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

Mever, M. van

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Marlien Admiraal-van Mever

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Promotores:
Dr. R. Ramautar
Prof. dr. T. Hankemeier

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" No Human Is Limited "

Eliud Kipchoge

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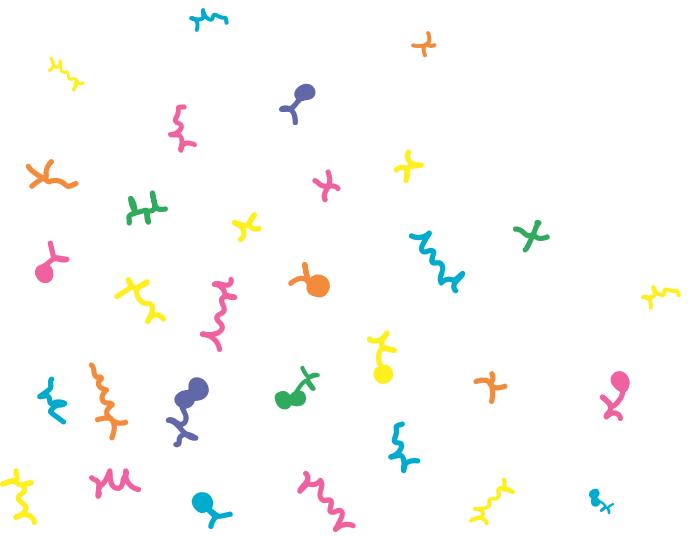


Chapter 1

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.

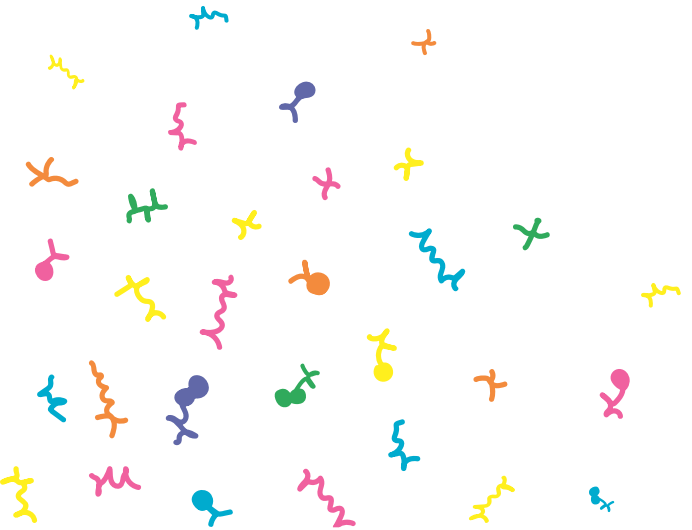
Another common misconception about CE-MS is that due to the low loading capacity, CE-MS is not suitable for trace-sensitive metabolomics. In **chapter 4**, we aim to improve the detection limits by developing a method that employs on-line preconcentration with sample stacking based on pH junction. The 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.



Chapter 2

Mass spectrometry based metabolomics of volume-restricted *in-vivo* brain samples: actual status and the way forward

Marlien van Mever*, Karen Segers*, Debby Mangelings, Thomas Hankemeier, Yvan Vander Heyden, Ann Van Eeckhaut[$] and Rawi Ramautar[$]

* Authors contributed equally

[$] Authors contributed equally

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Abstract

Brain metabolomics is gaining interest because of the aging of the population, resulting in more central nervous system disorders such as Alzheimer's and Parkinson's disease. Most often these diseases are studied *in vivo*, such as by analysing cerebrospinal fluid or brain extracellular fluid. These sample types are often considered in pre-clinical studies using animal models. However, the scarce availability of both matrices results in some challenges related to sampling, sample preparation and normalization. Much effort has been made towards the development of alternative, less invasive sampling techniques for collecting small sample volumes (pL till mid µL range) over the past years. Despite recent advances, the analysis of low volumes is still a tremendous challenge. Therefore, proper preconcentration and sample pretreatment strategies are necessary together with sensitive analysis and detection techniques suitable for low-volume samples. In this review, an overview is given of the state-of-the-art mass spectrometry-based analytical workflows for probing (endogenous) metabolites in volume-restricted *in-vivo* brain samples. In this context, special attention is devoted to challenges related to sampling, sample preparation and preconcentration strategies. Finally, some general conclusions and perspectives are provided.

Introduction

As the population is becoming older, central nervous system (CNS) disorders, such as Alzheimer's and Parkinson's disease, are becoming more prominent. The underlying pathological mechanisms of these neurological disorders are not yet well understood, and therefore more research is needed in order to develop novel drug targets and therapies. Brain metabolomics studies may reveal insights in the mechanisms of these CNS disorders.

Brain metabolomics approaches may be used to profile the (endogenous) small molecules, either in a targeted or non-targeted way [20, 21, 39]. Regularly, the study objects in *in-vivo* brain metabolomics are samples from small animal models, which result in sample volumes from the picoliter level to about 50 µL [40-42]. The detection of low metabolite concentrations is even more challenging in such small sample amounts. Therefore, adequate sample preparation and preconcentration steps are necessary, as there is a high need for sensitive techniques. Microextraction techniques for sample pretreatment, such as solid-phase microextraction (SPME) and liquid-phase microextraction (LPME), are emerging. Increased sample throughput can be obtained when coupling these techniques on-line with miniaturized separation techniques [41]. Nano- and micro- liquid chromatography (LC), capillary electrophoresis (CE) and chip-based systems are suitable for small-volume sample analysis. Another often-applied method for low sample amounts in metabolomics is gas chromatography (GC) [43]. GC is, besides LC, also suitable for on-line coupling with extraction techniques, while on-line derivatization is also possible. More specific, when mass spectrometry (MS) is applied as detection technique, isotope-coded derivatization (ICD) is gaining interest [44]. MS is indeed a very suitable detection technique for brain metabolomics, because of its higher detection sensitivity, compared to nuclear magnetic resonance (NMR) which is also often applied in metabolomics. Electrochemical and fluorescence-based detection techniques have also been used for the sensitive analysis of metabolites in brain samples [45, 46]. However, electrochemical detection is limited to electroactive substances, whereas fluorescence detection often requires a derivatization step in order to introduce a fluorophore. Therefore, these detection techniques (when used in combination with LC or CE) are more suited for targeted metabolomics studies.

In this review the focus is on the analytical challenges of different small-volume matrices studied in *in-vivo* brain metabolomics. Analytical challenges occurring during sampling, sample preparation and preconcentration are considered in detail, in addition to the challenges associated with coupling miniaturized separation

techniques to MS detection. Therefore, emphasis is given on novel miniaturized workflows, while traditional workflows in brain metabolomics will not be discussed. *In-vitro and ex-vivo* samples, such as cell lines, organoids and tissue samples, are also excluded from the discussion. More information about challenges related to sample collection, preparation, analysis and data normalization for tissue samples can be found in [47, 48] and for single cells in the following recent reviews [40, 49].

