Capillary electrophoresis-mass spectrometry based metabolomics approaches for volume-restricted applications

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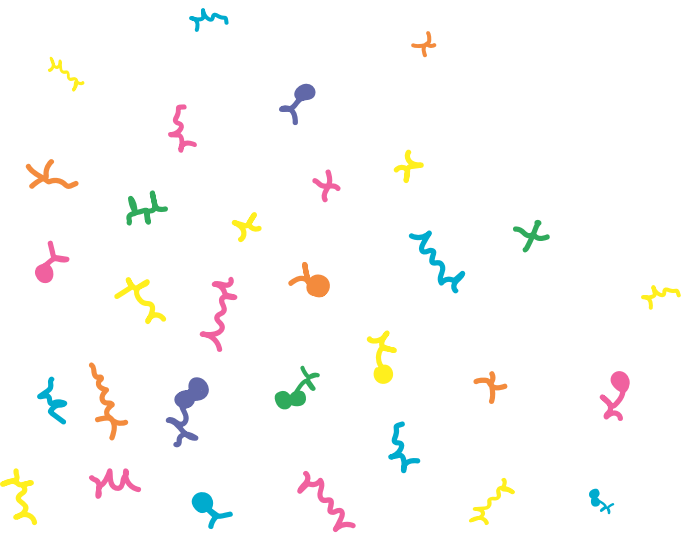

Chapter 1

Introduction and aim of the thesis

Over the past decades, a large paradigm shift has been observed towards a proactive manner of providing healthcare, in which a key focus is on preventing or (even) reversing the underlying pathophysiology of disease instead of using a purely reactive approach, i.e. action is taken once symptoms have appeared [1-3]. This proactive approach is driven by systems strategies and has led to a discipline that holds on to prediction, prevention, personalization, and participation (P4) [4], also known as 'precision medicine' [5]. By continuous health monitoring and creating physiological phenotypes, it is possible to obtain an in-depth understanding about how individuals are able to adapt to changes related to wellness and disease by identifying systematic changes over time, taking into account a persons' genetics, environment and lifestyle [2, 6]. In this regard, the focus of drug research has shifted from the 'single target–single drug' concept towards the understanding of complex and dynamic patterns of metabolite concentrations within a biological system. This way of metabolomics-driven health monitoring provides an important tool to gain insight into the complexity of human biology and the pathophysiology of diseases, and facilitates the development of tailored disease treatment and prevention, thereby potentially improving the quality of life for patients.

Metabolomics

Metabolomics encompasses the global profiling of small (endogenous) molecules within biological samples, and represents one of the most recent introduced 'omics' techniques. The metabolome i.e. the complete set of small molecules (<1500 Da) present within a biological system, is impacted by both internal and external factors and therefore provides a direct functional read-out of the physiological status of an organism, and can thus be used as a way to describe someone's actual health status [7]. Together with other 'omics' fields including (epi)genomics, transcriptomics and proteomics that include the global analysis of genes, RNA and proteins, respectively, a key aim of using metabolomics is to obtain insight into well-defined biomedical and clinical questions [8-10].

Metabolomics is considered a relatively young research field, but the study of biofluids to diagnose disease was already used early in history. For instance, thousands of years ago, urine was already used as a medical diagnostic tool to identify a variety of illnesses by investigating its color, turbidity, smell and taste [11]. In the early 20th century, a cascade of technological developments, including the invention of mass spectrometry (MS) detection, led to a significant increase in small molecule analysis in order to study pathophysiology of diseases. In 1948, Roger Williams and his coworkers were the first to suggest that organisms possess a unique 'metabolic fingerprint' that could be profiled by analyzing biological samples such as saliva and urine [12].

The term ‘metabolome’ was first introduced in 1998 [13], and the study of the metabolome i.e. metabolomics rapidly gained interest across the scientific research community in the years to follow.

As to date, the holistic view offered by metabolomics has already demonstrated its potential in diverse research areas and has had a profound effect on the development of diagnostics and therapeutics for various diseases [3, 14-19].

