Miniaturized metabolomics methods for enabling the study of biomass-restricted samples

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Chapter I

General introduction and scope

Clinical and pharmaceutical studies face a persistent obstacle with biomass-restricted samples, which impede the comprehensive deciphering of biological progresses and disease mechanisms. Traditional analytical methods often fall short when confronted with these challenges, necessitating the development of alternative approaches. Micro-flow LC-MS methods offer a compelling solution by enabling the analysis of minute sample volumes with high sensitivity and good precision. Biomass-restricted samples in this thesis refers to biospecimens with low concentrations or limited amount for analytical measurements.

In clinical studies, we seek a non-invasive sampling method that causes minimal pain or trauma to the patients. The development of micro-sampling methods including fine needle aspiration biopsy (tissue), fine microextraction techniques, dried blood spots(blood), microdialysis (cerebrospinal fluids), and so on, have achieved the goal for minimal sample amount (e. g. blood sampling volume <50 μL) [1]. However, the biomass requirements for biochemical and analytical analysis methods are normally larger than this to reach reliable quantification limits. Not to mention the common circumstance when multiple aliquots need to be made for comprehensive investigation with various analytical methods [2, 3]. Similarly, analyses with experimental animals are challenging as well, since the model animals have less blood/CSF/tissue, while pharmaceutical studies require sampling over multiple time points.

Development of miniaturized analytical methods for Metabolomics studies

Metabolomics studies typically employ analytical techniques to investigate changes in metabolites resulting from factors such as disease, diet, and exposure to specific environments. Liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) are commonly utilized due to their ability to analyze a wide range of compounds based on their physical chemical properties [4]. Although the analytical workflows using these techniques have been well-developed and contribute a lot to metabolomics, clinical and pharmaceutical studies [5-7], for some research when biomass-restricted samples are involved, the sensitivity of conventional methods can be an issue. To address this, miniaturized metabolomics has emerged as a promising approach. The common matrices for metabolomics studies include blood, urine,

feces, cerebrospinal fluids, and tissues. Miniaturized metabolomics methods are required for matrices such as body fluids from infants, small animal models, or other biospecimens obtained with micro-sampling devices. Advances in miniaturized workflows are crucial for enhancing our understanding of various diseases and pathological processes in biomass-restricted samples.

By operating at micro- to nano-liter flow rates, miniaturized LC-MS and CE-MS methods minimize sample dilution within the ionization source, thereby enhancing ionization efficiency. These techniques require only minute sample volumes to achieve equivalent or better limits of detection while maintaining broad coverage of metabolites [8]. Additionally, the reduced sample volume reduces source contamination and matrix effects [9]. The adoption of miniaturized techniques offers financial benefits and improves the welfare of experimental subjects. Reduced consumption of organic solvents for mobile phase and sample extraction significantly lowers costs. Furthermore, it mitigates the risk of researchers' exposure to toxic organic solvents like chloroform and acetonitrile (classified as class 2 solvents for toxicity in FDA guidance). In an era increasingly concerned with the welfare of patients, clinical study volunteers, and laboratory animals, minimizing sample volume translates to less harm inflicted on study subjects.

Miniaturized analytical techniques show promise for revolutionizing future clinical diagnostics and personalized medicine by offering minimally invasive procedures and the ability to detect important disease biomarkers even at lower concentrations. However, concerns about their robustness occasionally arise due to issues such as column clogging, tubing leaks, and spray emitter malfunctions. These challenges can be mitigated by implementing cleaner sample preparation methods and ensuring correct tubing connections within the system. The development of a miniaturized analytical method requires understanding the contributions from sampling, sample preparation, and LC/CE-MS instrumentation to sensitivity and robustness. By combining microsampling, miniaturized sample preparation method, and micro flow-LC-MS, CE-MS methods, the miniaturized workflow will be an optimal choice for studies with biomass-restricted samples, ultimately advancing our understanding of disease mechanisms and aiding in the discovery of novel biomarkers.