Volume-restricted sample matrices investigated in *in-vivo* metabolomics

Small sample amounts for *in-vivo* brain metabolomics are caused by a variety of reasons. For example, rodent models, especially mice, allow collection of only small sample volumes (low µL range) from a living animal. Furthermore, brain fluids, such as extracellular fluid (ECF), are scarcely available and are already volume-limited in larger animal models or human donors. Alternative animal models, such as zebrafish, are also gaining interest for metabolomics studies, leading to even lower sample volumes available for analysis. In order to have sufficient material for metabolomics studies, sometimes a pooling approach is applied. However, pooling will provide an average read-out and therefore information on metabolic changes at the individual level and/or time level, and thus heterogeneity, is lost. In this section, an overview of volume-restricted sample types investigated in *in-vivo* metabolomics, including the trends in their sampling strategies, will be given and discussed.

Brain fluid

Brain fluids, i.e. ECF and CSF, are of major interest in mental and neurological disorders. Especially CSF sampling, often performed via a lumbar puncture in the brain subarachnoid space, has proven to be an invaluable tool in clinical neurology. However, in animal model studies, CSF is still an underrepresented matrix due to its poor accessibility, invasive sampling and low sampling volumes [42]. Other challenges, related to lumbar puncture, are the presence of non-specific binding of apolar and/or larger molecules to the sampling catheter [50]. To allow serial CSF collection in conscious minipigs, a minimally-invasive method via catheterization of the subarachnoid space was proposed [51]. Šakić described a modified cisternal puncture method in *post-mortem* mice to enhance the quantity (most often still less than 40 µL) and quality of the CSF samples obtained [42].

A technique that is often used to collect brain ECF is microdialysis, an *in-vivo* sampling technique allowing continuous sampling in freely moving animals [52]. Traditionally, the sampling is performed at relatively high flow rates (~2 µL/

min) in order to provide adequate temporal resolution (typically 10-20 min) and sufficient sample volumes (≥30 µL) [53]. However, to get an accurate view on the rapidly changing neurotransmitter levels upon a perturbation, a higher temporal resolution of microdialysis sampling is preferred, providing lower sample volumes (few µL or less). An additional challenge is the high amount of inorganic salts in microdialysis samples, which makes the analysis with MS detection techniques challenging because of matrix-effect issues. Non-specific binding can also occur at the microdialysis probe [54]. Despite these limitations, microdialysis is still considered the gold standard for brain fluid collection. However, in order to be able to perform more localized measurements, to increase temporal resolution and to minimize tissue damage, promising results are obtained with microfabricated probes [55].

An alternative sampling method, i.e. push-pull perfusion, is regaining interest for neurochemical analysis, mainly due to the introduction of miniaturized probes and advances in microfluidics [55]. In the so-called micro push-pull perfusion sampling (µPPPS), the sample is withdrawn from one capillary using low flow rates (~50 nL/min), while perfusion fluid is infused from another capillary. The main advantage of µPPPS is its spatial resolution, which is higher than conventional microdialysis sampling (0.06 mm^2 for microdialysis vs. 0.004 mm^2 for low-flow push-pull perfusion) [56]. Compared to microdialysis probes, PPPS probes typically contain larger pores, making the latter technique better suitable for larger molecules, such as proteins. However, there are some challenges that arise with low flow push-pull sampling, e.g. using low flow rates at high temporal resolution (i.e. a few seconds) results in nanoliter samples, and Taylor dispersion, which is the broadening of sample zones during their transport through a microfluidic channel, also needs to be controlled. The latter issue could be resolved by implementing a segmented flow [55], which has been directly integrated with MS detection (**Figure 1A-B**) [57-59], showing great improvements in temporal resolution (few seconds). However, the current push-pull methods, similarly to microdialysis, are still not suitable for the determination of rather apolar analytes.

A recently developed variant of PPPS is cerebral open-flow microperfusion (cOFM) [60], which is based on push-pull perfusion, but uses a membrane-free probe to sample brain ECF. The absence of a membrane makes the technique suitable for larger and lipophilic compounds, while membrane-related problems, such as clotting, are prevented [61]. However, this also means that extra sample preparation steps are required before sample analysis. Additionally, the design and material of cOFM probes allow re-establishment of the blood-brain barrier (BBB) integrity, and thus long-time monitoring within brain ECF is possible.

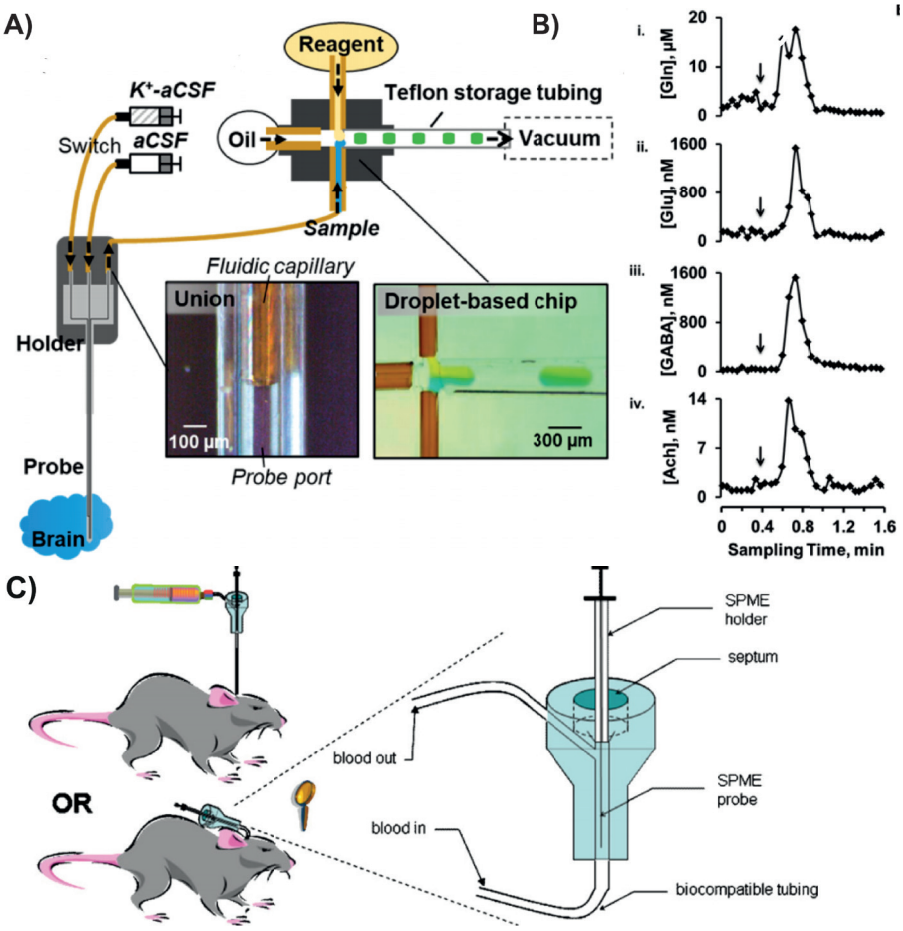

Figure 1. A) Monitoring brain chemical dynamics using micro push-pull perfusion sampling, droplet based microfluidics, and direct infusion MS assay, with (A) a schematic overview of a microfabricated push pull probe, and B) the averaged detection responses as measured by direct infusion MS of glutamine i), glutamic acid ii), GABA iii), and acetylcholine iv) during a microinjection of high potassium concentrations via the micro probe. **Figure** is adapted with permission from [23]. Copyright (2020) American Chemical Society. C) In-vivo SPME study on rats: placement of SPME devices and interface connection to the carotid artery, reused with permission from [89].