Metabolomics in neuroscience research

Metabolomics has emerged as a powerful tool in neuroscience research, as it has the potential to provide a deeper understanding of the mammalian brain and its function, furthering our understanding of the molecular mechanisms underlying neurodegenerative and neuropsychiatric diseases, such as Parkinson’s disease, epilepsy and schizophrenia [20, 21]. The early diagnosis of many central nervous system (CNS) disorders remains difficult due to the lack of specific symptoms at disease onset and the limited understanding of their etiology. The added value of metabolomics to understand CNS disorders has already proven itself the last few years in studies related to metabolic profiling of bodily fluids such as urine, plasma and cerebrospinal fluid (CSF), but also *post mortem* tissues. As an example, Wang *et al*. reported different metabolic profiles in serum of patients with epilepsy compared to healthy controls, in particular metabolic changes were observed that pointed in the direction of an energy deficit, inflammation, nervous excitation and neurotoxicity [22]. Therefore, this metabolomics study contributed to a further understanding of the pathophysiological processes underlying epilepsy.

Metabolomics analysis

Metabolomics studies can be divided into targeted and non-targeted approaches. Targeted metabolomics encompasses the analysis of a number of metabolites that belong to a specific metabolite class or metabolic pathway, and is predominantly hypothesis-driven. Furthermore, targeted metabolomics studies enable (absolute) quantification of the selected subset of metabolites, and thereby allow to obtain a precise snapshot of the biochemical pathway in question [23]. In contrast, a non-targeted metabolomics approach is often a hypothesis-generating study, and its goal is to maximize the number of metabolites that can be analyzed within a certain biological sample, typically detecting hundreds to thousands of metabolites per analysis. Generally, a non-targeted metabolomics study involves the comparison of metabolomics profiles from control and disease/treated groups by using multivariate data analysis strategies in order to identify the metabolic differences that might be relevant to a specific biological condition [24].

Present-day metabolomics studies are mainly performed by nuclear magnetic resonance (NMR) spectroscopy [25], and chromatographic techniques coupled to MS detection such as liquid chromatography (LC-)MS and gas chromatography (GC-)MS [26, 27]. NMR spectroscopy has been widely used for metabolomics studies due to its high accuracy and reproducibility and its minimal required sample preparation. Besides that, NMR is highly suited for absolute quantification purposes. However, compared to MS-based techniques, the sample requirement (typically in the range from 100 to 500 µL) and detection limits for NMR are rather high (detection limits of 10-100 times lower can be acquired with MS detection compared to NMR) [28]. Therefore, the use of MS-based techniques has gained interest for metabolomics studies the last decades [29]. GC-MS is a well-established analytical technique that is especially suitable for the analysis of volatile metabolites such as volatile organic compounds (VOCs) [30], but can also be applied in combination with derivatization strategies to analyze non-volatiles [31]. However, the fact that GC-MS is not directly suitable for the analysis of nonvolatile, polar, and/or thermally unstable compounds, makes sample preparation (including derivatization) a relatively long and labor-intensive manor. Reversed phase (RP)LC-MS is currently the most widely used analytical technique for metabolomics studies, mainly due to its robustness, ease of use and high versatility [32-34]. RPLC-MS separates metabolites based on hydrophobicity, and is therefore suitable for nonpolar (or weakly polar) metabolites, but its limitation is the lack of adequate retention of polar molecules on a RP stationary phase. For the profiling of polar metabolites, hydrophilic interaction liquid chromatography (HILIC)-MS is often considered due to its complementary separation mechanism to RPLC. HILIC-MS has shown great potential for metabolomics studies [35], however, a drawback of HILIC columns is the relatively long equilibration times that are required in between runs, and often lower robustness and column stability compared to RPLC.

One of the key challenges in (brain) metabolomics studies remains the reliable and sensitive analysis of limited amounts of biological samples. These challenges originate from the low volumes of available sample, but are also due to a large variety of biochemical species, wide dynamic range of their concentrations and in some cases, rapid turnover rates. Moreover, a key development in biomedicine, drug research and translational biology is to use patient-derived 3D cell culture systems on a microfluidic chip. Such systems offer the possibility to gain a deeper or basic understanding of the molecular mechanisms of complex diseases, to perform drug repurposing studies, etc. Additionally, the use of animal models could be reduced depending on the goal of the study. However, the amount of cells and medium/perfusate in such 3D microfluidic cell culture system is really low. Therefore, to enable the study of biomedical questions intrinsically dealing with low sample amounts with a metabolomics approach, the development of reliable

and sensitive analytical methods and workflows is (urgently) needed. In this thesis, the aim is to realize this ambition by developing microscale analytical workflows based on capillary electrophoresis-mass spectrometry and with a special emphasis on experimental procedures for generating metabolic profiles in volume-restricted samples.

