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

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Chapter VI

Conclusions and perspectives

Conclusions

Reversed-phase UHPLC-MS has become a routine analytical technique for both academic and pharmaceutical research in recent decades [1]. While widely employed for highly sensitive and high-throughput analysis, its detection and quantification ability are still compromised when analyzing biomass-restricted matrices. When the sensitivity of a method is not sufficient for a biomass limited sample like zebrafish larvae, single cells or minimal amount of body fluids, the commonly used strategy to reach the (required) detection limits is to pool several samples together, which results in sacrificing the heterogeneity of the sample set. However, collecting data for an individual (or single) sample is pivotal for metabolomics and for future personalized treatment strategies. Additionally, the development of non-invasive sampling techniques allows for the collection of a few picoliters to microliters blood or cytoplasm at a time, yet the lack of sensitive analytical methods for these trace amount samples leaves a gap between sampling and analysis. Addressing this volume-mismatch will further our understanding of disease pathology, drug metabolism and dose assessment.

The underlying idea of this thesis is to reduce sample dilution in the ionization source and improve MS sensitivity by down-scaling the flow rate to micro/nanoliter level. The robustness and applicability of micro flow-LC-MS and CE-MS for the analysis of biomass-restricted samples were demonstrated. While nano flow LC-MS provides superior sensitivity, it often sacrifices robustness compared to conventional LC-MS methods. Micro flow LC-MS offers a balanced approach, providing sufficient sensitivity for biomass-restricted samples while offering greater robustness compared to nano flow methods. Additionally, CE-MS is an ideal technique for volume-limited samples due to its zero dead volume, which maximizes system efficiency. The use of sheathless CE-MS further enhances sensitivity, making it a perfect fit for analyzing small sample sizes without compromising performance.

In this thesis, miniaturized MS-based analytical methods are established to address the volume-mismatch issue between sampling and analysis, as well as enabling the analysis of concentration-limited compounds in matrices including human plasma and cerebrospinal fluids. The significance of miniaturized MS-based workflows, including sampling and sample preparation methods, separation techniques and ionization sources, was emphasized

at the beginning of the thesis (**Chapter 2**). The applications showcasing recently reported miniaturized methods were discussed. In **Chapters 3** and **4**, micro-LC-MS methods were developed and successfully applied to biomass-restricted samples after evaluating various MS ionization source designs. To enhance the robustness and sensitivity, a spray needle was developed for positive mode analysis of endocannabinoids. For analysis of oxylipins in negative ionization mode, an OptiFlow ionization source was utilized for obtaining optimal performance. These two methods demonstrated that robustness is no longer an obstacle when the correct instrumentation is selected.

CE-MS has its advantage in analyzing polar and charged compounds, especially for volume-limited samples [2-4]. A sheathless CE-MS method was developed in **chapter 5** with high sensitivity for the quantification of creatinine in residual children plasma. Although the fixed length of the sheathless capillary compromises flexibility to some extent, this method managed to improve analysis throughput by employing a multi-segment injection strategy, enabling seven samples to be measured in one electrophoretic run. The measured creatinine values correlated well with the results from clinical measurements.

Sensitivity enhancement

Miniaturized analytical methods operate on a significantly smaller scale compared to conventional methods, utilizing reduced flow rates and sample volumes. This miniaturization enhances sensitivity with low flow rates, which reduces the dilution within ionization source, making micro-flow LC-MS ideal for analyzing biomass-restricted samples. By optimizing chromatographic conditions and mass spectrometry parameters, micro-flow LC-MS enables the precise quantification of analytes even at ultra-low concentrations.

In **Chapter 3**, our focus was on the detection and quantification of endocannabinoids in 250 µL human CSF. The trace levels of endogenous concentrations make this challenging for conventional methods. **Chapter 4** presents a negative quantification method for oxylipins in 5 µL human plasma. Both of these methods were developed with 4 µL/min flow rate and were compared with conventional flow LC-MS methods (i.e. flow rate above 0.5 mL/min). The sensitivity for endocannabinoids and their analogues improved 5 to 22 times, notably enhancing the ionization efficiency of AEA and 1-AG/2-AG by about nine

times compared to conventional flow rates (550 µL/min). For oxylipins, sensitivity increased by 1.4 to 180.7 times with 4 µL/min compared to 700 µL/min. Although enhancement values varied for each compound, comparing results indicated that micro flow-LC-MS significantly enhances detection of both concentration-limited and volume-limited sample compared to conventional LC-MS.