Another *in-vivo* sampling technique that is gaining more interest for monitoring brain chemistry is solid phase microextraction (SPME) [62, 63]. In (direct immersion) SPME, a thin probe (thickness ~200-300 µm) containing a biocompatible outer coating (often a C18 sorbent combined with polyacrylonitrile) is used to extract analytes through passive diffusion (**Figure 1C**), directly from brain tissue, plasma or extracellular fluid in a minimally invasive manner. SPME is complementary to microdialysis and PPPS, because it can be used to extract hydrophobic analytes, as it has proven to be effective for the analysis of a wide range of metabolites, including low abundance steroids and lipids. Recoveries of highly polar compounds, on the other hand, are still low for SPME [64].

The small size of the SPME probe and the selectivity of its coating allow a highly selective extraction of analytes of interest, isolating them from macromolecules or waste in the sample. Furthermore, for *in-vivo* SPME, the same set-up as for microdialysis can be used, meaning that SPME can be straightforwardly implemented into already existing workflows [65]. Additionally, because of their complementary nature, SPME and microdialysis sampling could be performed in parallel within the same test subject in order to capture as much chemical information as possible [66]. An important limitation of SPME is still the lack of fundamental understanding of the extraction process using biocompatible coatings, which show low recovery of compounds and resulting in a low commercial availability of these coatings [62, 67]. However, progress is ongoing in this area and it is therefore anticipated that SPME sampling will obtain a more prominent place in *in-vivo* brain metabolomics studies.

Blood

Because of the challenges with extracellular brain fluid and tissue collection, blood samples are more commonly considered for clinical and diagnostic purposes. However, they do not reflect the actual brain concentration. Whole blood collection requires the need of well-trained personnel and results often in large volumes in humans and larger animals (up to 5 mL). Such volumes cannot be sampled from all living animals because of increased animal welfare concerns and the limited available blood volume present per kg body weight (55-70 mL/kg body weight). For instance, in mice only a maximum of 20 µL blood sample is allowed to be taken in a serial sampling strategy [68, 69]. Therefore, alternative sampling strategies are necessary for small-volume blood collection. They may also be useful for humans or larger animals because of their less invasive character and possibility of "self-sampling". Different alternative blood collection and microsampling techniques are introduced, such as dried blood spots (DBS), volumetric absorptive microsampling (VAMS) and capillary microsampling (CMS) [41, 70]. An important challenge for DBS is the fluctuations in haematocrit content, leading to an uncertain sample volume. VAMS and CMS result in a precise and accurate blood volumes by making use of an absorbent polymeric tip (10, 20 or 30 µL) or glass capillary (1-35 µL), respectively. CMS is not (yet) widely used in metabolomics but the suitability of VAMS in non-targeted metabolomics was already shown by Volani et al. [71]. They evaluated the type of extraction solvent and the sample stability, thereby concluding that the VAMS samples should be stored at -80 °C instead of at room temperature. Furthermore, a targeted study with VAMS as a sampling technique was developed and validated for the measurement of 36 metabolites [72]. The extraction of organic acids and amino acids from the VAMS devices was performed with acetonitrile-water (60:40, v/v) in only 10 min. Moreover, the internal

standard was added the absorptive tip before blood sampling, allowing to correct for variations in the extraction process and/or potential metabolite changes upon long-term storage [72]. In general, VAMS is not widely applied in metabolomics and even not in brain metabolomics, but it shows a lot of potential as demonstrated by the above studies. More information on DBS, VAMS and CMS can be found in [41, 70].

Sample pretreatment and preconcentration

Sample preparation of biological matrices is a crucial step in metabolomics. This step is often most time consuming and labour intensive. The main goal of the sample preparation is to reduce matrix interference, increase selectivity for the metabolites of interest in (semi) targeted analyses, preconcentration and stabilization of the sample, and to get the compounds of interest in the (proper) dynamic range of MS-based metabolomics. These improvements are necessary to overcome the challenges of crowded spectra, sensitivity issues, identification and structure-determination problems. The most easily and quickly applied off-line sample preparation methods that are still often applied in brain metabolomics are simple dilution, protein precipitation and centrifugation or filtration. However, matrix effects have a high chance of occurrence using these sample preparation techniques. It is even seen that a more selective sample preparation such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE), can result in an increased coverage in non-targeted metabolomics and better reproducibility of the method [73] due to the reduced matrix effects.

However, the traditional LLE and SPE techniques often require relatively large sample volumes. Other limitations include the high consumption of organic solvents, use of salts and buffers and waste production. Moreover, the manual operation could be quite laborious and time-consuming. A strategy to make metabolomics workflows better suited for low-volume samples and to overcome the disadvantages of the traditional techniques is to consider the potential of micro-extraction techniques [74-76]. In this context, LPME and SPME are ideal approaches and have recently been reviewed for metabolomics applications by Hemmati *et al.* [41]. In the present review only the recent advances and (remaining) challenges of microextraction techniques for brain metabolomics are highlighted. Moreover, alternative miniaturized SPE techniques are briefly discussed.

Another strategy to improve detection sensitivity and selectivity of neurochemicals is to change the physicochemical properties of analytes by performing a derivatization step. Recent advances on this topic are also discussed.

Micro-extraction techniques and micro-SPE

As already mentioned, two well-known micro-extraction techniques are LPME and SPME. A major advantage of both is their ease of automation, thereby integrating sampling, extraction, concentration and injection, resulting in on-line coupled techniques. Especially in case of *in-vivo* sampling, this on-line coupling is greatly advantageous in terms of minimizing handling errors and increasing sample stability. In spite of these advantages, SPME and LPME are still often applied off-line.

Different LPME techniques have already been widely discussed in the literature [41, 74, 77]. However, in brain metabolomics the most often applied LPME technique is dispersive liquid-liquid microextraction (DLLME; **Figure 2A**) [41]. The principle of DLLME and its application in metabolomics are discussed in the following reviews [41, 74, 75]. In general, a small volume of organic solvent mixed with a dispersant solvent is rapidly injected in the aqueous sample, resulting in tiny droplets of extraction solvent in the sample. The major advantage is that analytes can easily penetrate into the solvent resulting in a high preconcentration factor. This technique was for example used by Zheng et al. [34]. They have developed a stable isotope-labelling derivatization-ultrasound-assisted DLLME for the simultaneous determination of monoamine neurotransmitters and their biosynthesis precursors and metabolites in rat brain microdialysis samples [78]. This approach overcomes the severe matrix effects as well as the limitations of isotopic labelling present in traditional dilution approaches. The extraction of hydrophilic compounds with DLLME is still challenging but can be achieved by adapting the pH. Another LPME method in metabolomics is electromembrane extraction (EME). This method has the advantage that the sample clean-up can be selectively tuned by modifying the electrical field. However, the method is already applied for plasma [41, 77, 79] and for the analysis of spiked neuropeptides in CSF [80]. Those investigations and applications makes EME a very attractive tool for brain metabolomics. A further discussion of the technique and their main objectives with still some points of research are recently discussed by Huang *et al*. [81]