Capillary electrophoresis–mass spectrometry for metabolomics

Capillary electrophoresis (CE)-MS is a powerful analytical technique for the profiling of polar ionogenic compounds in biological samples. Its separation mechanism is based on differences in migration velocity of analytes under the influence of an electric field, also referred to as the analyte's electrophoretic mobility (μ_{eff}). Separation of analytes is thus achieved based on difference in charge-to-size ratios, thereby providing complementary information to other (chromatography-based) separation techniques.

Due to its microscale character and orthogonal separation mechanism, CE-MS has noticeably gained attention as a suitable technique for metabolomics. The first studies that used CE-MS for the metabolite profiling of biological samples was already performed in 2002 by Soga and co-workers [36, 37], and in the following two decades CE-MS was used by multiple research groups for the analysis of a range of biological matrices. When it comes to neuroscience research, the prerequisite of low sample amounts to perform nanoliter injections from a few microliter in the sample vial makes CE-MS especially a very suitable technique, since brain metabolomics studies often deal with scarcely available and valuable samples such as brain fluids.

However, even though CE-MS has been used by various research groups now, it is still inadequately represented compared to chromatographic techniques such as LC-MS and GC-MS. This is mainly due to the perception that the technique is less robust, less sensitive and difficult to use, among other (mis)conceptions [38]. Therefore, in this thesis, we aim to address these misconceptions by showing the actual utility of CE-MS for metabolomics for the reliable, efficient and sensitive analysis of biological samples.

Scope and outline of the thesis

The aim of this thesis is to develop innovative CE-MS methods and workflows for metabolomics studies, with a special focus on material-limited biological samples. The underlying idea is that CE can be made as suitable as LC-MS for metabolomics. This will be achieved by on one hand, the development of novel microscale analytical workflows employing CE-MS, and on the other hand, by showing the utility of CE-MS for metabolomics for various biological applications related to neuroscience research.

The aim of the first part of this thesis is to highlight the potential of MS-based analytical workflows for the metabolomics study of the central nervous system, with a focus on *in-vivo* studies that deal with intrinsically low sample volumes (**chapter 2**). **Chapter 2** covers *state-of-the-art* metabolomics workflows, including recent trends and challenges considering sampling, sample preparation and preconcentration strategies as well as sensitive analytical technologies suitable for material-limited samples.

In **chapter 3** we address one of the most common misconceptions about CE-MS; which is that CE-MS is perceived as a technique that lacks reproducibility. Through an interlaboratory study that encompasses 13 independent laboratories from 11 countries, we investigated if it is possible to overcome migration time variability and thereby increase reproducibility in CE-MS when migration times are converted to the effective mobility scale. Additionally, the suitability of the effective mobility a direct parameter of metabolite identification is studied.

Another common misconception about CE-MS is that due to the low loading capacity, CE-MS is not suitable for trace-sensitive metabolomics. In **chapter 4**, we aim to improve the detection limits by developing a method that employs on-line preconcentration with sample stacking based on pH junction. The applicability of the method was demonstrated for the direct profiling of endogenous metabolites in volume-limited rat brain microdialysis samples.

The majority of CE-MS-based metabolomics studies are focused on the analysis of polar cationic compounds, as the profiling of anionic metabolites often results in relatively low sensitivity and poor repeatability. Various efforts have been made to improve anionic metabolite profiling by CE-MS, and **chapter 5A** covers recent methodological developments, including an overview of recent applications in different research areas. **Chapter 5B** reports on a novel chemical derivatization procedure that provides a permanent positive charge to acidic metabolites, allowing their analysis using the same conditions as used for basic metabolites.

The applicability of the method was demonstrated for the analysis of energy metabolism-related metabolites in low numbers of HepG2 cells.

The last section of this thesis is focused on the actual application of CE-MS for non-targeted metabolomics. In **chapter 6,** a CE-MS workflow is presented for metabolic profiling in extracts from individual zebrafish larvae and pools of small numbers of larvae. Using this optimized workflow, the effects of cortisol on stress metabolism were studied. Multivariate data analysis was employed to detect differential metabolites.

Finally, **chapter 7** provides a general conclusion and discussion on the developed CE-MS workflows for material-limited biological samples. Further possible developments and potential directions are also discussed.