In **Chapter 5**, a sheathless CE-MS method was developed for the profiling of hydrophilic compounds in residual plasma samples. The method was validated for quantification of creatinine in 5 µL plasma. Results from 25 adults and 9 children samples were compared with the golden standard enzymatic colorimetric assay used in clinics. Good alignment was observed between these two methods. This method holds promise as an alternative quantification tool in clinics, particularly in pediatric contexts, due to its significantly improved sensitivity and minimal sample volume requirement.

By establishing these three methods with micro flow LC-MS and sheathless CE-MS, we have demonstrated with solid data that miniaturized analytical methods significantly enhance sensitivity regardless of whether under positive or negative ionization, and whether for hydrophilic or hydrophobic compounds.

On the other hand, the required volume in the injection vials for LC and CE injections could be further miniaturized. For the micro-flow LC-MS and CE-MS methods used in **Chapter 4&5**, 5 µL of plasma was used as the starting volume, and samples were reconstituted in 20 µL and 25 µL solvents before injection due to lack of proper miniaturized vials, respectively, which resulted in unnecessary sample dilution. Optimization of injection strategy such as using online sample preparation and loading samples directly to the column could be benefit for higher sensitivity.

Robustness

Miniaturized techniques necessitate the downscaling of various components such as LC columns, CE capillaries, tubing, pumps, spray needles, and ionization sources in MS. Optimizing these miniaturized components enhances ionization efficiency and sensitivity. However, system robustness can be compromised, as smaller components are more prone to clogging by matrices. In addition, miniaturization can lead to other issues like discharge for nanospray in negative mode if conditions are not properly chosen. To mitigate this issue,

it is crucial to focus on two aspects: ensuring clean sample preparation and developing robust analytical systems.

For metabolite analysis, clogging is often caused by the residue proteins or salts from the matrices. In **Chapter 5**, an intensive sample preparation method was used for CE-MS analysis, including protein precipitation (PP) and one-hour centrifugation with an ultra-filter. This extraction procedure ensured good recovery (>87.6%) for creatinine and robust analysis using the capillary cartridge with a 30 µm inner diameter (i.d.). Given the CE-MS flow rate of only a few nanoliters, an ultraclean sample preparation is pivotal. Over 700 samples were injected using the same capillary cartridge during the method development and application to human plasma, proving the robustness of the sample preparation procedure. The intra- and interday precision of this method was below 3.2%.

Compared to the CE-MS method with nano-flow rate (around 30 nL/min), micro flow LC-MS methods in **Chapter 3&4** were developed using micro-level flow rate (4 µL/min), wider inner diameters of tubings (50-60 µm) and column (i.d. 300 µm). These properties decrease the chance of clogging during sample analysis. To further improve method robustness, the focus was on selecting the optimal instrument and plumbing parts. The spray emitter was modified for positive mode analysis in **Chapter 3**. Along with well-fitting sleeves and zero dead volume connections, the modified needle enabled the quantification of endocannabinoids in 288 human CSF samples with precision under 13.7%. For the analysis of oxylipins in **Chapter 4**, discharge issues in electrospray sources with negative mode makes it more challenging than positive ionization method. An OptiFlow ionization source with SteadySpray electrode was selected for optimal performance as well as avoiding the corona discharge during analysis.

With the development and application of these three miniaturized methods, we can conclude that the poor robustness of miniaturized techniques is a misconception. By employing efficient sample preparation to obtain clean sample injection solutions, appropriately optimized instruments, and plumbing parts, micro flow LC-MS and CE-MS methods are promising for various metabolomics, clinical, and pharmaceutical studies involving large cohort and diverse sample types.

Future efforts to improve the robustness of micro-flow LC-MS should be putting on eliminating too many connections in the system to avoid dead volume and possible leakage.

For sheathless CE-MS, the capillary cartridge should be more robust if extra coating can be applied on the fused silica tubing to make it less fragile.

The optimal choice for biomass-restricted samples

MS-based metabolomics has been a crucial part of biomarker discovery for the in-depth deciphering of various disease pathophysiologies. However, conventional methods fall short in dealing with biomass-restricted samples due to the higher sensitivity required. For the application in academic and clinical studies, high coverage for biomarker detection, good precision and accuracy for quantification are key requirements. In this thesis, in order to show the applicability of miniaturized methods, conventional LC-MS methods and clinical enzymatic method were used as standards for comparison. The established methods showed their robustness and accuracy when applied to various matrices. Among all biomass-restricted samples, those from infants and children are the most valuable due to their limited total blood volume and vulnerability.