SPME was previous discussed as an *in-vivo* sampling technique. The possibility of sampling together with applying a sample clean-up and pre-concentration is a main advantage of SPME. Another great possibility is the direct coupling of SPME to MS devices, as has been shown by Nazdrajić et al. [82], where direct immersion SPME was directly coupled to microfluidic open interface (MOI)-MS for direct analysis of metabolites in whole human blood [83]. Furthermore, SPME is more widely applied than LPME as an extraction technique in brain metabolomics [74]. Conventional SPME contains fiber coatings with tuneable structures but shows small absorption capacities and low extraction rates, especially for hydrophilic compounds. Therefore, thin-film microextraction (TFME) was developed. TFME contains a sheet

of a flat-film resulting in a higher area-to-volume ratio and consequently a higher uptake rate and extraction recovery, even for hydrophilic compounds. Principles, advantages and applications are discussed in the review of Hemmati *et al*. [41] and of Olcer *et al*. [84]. TFME shows a lot of potential but has, to the best of our knowledge, not yet been used in brain metabolomic studies. SPME still has some limitations, such as the instability of the fiber and low recovery, and results in higher variability than LLE and SPE. The same sorbents as for SPE could also be considered in SPME as coating for the fiber. Still, inter-device variability is observed in SPME of commercial and in-house made devices. Therefore, the development of highly reproducible new fiber coating technologies will be of interest to generate more accurate and reproducible results in SPME. Reproducibility could also be encountered by considering the pre-loading of an internal standard onto the fiber coating to control sampling and matrix related variabilities [85].

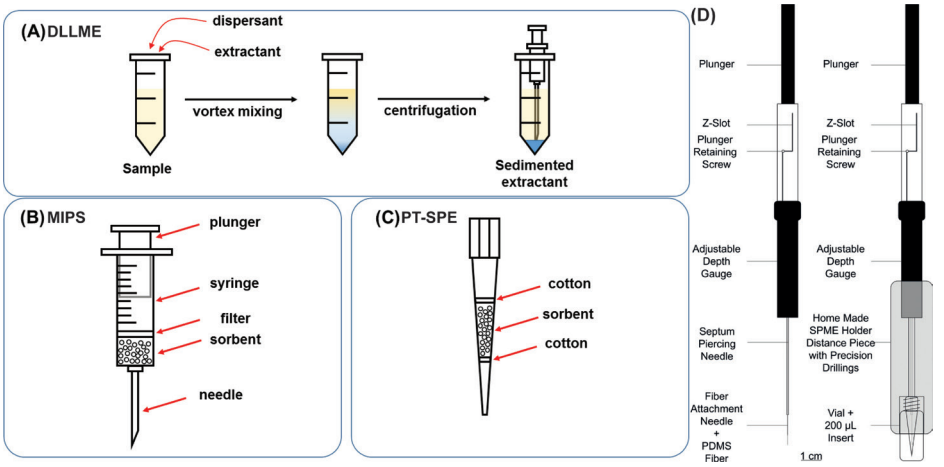

Figure 2. Schematic overview of miniaturized sample preparation techniques suitable for low-volume samples. A) Dispersive liquid-liquid microextraction, B) Microextraction in a packed syringe and C) Pipette-tip solid-phase extraction. **Figure**s A, B and C re-used with permission from [39]. D) shows a SPME device with a modified distance piece to get reproducible immersion depths necessary for coupling SPME to GC-MS, reused with permission from [84].

In recent years, (more robust) miniaturized SPE techniques (micro-SPE) are upcoming, such as microextraction packed in pipette tips, better known as Pipette-tip solid-phase extraction (PT-SPE) or in syringes (microextraction in a packed syringe (MEPS)). A schematic representation of PT-SPE and MEPS is shown in **Figure 2B and 2C**. Here, the typical steps known in SPE need to be performed such as conditioning, loading, washing and eluting. Both micro-SPE techniques, PT-SPE and MEPS, contain a small volume of solid-phase sorbent packed in a gas tight syringe or pipette tip and result in higher extraction recoveries than SPME [74, 75]. Well-known sorbents are C_{18} or C_8, while alternatives are mixed mode cation/anion-exchangers and weak anion/cation-exchangers. At the moment, C_{18}

and mixed-mode (hydrophilic/lipophilic) extraction phases have been used in brain metabolomics [64, 86]. However, different new sorbent materials emerge fast, such as nanoparticles, molecularly-imprinted polymers (MIPs), metallic nanoparticles and metal-organic frameworks [74, 77, 87]. The MIP cartridges are only used in targeted studies because of their high selectivity [87].

Fractionation of samples provides the possibility to extract each fraction with a different selectivity. However, fractionation in still valuable aliquots is difficult when low sample volumes are acquired. The applicability on low-volume samples (20 µL plasma) was recently discussed by van der Laan et al. [88]. A comprehensive fractionation approach of serially coupled SPE columns (reversed phase, mixed-mode anion and cation exchanger) was developed and showed reduced ion suppression as well as increased sensitivity for multiple metabolite classes. This highlights an emerging need for multiplatform sample preparation protocols to enhance the metabolome coverage. In the future, the development of pre-fractionation techniques will be more prevalent [76]. The possibility of fraction analyses on different platforms will result in an increased metabolite coverage of studied samples and detection of novel markers with a higher certainty.

Derivatization strategies

Chemical derivatization, which includes altering the physicochemical properties of analytes by the introduction of a chemical moiety, is an emerging strategy for metabolomics studies [75, 89]. It can be used to allow, for instance, the separation and detection of compounds with differences in polarity, acidity/basicity and concentration using the same analytical method. Additionally, derivatization may be used to stabilize metabolites of interest by preventing oxidation, for example, of monoamine neurotransmitters.

Especially for GC-MS based metabolomics studies, chemical derivatization has been applied for several decades in order to improve volatility, separation and detection sensitivity of metabolites [90]. Here, typically a two-step procedure is executed, including oximation and silylation. Often reported issues with regard to this two-step derivatization procedure is the lack of reproducibility during batch derivatization. However, Miyagawa *et al.* [91] obtained reproducible peak areas for 52 selected metabolites using sequential derivatization and interval injection. Furthermore, silylation is however not suitable for some metabolites, such as amino acids, as unstable silylated derivatives are obtained. Silylation reactions require anhydrous reaction conditions, meaning that no water should be present in the sample. Alternatively, methyl chloroformate can be used as derivatization reagens for alkylation of polyfunctional amines and organic acids.

These alkylation reagents are inexpensive and the reaction occurs instantaneously without heat or water exclusion. However, most of them are toxic or hazardous.

Still, in non-targeted metabolomics, it is practically impossible to achieve (complete) derivatization for all compounds. Therefore, the optimization of the derivatization steps in GC is still an active research domain [92]. An already developed derivatization approach is known as solid-phase analytical derivatization (SPAD) [93]. Here, derivatization and SPE are combined resulting in a workflow which is less labour intensive and provides increased selectivity, automation possibilities and compatibility with a wide range of matrices. Moreover, recent developments in GC derivatization can be found in [90]. However, an extensive discussion of derivatization agents and procedures is out of the scope of this review and is already discussed in the aforementioned review of Beale *et al*. [90]. As general drawback, derivatization might lead to incomplete derivatized products, by-products and multiple derivatives which makes the structure elucidation process more complicated. Therefore, optimization and standardization is required for each application.