Biomass-restricted studies

-Pediatric studies

Pediatric studies involve the examination of medical conditions, treatments, and interventions specifically tailored to children, from birth through adolescence. These studies encompass a wide range of disciplines, including pediatric oncology, neonatology, developmental biology, pharmacology, psychology, and public health [10-12]. The difference of how children react to disease or treatment can be differ among various age stages [13]. In order to address the unique physiological, developmental, and psychological needs of children, as well as help with the diagnosis and treatment in this vulnerable population, analysis using pediatric samples is necessary.

Blood is one of the most frequently analyzed matrices in pediatric studies. It provides valuable information about the child's overall health, including levels of various biomarkers, hormones, nutrients, and medications. The volume of blood that can be safely obtained from children, especially infants and newborns, is limited since they possess lower total blood volume (TBV) [14]. In a newborn, the total volume of blood is estimated at 80–90 mL/ kg body weight. According to the European regulatory guidelines, trial-related blood loss should not exceed 3% of the total blood volume during a period of 4 weeks and should not exceed 1% at any single time [15]. A study including 141 children from six months to 12 years old also suggested repeated sampling totaling up to 3% of blood volume is safe [16]. According to the WHO, the average birth weight of a full-term male baby is 3.3 kilograms (kg). The average birth weight of a full-term female is 3.2 kg. This means there is a total volume of around 276.3 mL blood per full-term baby, the volume that could be sampled a single time is not more than 2.8 mL (1% TBV), and not more than 8.3 mL for repeated sampling (3% TBV). The volume will be only less if preterm and children with health issues are taken into account. However, in extreme circumstance, medical intervention is required in preterm infants, and they could lose almost one-third of their total blood volume in the first month of life due to multiple blood draws for laboratory investigations, and procedures [17, 18], which leads to extra trauma to the patients. Blood samples can be obtained via heel puncture or fingerstick, which is painful for the vulnerable infants and newborns.

Microsampling techniques, which allow minimally invasive sampling using microneedles, can ease the pain from blood sampling as well as reduce excessive blood loss. However, the smaller sample volume obtained may pose challenges for conventional analytical measurements.

Given the volume limitations inherent in pediatric samples, to ease the pain from the sampling procedure as well as lower risks from too much blood loss, analytical methods with higher sensitivity for trace volume samples is urgently required.

-Animal studies

Experimental animals can serve as models for studying complex biological processes and disease mechanisms that often cannot be adequately replicated in vitro or in silico. By studying diseases and treatments in animals, researchers gain valuable insights into the underlying mechanisms of disease pathology, drug metabolism, and pharmacokinetics [19-22].

During pharmaceutical development, before the candidate drug can be tested in clinical studies on humans for treatment purpose, it must undergo rigorous preclinical testing in animal models to assess its safety and efficacy, including potential side effects, toxicities, allowing researchers to refine dosing regimens and prioritize drug candidates for further development. For PK/TK purposes, multiple sampling over 24 hours is required. Apart from pharmaceutical studies, to gain a comprehensive understanding of certain health-related conditions, samples from animal models are commonly analyzed by multiple platforms including metabolomics, proteomics and transcriptomics. However, the sample amount acquired from some small animal models is inadequate for all of those analysis.

Take mouse as an example, a mouse with body weight of 25 g has a total blood volume around 1.8 mL, a single sampling above 15% TBV (>270 µL) and multiple sampling more than 20% TBV (>360 µL) are not recommended since hypovolemic shock may occur [23] and affect the following sampling timepoints. For metabolomics studies, the interference of such acute stress caused by sampling could also cause changes in the metabolite profile and deliver unreliable data [24]. Moreover, excessive sampling is against animal welfare and the 3R (replacement, reduction, refinement) principle [25]. While multiple blood sampling

from tail vein is normally applied on experimental mice/rats in pharmaceutical study, the biomass of blood sample could be limited per collection point.

The zebrafish and zebrafish larvae exhibit a correlation between neuroanatomical and physiological features that is comparable to what has been observed in mammals. This similarity enables the creation of dependable and pertinent experimental models for researching neurological disorders [26, 27]. As an ideal alternative experimental model to mammals, the analysis of zebrafish or zebrafish larvae using LC/CE-MS usually requires pooling several samples, resulting in a loss of data on the heterogeneity between animals [28].

