects of sample handling of complex and biomass-limited samples towards fully automated workflows, namely, sample transfer and sample preparation. The aim of this thesis is to develop novel modules for sample transfer, sample preconcentration, and sample enrichment that can be directly integrated into different metabolomics-based workflows. The underlying idea is that combining zero-dispersion sample transfer with sample preconcentration or sample enrichment is crucial for the analysis of volume-limited samples at a high throughput. Additionally, all developments should fit within the ecosystem of commercially available robotic autosamplers for maximum compatibility with industry-standard automation, as a step towards large-scale implementation of metabolomics.

Firstly, **Chapter 2** provides an overview of the developments towards high-throughput metabolomics, from sample preparation to data processing and data sharing in public databases. This review underlines the trade-off between speed of analysis and depth of information, emphasizes the need for automation towards an integral approach for complex samples, and, finally, discusses the challenges and recent developments for sample handling and preparation.

The aim of the next two chapters is the development of a novel segmented-flow analysis – nuclear magnetic resonance (SFA-NMR) system based on a novel fluoropolymer cell, fluorocarbon oils, and fully fluorinated flow path developed for industrial biotech screening. **Chapter 3** describes the development of this system, including optimization of the fabrication process for the

fluoropolymer flow cells, and its application to biofluids. Important characteristics, such as spectral quality of the fluoropolymer flow cell, as well as carryover and repeatability were evaluated to demonstrate the potential of this technology. In **Chapter 4,** the fluid mechanics of the segmented flow in the flow cells was investigated. Fundamental understanding of the balance between competing forces allows to predict fluid behaviour and, consequently, adapt the design to enable the use of a wide range of organic solvents in flow-NMR.

The aim of the following two chapters is the development of modules for enrichment of low-abundant analytes – either from inherently low-concentrated samples or diluted from previous sample handling and preparation steps. In **Chapter 5,** the aim is to achieve enrichment of analytes by controlled evaporative solvent removal. For this purpose, a preconcentration module was developed using machine vision controlled solvent evaporation from a hanging droplet. The preconcentrating effect of evaporating solvent is demonstrated. Moreover, practical limits of the developed setup are explored with a theoretical model of droplet evaporation. A proof of concept is shown with direct transfer of the preconcentrated droplet into conventional LC-MS equipment. In addition, the potential of transferring the drop concentrated drop to GC injection is demonstrated. **Chapter 6** focuses on the enrichment of charged analytes using an automated electro-driven approach based on membrane-free, three-phase electroextraction into a sub-microliter droplet. The stability of the extraction process was evaluated with machine vision and process monitoring, and compared to theoretical models. The applicability and potential of this approach was demonstrated by coupling the sample preparation step to LC-MS for the detection of nonpolar analytes in human plasma and urine.

Finally, in **Chapter 7**, general conclusions are presented and future perspectives are discussed. Moreover, reflections on the potential of the developed modules for automated metabolomics-based workflows towards a Lab 4.0-approach are provided.

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