For LC-MS, chemical derivatizations with for instance dansyl chloride [92], benzoyl chloride [94] and dimethylaminophenacyl bromide (DmPABr) [95] have been successfully applied on low amounts of sample material, showing great potential for future brain metabolomics studies. Recently, a multi-platform study was published where relatively small CSF samples (30 µL) were derivatized using PITC (phenylisothiocyanate)-derivatization by a commercial Biokrates kit (BIOCRATES Life Sciences AG, Innsbruck, Austria), and analysed by NMR, LC-MS and GC-MS [96].

An interesting trend in derivatization strategies is ICD [44], where an isotopic labelled version of every derivatized analyte is produced. Using ICD, it is possible to overcome the dependence on isotope labeled analogues, which are not available for all metabolites and which are expensive. Another advantage of introducing a single internal standard for every metabolite is its large improvement in metabolite quantification [94, 95]. A drawback of ICD labelling is that the derivatization usually occurs after the sample pretreatment, which means that for the latter no correction by the internal standard recovery is made and thus needs to be accurate, efficient and standardized. For more information about ICD we refer to a recent review of El-Magrahbey *et al*.[44].

Mass spectrometry-based approaches for metabolomics of volume-restricted samples

MS is the most suitable detection technique for low-volume samples containing low concentrations of metabolites. Direct infusion-MS (DI-MS) would provide the highest throughput for analysing metabolites in (volume-limited) biological samples. However, a trade-off exists between sample throughput and metabolic coverage. Using DI-MS, matrix effects (i.e., compounds affecting each other's ionization efficiency) may compromise quantification, and in case of ion suppression of low-abundant metabolites it may result in a loss of relevant sample information as these compounds are not detected anymore. Sample preparation is therefore very critical when employing DI-MS for metabolomics studies. Moreover, DI-MS does not allow resolving isomeric compounds with identical fragmentation patterns. Therefore, most often, a separation technique is used before the sample compounds reach the detector. The separation technique needs to be suitable to analyse the low-volume samples with sufficient sensitivity. This brings the discussion towards miniaturized techniques and especially the inclusion of miniaturized columns and capillaries, as applied in LC, CE and GC. These approaches are considered in more detail in the following sections by devoting attention to advantages, trends and challenges for small-volume samples. DI-MS is not covered here as our survey of the scientific literature did not reveal that such an approach has been developed and/or used for metabolite analysis of volume-restricted *in-vivo* brain samples.

Miniaturized LC-MS and chip-based systems

LC-MS is still the most common applied platform in metabolomics. This technique is suitable to measure polar and apolar abundant metabolites in different sample matrices. However, due to the high flow rate, sensitivity issues often occur. Miniaturization of the LC part may overcome the sensitivity issues because of the lower flow rate required, resulting in less sample dilution and increased ionization efficiency [73, 97, 98]. The lower flow rate allows coupling LC with electron ionization (EI). EI is more robust and is less prone to matrix effects than electrospray ionisation (ESI) [97, 98].

Moreover, low volumes of reagents and samples are also required in miniaturized LC [73, 97], which therefore is favourable for the analysis of volume-restricted samples obtained in *in-vivo* brain metabolomics studies. The miniaturization in LC is driven by column adaptations resulting in capillary (i.d. 0.1-0.5 mm) to nano (i.d. < 0.1 mm) columns with reduced column dimensions [97, 98].

A further reduction in dimensions is achieved with open tubular columns (0.05-0.005 mm i.d.) [98, 99]. The possibilities of open tubular columns are widely studied and extensively described in reviews by Vasconcelos Soares Maciel *et al.* [98] and Mejía-Carmona *et al.* [99] . These columns show several advantages, such as higher efficiencies compared to capillary and nano columns, the possibility to work at higher flow rates without an increased pressure and less waste production. Their application in research fields, such as metabolomics, did not reach its climax due to the absence of commercially available open tubular columns for LC [99]. At the moment, a limited amount of companies develop these columns for LC, while research groups made many efforts to prepare their own open tubular columns in order to show their robustness, efficiency and superior performance.

The decreased column dimensions require some instrumental adaptations [73, 97, 98], which in turn can have beneficial effects. For example, the position of the ESI emitter is closer to the MS inlet in nano liquid chromatography nano electrospray ionization, resulting in an additional gain in sensitivity [73].

Besides this instrumental benefit, some challenges are also related to nano- and capillary LC instruments [73, 97]. Indeed, robustness issues, such as retention time shifts and reproducibility issues of the peak intensity, occur in these set-ups. Consequently, the number of papers using nano- or capillary LC for brain metabolomics is limited, while contradictorily it is often applied in brain proteomics [73, 97, 100]. In a study by Lin *et al.* [101] nano-LC-MS/MS is applied for proteomics and UHPLC-Time of flight (TOF) for metabolomics, in order to better understand Alzheimer's disease. In case of protein and peptide analysis, sensitivity issues may occur due to the presence of charge state distributions. The use of superchargers could be of interest to overcome this sensitivity problem. A few studies demonstrate the influence of supercharging agents on the charge state of neuropeptides [102, 103]. The exact mechanism of supercharging agents is not known yet but their use could be of interest in brain metabolomics to increase sensitivity, especially in the analyses of low volume-samples.

Due to the reduced column dimensions, emitters and connections are more prone to blockages and therefore the sample preparation is crucial in these miniaturized LC set-ups [73, 98]. Miniaturized sample preparations and preconcentration steps are already discussed in Section 3. Another, not yet, discussed on-line LC sample preparation approach is a column-switch set-up. This results in a rapid analysis, sample enrichment and purification and an extended coverage of the metabolome. This method was applied in [104] to study Alzheimer's disease in plasma using a nano-LC system with a nano pre-column for clean-up and preconcentration. The instrumental set-up and a chromatogram are shown in **Figure 3**. Actually this type

of column-switch set-up is not commonly applied anymore in brain metabolomics. This is the result of other upcoming techniques, such as in-tube SPME, which can be coupled to LC. More information about column switching instrumentation can be found in [105].

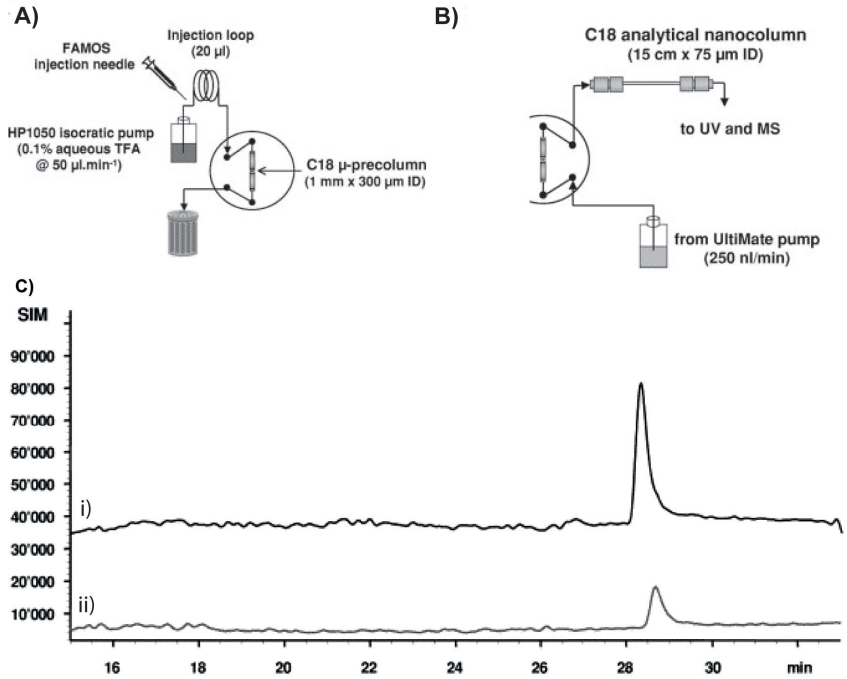

Figure 3. Column-switch instrumental set-up in capillary LC; A) sample clean-up, B) analytical separation and C) chromatograms of Aβ1-40 peptide are shown. In the upper part of (C) i) 20 µL of the solution was injected and compared with ii) 10 µL injection. **Figure**s are taken with permission from [68].