In **Chapter 5**, the comparison between CE-MS and golden standard enzymatic method indicated the applicability of CE-MS for quantifying creatinine in plasma. The quantification accuracy was below 8.5% for concentrations from 1 μM to 10 μM, aligning well with the enzymatic method. For clinical utilization, this means significantly less sample volume from children is required with equivalent measurement ability, which also leads to reduced pain from the sampling procedure.

Beyond higher sensitivity, miniaturized LC-MS and CE-MS enable wider compound coverage than current biochemical methods in clinics. **Chapter 3 & 4** determined 16 endocannabinoids and 66 oxylipins in biomass-restricted samples, respectively. The CE-MS method also allows for profiling a broad range of hydrophilic compounds. The metabolomics data provided by these methods could aid in more comprehensive monitoring of healthy or diseased conditions, enabling pharmacokinetic profiling, biomarker discovery, and the development of personalized medicine.

In addition, LLE was used for the extraction of endocannabinoids and oxylipins for micro-flow LC-MS analysis. In this case, only extracts from organic layer were injected and the aqueous extracts were disposed, while both of them could be utilized by injecting the aqueous extraction to CE-MS. In this way, more comprehensive information could be obtained from one aliquot of sample, and enables higher throughput analysis. Miniaturized

SPE could also be considered as hydrophilic and hydrophobic compounds could be eluted separately from the SPE column, hence preventing the contamination from proteins.

Future perspectives

Combine miniaturized analytical methods with other miniaturized techniques

For the analysis of biomass-restricted samples, sensitivity enhancement is crucial when designing the experimental workflow. However, the volume mismatch between sample handling and the minimal requirement for injection still jeopardizes the measurement. While sample preparation is imperative for miniaturized MS-based techniques, two aspects can be improved to avoid unnecessary sample loss, which is especially critically when small volumes are in contact with large surfaces. One of them is reducing sample transfer steps during sample preparation. In this thesis, PP and LLE were used for CSF and plasma sample preparation. Sample loss due to manually transferring is inevitable, pipetting error could also be significant when the required volume is below 5 µL depends on the viscosity of matrix sample[5]. By using deuterated internal standards and online sample preparation techniques, the repeatability and robustness could be improved.

So far, some online sample preconcentration methods have been developed to avoid unnecessary sample loss and gain more signal abundance for MS analysis. In our lab, a three-phase electroextraction (EE) method was reported for the fast online analysis of trace-level pharmaceuticals in plasma [6]. By applying 40-400V extraction voltage, acidic compounds were concentrated to a 0.3 µL aqueous acceptor droplet and directly injected to LC-MS. The enrichment factor ranged from 70 to 190 for the targeted compounds. This electroextraction is currently fully automated and promises to gain even higher sensitivity by connecting to micro flow LC-MS. Given that the electromigration-based separation principle is similar to CE, this method is also suitable for coupling with CE-MS [7].

Another potential online preconcentration approach is a hanging droplet evaporator. This technique concentrates the sample on-the-fly in a hanging droplet exposed to heated nitrogen gas flow enriching up to tens of microlitres into a hanging droplet of less than one microlitre, without evaporation to dryness. The solvent evaporation rate and droplet volume are measured and controlled by a camera and real-time image processing. A 15-fold preconcentration can be obtained in less than 3 min. **Figure 1** shows the SRM

chromatograms obtained with an evaporator module coupled to a micro flow LC-MS system. A 10 µL endocannabinoid standard solution was evaporated into a 2.5 µL droplet and injected. The repeatability for 12 injections were 2.7% for LEA and 3.0% for EPEA. Although further optimization is still required for better robustness, the combination of online evaporation and micro-flow LC-MS is anticipated to benefit the analysis of biomass-restricted samples. For examples, samples can be enriched via evaporation without the need to redissolve the analytes after evaporation.