While animal research remains essential for advancing medical science, efforts to refine experimental techniques, minimize suffering for better experimental animal welfare, and explore alternative models are ongoing. As an example, a pharmacokinetic study of insulin in rat plasma combined microsampling and micro flow-LC-MS analysis, which proved the advantage of miniaturized analytical workflow over conventional method. A 47-fold sensitivity increasement enabled the analysis of volume-limited samples [29].

- Miniaturized method development for concentration-limited compounds

Miniaturized analytical methods offer a significant advantage in sensitivity enhancement, enabling the detection and quantification of compounds including drugs and metabolites in trace level concentrations [30-32]. Upon analyzing concentration-limited samples, the method development strategy is the same as conventional methods. For hydrophobic compounds like lipids, the micro flow-RPLC-MS method provides benefits such as high-resolution separation, sensitivity, and robustness. However, RPLC is not the ideal separation method for polar compounds such as amino acids and their derivatives. In contrast, hydrophilic interaction liquid chromatography (HILIC) is a valuable tool for analyzing polar compounds, although miniaturizing it is comparatively challenging compared to RPLC. The inclusion of salts in HILIC mobile phases decreases retention and improves peak shape, but also leads to ionization suppression and the risk of clogging in micro-LC column and spray emitter. Conversely, capillary electrophoresis (CE), which is sometimes overlooked, allows for the measurement of polar compounds by separating them

based on size and charge within an electric field. The advancement of sheathless CE-MS avoids dilution in ionization source and highly improves its sensitivity. The low-nano liter injection volume makes it ideally suited for miniaturized sample analysis [33].

Proper sample preparation methods are crucial for concentration-limited analytes in miniaturized metabolomics workflow. To avoid clogging systems and improve the detection sensitivity, a clean and efficient sample preparation procedure is required. In theory, a consistent extraction recovery through different concentration levels is good enough for bioanalysis. However, for concentration-limited compounds, sample loss such as due to adsorption on wall of sample vials could be fatal to the quantification. Online sample preparation techniques such as solid-phase microextraction coupled with miniaturized systems could help overcome the sample loss during sample transfer as well as increase throughput [34]. On the other hand, up-concentration strategy by evaporation or electro-extraction is commonly used for sensitivity improvement. While miniaturized LC-MS or CE-MS systems are easier to be clogged by matrix samples, extra attention should be paid on sample clean up.

Scope and outline of the thesis

Quantification of trace level compounds is always challenging to conventional analytical techniques. The demand of using limited volume samples further emphasizes that more sensitive and robust analytical methods are urgently required. As a concentration-sensitive detector at the flow rates used in this thesis, MS exhibits the same sensitivity when samples with identical concentrations are sprayed into the MS via the electrospray interface. In this thesis, the hypothesis is that by using micro/nano flow rates, we can significantly reduce sample dilution in the MS ionization source when working with biomass-restricted samples and a rugged source design is needed for clinical applications. In addition, when further decreasing the flow rate as with sheathless CE-MS, the MS detector becomes mass sensitive as the ionization becomes more efficient, and the detection limits become more favorable for biomass-limited samples.

In order to verify this hypothesis, we investigate how miniaturized analytical methods can enhance the sensitivity for biomass-restricted samples in metabolomics studies. This will be achieved by selecting and optimizing analytical techniques based on the properties of matrices and targeted compounds. Micro-flow LC-MS and sheathless CE-MS methods are established respectively for the analysis of lipids and amino acids, and these methods are applied on various biomass-restricted biospecimens to prove their applicability in biological and clinical studies.

To address the biomass mismatch challenge during quantitative analysis, and increase the sensitivity of analytical methods, this thesis discusses the development of miniaturized analytical methods using micro-flow LC-MS and CE-MS and investigates their potential for clinical studies with biomass-restricted samples. An overview structure of the thesis and the aim of each chapter is shown in **Figure 1**.

A robust system with stable ionization spray and high ionization efficiency is the key for method development and application on biological studies. At the start of the thesis, the focus is on performance evaluation of current commercial instrumentation for miniaturized analysis, including LC, MS, and ionization sources designed for low flow rates. An overview of the state-of-art analytical techniques for biomass-restricted analysis is presented in **chapter 2**, giving a critical overview of current microscale analytical techniques for the analysis of small-volume biological samples with a metabolomics approach. Technological developments are highlighted and relevant applications are discussed. However, the robustness of miniaturized techniques needs to be further improved for their application in clinical studies.