Newer approaches are pillar-array columns and chip-based liquid chromatography. Both are still not commonly applied in metabolomics, although they have severaladvantages. The efficiency of microchips is better due to a reduction of the void volume problems seen in nano- and capillary LC. However, there are still challenges related to the LC chip devices, such as low reproducibility [98, 106]. Researchers try to further integrate all compartments on a chip and some commercial LC-chip devices are already on the market. Examples are the HPLC-Chip/MS devices from Agilent and the more integrated system from Waters, Ion-Key, which will lead to more robust applications. The suitability of the micropillar array columns for proteomics is, among others, demonstrated by Tóth *et al.* [107]. Compared to packed bed columns, the pillar columns resulted in a better identification for peptides and proteins due to reproducible retention times.

The technique is considered exceptional because of the low back-pressure, even distribution of compounds, precision of the retention time and the long lifetime of the columns. Therefore, this type of miniaturized and chip-based approaches are of great interest for volume-limited metabolomics studies.

CE-MS and microchip CE-MS

CE-MS is a microscale analytical technique that has gained popularity for metabolomics studies in the last decade, mainly because of its complementary separation mechanism to chromatographic techniques, such as LC and GC. CE-MS can be considered an attractive tool for the analysis of volume-limited or scarcely available samples as only nanoliter injections are typically employed from just a few microliters of sample. Generally, a classical co-axial sheath–liquid interface is used for coupling CE to MS, but improvements in interfacing designs have emerged over the last decade [108], which mainly resulted in methods with an improved detection sensitivity.

Even though CE-MS is still an underrepresented technique in brain metabolomics studies, it was recently employed for the analysis of low-volume plasma samples from mouse disease models for epileptic seizures [68] and hemiplegic migraine [109], showing its potential for biomarker studies. However, a limitation of the low sample-loading capacity of CE (referring here to capillary zone electrophoresis), especially in conjunction with a classical sheath-liquid interface, is that the detection sensitivity can be compromised. Therefore, a number of on-line sample preconcentration methods have been developed to increase sample-loading volumes without hampering the intrinsic high separation efficiency of CE. Examples of in-capillary preconcentration strategies are pH mediated stacking, dynamic pH junction, field-amplified sample injection (FASI), field-amplified sample stacking (FASS), sweeping, large-volume sample stacking, transient-isotachophoresis and electrokinetic supercharging (EKS) [110]. Preconcentration based on dynamic pH junction already showed to be compatible with high-salt content urine samples, and the technique has recently been optimized for microdialysis samples [111]. In the method, only 5 µL of microdialysate was needed for direct CE-MS analysis (of which 291 nL was injected), thereby allowing multiple injections from the same sample vial, showing its potential to improve microdialysis sampling times. In another study, preconcentration based on electrokinetic supercharging was successfully coupled with ESI-MS/MS [112], thereby showing an about 5000-fold sensitivity improvement for neurotransmitters analysis as compared to a CE-MS method using a conventional hydrodynamic injection (**Figure 4**), reaching LODs as low as 10 pM. However, especially when performing electrokinetic injections, the sample nature is of critical importance for the selectivity and repeatability of

injections. Therefore, various chromatographic-based sample preparation systems coupled on-line or in-line to CE-MS have been developed [113]. Besides improved repeatability, also extra preconcentration could be achieved by adding an extra sample preparation step. With the ability to achieve these low detection limits, CE-MS could play a prominent role in future brain metabolomics studies.

Furthermore, the introduction of novel, high-throughput methodologies employing CE-MS has high potential for brain metabolomics studies. For instance, the introduction of multi-segment injection a few years ago greatly improved the throughput of CE-MS analyses (<5 min per sample), and even though it has not been used yet for brain fluids and/or tissues, it already showed its suitability for the metabolomic analysis in DBS samples [114]. Another study, focusing on increased throughput, presented a method where microdialysis was on-line coupled to CE in order to investigate branched chain amino acids as possible biomarkers [115]. Temporal resolution of microdialyis sampling was about 60s, and CE coupled to laser-induced fluorescence analysis was performed in less than 30s. This assay shows the potential of on-line coupling of brain-fluid sampling directly to CE for high-speed brain metabolomics.

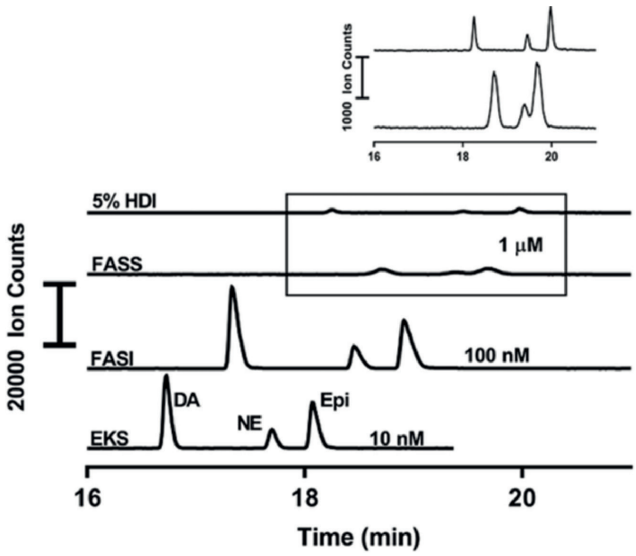

Figure 4. Comparison of electrokinetic supercharging (EKS) method with conventional hydrodynamic injection (HDI) and other common forms of preconcentration in CE-MS e.g. field-amplified sample injection (FASI) and field-amplified sample stacking (FASS). **Figure** is reused with permission from [76].

Another promising technique for brain metabolomics applications is microchip CE (MCE). In MCE, high-speed separations of ultra-low sample volumes can be performed while maintaining the high separation efficiency of CE. However, MCE coupled to MS is still at its infancy, and without the availability of commercial MCE-MS devices, this will remain an attractive research field [116].

GC-MS

GC-MS is the most efficient, robust and reproducible analytical platform in metabolomics. The combination with well-established libraries makes the technique the most widely used in metabolomics [75]. Two ionization sources are used to hyphenate GC with MS, EI and chemical ionization (CI) [90]. In metabolomics studies EI is more often applied than CI. As previously discussed, an advantage of using EI is that it is less prone to matrix effects. Another benefit of EI is that it will produce fragments of each analyte and the fragmentation is robust, which makes the data suitable to compare with known databases, such as for example National Institute of Standards and Technology (NIST).