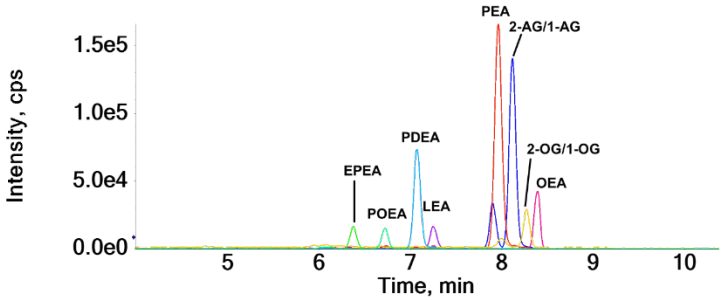

Figure 1. Typical SRM chromatograms from endocannabinoids standard solution obtained with an online evaporator module coupled with micro flow LC-MS

Further improvement in instrument design

Down-scaling flow rates and plumbing volume enhances sensitivity. This benefit has been proven and increasingly utilized in proteomics study. However, the application of miniaturized methods for small molecule is still rare due to concerns about method robustness and relatively long analysis time. To address this issue, many users have modified their own micro- or nano-flow systems, including tubing, connections, columns, flow generators, ionization sources, and CE-MS interfaces (**Chapter 2**). In **chapter 3** of this thesis, a commercialized spray needle was modified to avoid clogging from matrix samples. Additionally, the sizes of tubing and connection fittings were carefully selected to prevent possible leakage and dead volume within the system. These innovations enable more sensitive and robust analysis with miniaturized techniques. However, the lack of standardized (or ready-for-use) techniques including miniaturized ionization sources, spray emitters, miniaturized LC tubings and connections has hindered their widespread application in academic and clinical studies. In-house designs of these instruments also require extra training for other researchers to operate.

Efforts have been made in recent years to standardize miniaturized device design. Examples include the EVOSEP+ method from EVOSEP (Denmark), which is a standardized method for detecting stanozolol in equine hair and urine, specially designed for a ReproSil-Pur C18 (8 cm x 100 µm, 3 µm) column. Additionally, chip-based LC-MS devices have been developed by companies such as Agilent (HPLC-Chip/MS), Waters (TRIZAIC™ nano tile), Sciex (cHiPLC®-Nanoflex), and New Objectives (PicoChip) [8]. These devices require appropriate connection among the pumps, columns, and detector to fully release their advantages in miniaturized analysis. Further development of miniaturized techniques should focus more on the communication and connection among all the parts, minimizing the volume within system to improve the throughput. Moreover, the design of more user-friendly and robust instruments should be promoted for their broader adoption and reproducibility in research and clinical settings.

Translation to clinical studies

Miniaturized LC-MS and CE-MS methods offer a transformative solution to the analytical challenges posed by biomass-restricted samples in clinical and pharmaceutical studies. By leveraging their high sensitivity, these miniaturized methods enable researchers to extract maximal information from minimal sample amount, thereby advancing our understanding of disease mechanisms, guiding therapeutic interventions, and accelerating drug discovery and development.

In this thesis, the potential of CE-MS for clinical analyses was demonstrated for creatinine analysis by comparison of the findings with the gold-standard enzymatic assay employed in clinical chemistry labs. The reliable quantification ability of CE-MS using only 5 µL of plasma as starting volume, instead of the traditional 100 µL (due to the void volume), showed its potential for translation to clinical, especially pediatric, studies. In addition to higher sensitivity, the multi-segment injection strategy allowed seven samples to be analyzed in a single run, contributing to the high-throughput analysis of targeted compounds for clinical practice.

Similarly, micro-flow LC-MS methods were capable of measuring oxylipins and endocannabinoids in biomass-restricted samples with higher sensitivity compared to conventional methods. Their applications on biomass-restricted human CSF and plasma

proved their capability of adapting to future clinical practice. Additionally, in both academia and clinical settings, the welfare of patients and experimental animals is a primary concern. Consequently, microsampling techniques such as microdialysis, volumetric absorptive microsampling (VAMS), and capillary blood sampling are becoming more prevalent [9]. Miniaturized methods are therefore worth promoting for use in both academic and clinical contexts. Moreover, the cost of analyzing samples is lower with miniaturized flow rates due to significantly reduced solvent consumption.

Repeatability and accuracy are crucial for the application of analytical methods. Micro-flow LC-MS methods and CE-MS methods developed in this thesis highlight the importance of clean and efficient sample preparation procedure and connection of robust instruments to ensure robust, accurate, and sensitive measurements. While the high sensitivity of miniaturized techniques is widely known, future studies should focus on standardization of methodologies, optimization of online or automated sample preparation procedures, and development of robust and high-throughput instruments to realize their full potential in clinical and pharmaceutical research. Furthermore, interdisciplinary collaboration and the integration of miniaturized methods with complementary analytical techniques hold promise for addressing complex analytical problems and driving innovation in the field.

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