After comparing the currently available micro-flow LC-MS techniques, investigation of their practical performance is done in **Chapter 3&4**. The aim of **Chapter 3** is to develop a new micro-LC-MS approach for the selective and sensitive determination of endocannabinoids and its analogues in human cerebrospinal fluid (CSF). Such a method is needed in order to study the role of the endocannabinoid system in various brain disorders, as current LC-MS approaches are not sensitive enough or lack the analytical performance for this purpose. In particular, a modified micro-ESI spray needle is used in this study (Shimadzu Mikros), which enhances the analytical performance and durability of our

method for the quantitative determination of endocannabinoids in human CSF samples. The developed workflow is successfully used for the determination of endocannabinoids in 288 human CSF samples, thereby clearly showing the utility of this approach for endocannabinoids profiling in biomass-restricted clinical samples.

Low flow LC-MS in negative mode is often not robust due to ionization discharge. In addition to the quantification of concentration limited compounds, the applicability of micro-LC-MS in volume-limited plasma under negative mode is discussed in **chapter 4**, where a sensitive method dedicated to the study of oxylipins in 5 µL human plasma is developed. The method is validated and compared to a published conventional UHPLC-MS method, particularly in terms of sensitivity enrichment. The same triple quad MS is used for both methods to ensure a fair comparison. In order to demonstrate its applicability, the method is applied to 40 human plasma samples from a healthy aging study.

The coverage of compounds is important for metabolomics study in biomarker discovery. As reverse-phase LC-MS is utilized in the previous chapters for hydrophobic compounds. The potential of a miniaturized analytical method for polar compounds in volume-limited samples is discussed in **chapter 5**. We focus on developing and assessing the utility of a sheathless CE-MS-based analytical workflow for the determination of creatinine in residual plasma samples with the aim to assess whether this approach is suited for the reliable quantitative determination of endogenous metabolites in small-volume samples. The latter is a requirement before actually testing the analytical workflow on neonatal samples, which will be the logical next step/follow-up work. In this study, we demonstrate that with a starting amount of only 5 microliter of human plasma, we can quantitate creatinine in a reliable way, and compare this with a gold standard assay for creatinine analysis, which requires 100 microliter in clinical labs. Apart from creatinine, we can analyze many other metabolites with sheathless CE-MS in the residual plasma sample, in contrast to the limited scope of the gold standard assay, opening up the possibility to study the role of metabolites and metabolite profiles in neonatal healthcare.

Finally, **chapter 6** offers a general conclusion of the studies described in this thesis. Perspectives and recommendations on further improvement and applications of the proposed miniaturized analytical methods are also discussed.

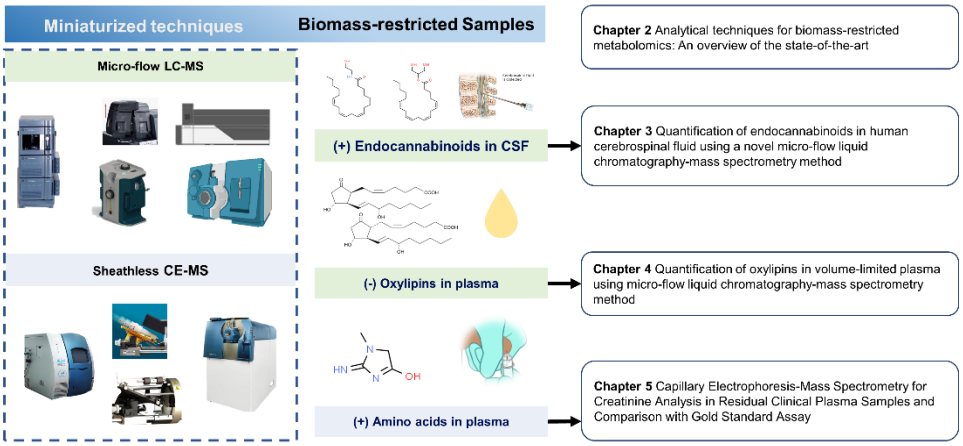

Figure 1. Overview structure of the thesis and the aim of each chapter.

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