The technique is applicable for the analysis of polar small molecules, for instance neurochemicals, present in different matrices, such as CSF, ECF and blood products (e.g. DBS). The study by Motsinger-Reif *et al*. [117] for example uses GC-TOF to analyse 30 µL CSF samples to find biomarkers to discriminate between Alzheimer's disease patients and control groups. In general, samples from brain metabolomics have volumes of approximately 40 µL or less. The low-volume samples can easily be analyzed by GC-MS thanks to the small open tubular capillary columns, where only 2 µL are necessary for injection [98].

The above mentioned sample preparation techniques, e.g. SPME and MEPS, can also be used for GC analysis [90, 118]. However, chemical derivatization is necessary to increase the volatility and thermal stability of the neurochemical products. Derivatization is already discussed in Section 3, while a more extensive discussion on different derivatization strategies (off-line, in-line and in-liner) and agents are described in [90]. Still most often a two-step off-line derivatization (including sylilation and oximation), performed on approximately 40 µL sample [91, 118], is applied in brain metabolomics [119]. The approach could also be suitable for lower volumes by adapting the volumes of derivatization agents. As already mentioned, it is necessary to investigate the most adequate derivatization procedure and combination of agents to obtain the best sensitivity and selectivity for targeted metabolites [90].

Nowadays there are few novel trends in GC instrumentation or column development. Recent applications consider the more novel sample preparation techniques and preconcentration of low-volume samples. Guntner *et al*. developed an in-house modified SPME device (**Figure 2D**) and coupled it to GC-MS for the analysis of propofol in microdialysates [120].

The future focuses on the development of miniaturized sample preparation/ preconcentration methods, of standardized protocols for temperature control in GC-MS analyses and of derivatization processes [119]. The 2D GC technique (GCxGC-MS) may result in an increased peak capacity (resolution power) leading to an improved spectral quality and sensitivity, the advances of GCxGC-MS for metabolomics is discussed in the recent review of *Higgins Kepler et al.* [121]. Increased sensitivity would be of interest for brain metabolomics to reveal low concentrated metabolites in the low volume samples. Although GCxGC-MS is already applied in different metabolomics studies, such as for the profiling of human serum samples of patients with a neurodegenerative disorder [122]. The analysis of the obtained complex data remains very challenging and time-consuming. Therefore, automation of the data analysis procedure needs to be further optimized [123]. The suitability of 2D GC for metabolomics has been demonstrated by Yu *et al.* [124] who optimized a 2D GC method for different matrices (tissue, serum and urine). The authors showed an increase in metabolite identification with their 2D method due to an improved separation of amongst others lactate and pyruvate. This was also seen in the aforementioned study of Winneke et al. [122]. They compared the analysis of GC-MS with GCxGC-MS, with the result of more detected peaks by the latter method.

Conclusions and perspectives

Brain metabolomics is a research area that has greatly gained popularity in last years. The ability to perform *in-vivo* metabolomic profiling provides valuable real-time information about the biological status of the brain, avoiding the risk of metabolite degradation, which happens when analysing *post-mortem* brain tissues. However, *in-vivo* neuromessenger analysis is still found to be a huge analytical challenge, mainly because of the poor accessibility to brain fluids, the highly invasive sampling and the wide range and fast fluctuation of neuromessenger concentrations.

Miniaturization of sampling probes and advances in microfluidics have led to major improvements in spatial and temporal resolution of brain-fluid sampling, drastically reducing tissue damage and sampling invasiveness. The miniaturization of plasma collection and the possibility of self-sampling for patients play also an important role in sample accessibility. As a result of the miniaturization of sampling strategies, which lead to lower sample volumes, miniaturized sample extraction strategies (micro-SPE) and mirco-extraction techniques (LPME and SPME) are more frequently applied for brain metabolomics purposes. In this regard, a trend towards the on-line coupling of micro-extraction techniques to analytical devices is observed, resulting in lower costs and higher throughput.

For the low-volume samples from brain metabolomics studies, microscale separation techniques, such as CE-MS, and nano- and capillary (UHP)LC-MS will probably play a more prominent role in the future. However, there is still room for improvements in the sensitivity and high throughput capabilities of these techniques. Other approaches to gain a better coverage of the metabolome, such as the coupling of different column chemistries in parallel or in series, and the inclusion of two- and multidimensional techniques are also considered. Multiplatform analysis is the only suitable way to cover in the broadest way the entire metabolome, which is perhaps the most challenging perspective for these limited-volume samples.

Another interesting feature of analyzing small amounts of sample is that large scale animal studies can be reduced as pooling across individual animals will no longer be required to achieve an analyzable sample, thus increasing the 3Rs credentials of metabolomics, i.e. Reduction, Refinement and Replacement of animal testing.

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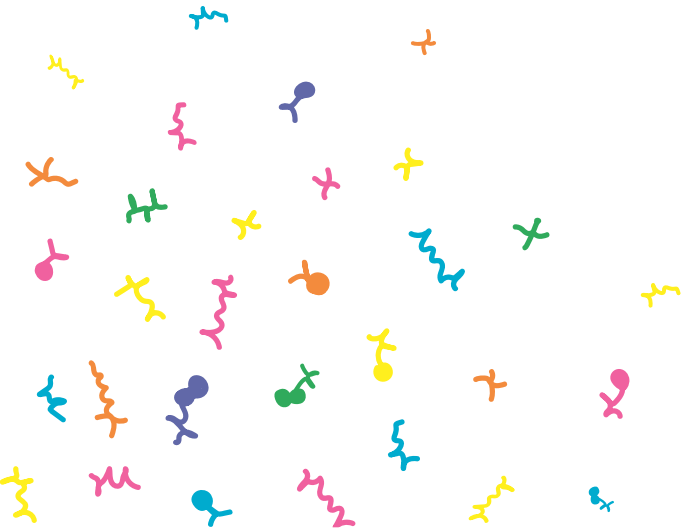

Chapter 3

Capillary electrophoresis-mass spectrometry at trial by Metabo-ring: effective electrophoretic mobility for reproducible and robust compound annotation

Nicolas Drouin*, Marlien van Mever*, Wei Zhang, Elena Tobolkina, Sabrina Ferre, Anne-Catherine Servais, Marie-Jia Gou, Laurent Nyssen, Marianne Fillet, Guinevere S.M. Lageveen-Kammeijer, Jan Nouta, Andrew J. Chetwynd, Iseult Lynch, James A. Thorn, Jens Meixner, Christopher Lößner, Myriam Taverna, Sylvie Liu, N. Thuy Tran, Yannis Francois, Antony Lechner, Reine Nehmé, Ghassan Al Hamoui Dit Banni, Rouba Nasreddine, Cyril Colas, Herbert H. Lindner, Klaus Faserl, Christian Neusüß, Manuel Nelke, Stefan Lämmerer, Catherine Perrin, Claudia Bich-Muracciole, Coral Barbas, Ángeles López Gonzálvez, Andras Guttman, Marton Szigeti, Philip Britz-Mc-Kibbin, Zachary Kroezen, Meera Shanmuganathan, Peter Nemes, Erika P. Portero, Thomas Hankemeier, Santiago Codesido, Víctor González-Ruiz, Serge Rudaz and Rawi Ramautar

*Authors contributed equally

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Abstract

Capillary zone electrophoresis-mass spectrometry (CE-MS) is a mature analytical tool for the efficient profiling of (highly) polar and ionizable compounds. However, the use of CE-MS in comparison to other separation techniques remains underrepresented in metabolomics, as this analytical approach is still perceived as technically challenging and less reproducible, notably for migration time. The latter is key for a reliable comparison of metabolic profiles and for unknown biomarker identification that is complementary to high resolution MS/MS. In this work, we present the results of a Metabo-ring trial involving 16 CE-MS platforms among 13 different laboratories spanning two continents. The goal was to assess the reproducibility and identification capability of CE-MS by employing effective electrophoretic mobility (μ_{eff}) as the key parameter in comparison to the relative migration time (RMT) approach. For this purpose, a representative cationic metabolite mixture in water, pretreated human plasma and urine samples spiked with the same metabolite mixture, were used and distributed for analysis by all laboratories. The μ_{eff} was determined for all metabolites spiked into each sample. The background electrolyte (BGE) was prepared and employed by each participating lab following the same protocol. All other parameters (capillary, interface, injection volume, voltage ramp, temperature, capillary conditioning and rinsing procedure, etc.) were left to the discretion of the contributing labs. The results revealed that the reproducibility of the μ_{eff} for 20 out of the 21 model compounds was below 3.1% vs. 10.9% for RMT, regardless of the huge heterogeneity in experimental conditions and platforms across the thirteen labs. Overall, this Metabo-ring trial demonstrated that CE-MS is a viable and reproducible approach for metabolomics.

Introduction

The *state-of-the-art* mass spectrometry (MS) instrumentation used in metabolomics typically provides a read-out of thousands of molecular features in a given biological sample within a single run when rigorous data filtering is not applied to reject a large fraction of spurious signals, redundant ions and background contaminants [125]. The annotation of these features to specific compounds is currently one of the key challenges in metabolomics and is often performed using library-based approaches, corresponding to annotation at various confidence levels according to international guidelines (e.g. Metabolomics Society) [126-129]. Ideally, only parameters presenting good reproducibility and low bias, such as exact mass, should be used and crossed to reach high identification confidence [127, 130]. However, other important parameters, such as retention time in liquid chromatography (LC), may lack consistency from one laboratory to another due to the difficulty to standardize all the operating parameters. This is particularly the case for the analysis of polar compounds using hydrophilic interaction liquid chromatography (HILIC) conditions where the chemistry and the age of the column, as well as the injection solvent and sample matrix including the preparation of the mobile phase, may yield variable conditions in separation. To tackle this challenge, several laboratories have resorted to experimentally building metabolomic libraries for compound identification purposes in-house, an often time-consuming and relatively expensive effort.

Capillary zone electrophoresis (CE) coupled to MS is highly suited for the profiling of polar and charged metabolites, notably for compound classes such as nucleotides, sugar phosphates, organic acids, nucleosides and amino acids [131-137]. In metabolomics, the use of CE-MS is considerably underrepresented in comparison to other analytical techniques [138, 139]. Over the past few years, various research groups have shown the utility of CE-MS for biomarker discovery studies using both large and small sample sets. For example, Harada *et al*. assessed the long-term performance of CE-MS for metabolic profiling of more than 8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52-month period [140]. Mischak and co-workers have profiled native peptides in more than 20,000 human urine samples by CE-MS with an acceptable interlaboratory reproducibility [141-144]. Onjiko *et al*. used CE-MS to phenotype cell types in single cells of developing frog embryos [145]. Very recently, CE-MS has shown good mutual agreement (mean bias <15%) for reliable quantification of various plasma/serum metabolites and fatty acids as compared to reversed-phase LC-MS and GC-MS[146, 147].Overall, all these studies demonstrate the usefulness and added value of CE-MS in the field of metabolomics.

However, the separation science community still perceives this analytical technique as technically challenging and less reproducible, especially in terms of migration time among comparative metabolic profiling studies using gas and LC [148-150].

Migration-time reproducibility is of utmost importance for reliable metabolomics. It aids comparison of metabolic profiles, including scrutinizing samples for subtle changes in profiles/patterns in comparative metabolomics studies, and facilitates the identification of unknown metabolites. In CE-MS analysis, variability in migration time arises from fluctuations in the electro-osmotic flow (EOF), temperature, physicochemical properties of solvents and the capillary, often due to frequently matrix-induced capillary surface interactions, among other factors. In contrast to chromatographic-based separation techniques, (open-access or commercial) software tools for effectively correcting shifts in migration times are still lacking, despite a high need to improve overall data robustness for enhancing analytical robustness. Nemes *et al*. employed non-linear time warping to reduce relative errors from ~5–10% to ~0.3% for migration times, thus substantially aiding metabolite identifications [151]. More recently, González-Ruiz *et al*. tackled this challenge by developing a software, called ROMANCE, which converts the migration time scale into an effective electrophoretic mobility (μ_{eff}) scale [152]. The approach demonstrated effective correction of EOF-caused shifts in migration times, albeit in a small cohort of samples, thus being able to improve the reproducibility of the migration index below 1.5%. The use of μ_{eff} raises a potential to aid compound identification in biological samples, notably when using metabolite libraries of electrophoretic mobilities. Such chemical libraries prove especially useful in CE, where migration times may exhibit higher variability.

In 2018, Drouin *et al*. published the first μ_{eff} database for 458 endogenous metabolites [153], as well as its use to identify compounds across different laboratories. However, the utility of this approach was examined only by a single individual responsible for preparing the BGE and analysing the samples in two different laboratories. Based on this study, we present the results of the Metaboring study in which μ_{eff} on migration-time reproducibility and identification capability in CE-MS-based metabolomics was assessed. The study encompassed 13 independent laboratories from academia and companies spanning 11 countries of 2 continents. All participants used the same batch of samples, consisting of a representative metabolite mixture, human plasma and urine (both matrices used at zero-, five- and tenfold diluted form) spiked with the same representative metabolite mixture. Each participating lab prepared and employed the same background electrolyte (BGE) on the basis of a protocol. All other parameters (capillary, interface, injection volume, voltage ramp, temperature, type of instrument used, and capillary conditioning and rinsing procedures, etc.) were left

to the discretion of the participating labs. The key parameters assessed by this Metabo-ring were the reproducibility of relative migration time (RMT) and the μ_{eff} across the laboratories, which was determined for a representative set of cationic metabolites in each sample.

Experimental section

Chemicals and reagents

Acetonitrile (ACN) and dichloromethane were purchased from Actu-All Chemicals (Oss, the Netherlands) and Biosolves (Valkenswaard, the Netherlands), respectively. Spermine, thiamine, choline, tryptamine, creatine, L-neopterin, trans-4-hydroxy-L-proline and inosine were obtained from Sigma Aldrich (Steinheim, Germany).L-arginine, L-isoleucine, L-leucine, L-tryptophan, L-proline, L-glutamine, L-lysine, histamine, adenosine and procaine were supplied from Fluka (Buchs, Switzerland). Agmatine, adenine, and serotonin were purchased from Alfa Aesar (Kandel, Germany). Paracetamol and nicotine were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Carl Roth (Karlsruhe, Germany), respectively. MS-grade water was provided through a Milli-Q Advantage A10 water purification system (Merck, Darmstadt, Germany).

Study design

The goal of this study was to assess the migration-time reproducibility of CE-MS for metabolomics and determine the most suitable approach for metabolite annotation by comparing RMT versus μ_{eff} in standardized condition of BGE. Changes in BGE composition (and thus pH and ionic strength) are one of the main sources of variability in separation in CE across laboratories. BGE in CE-MS-based metabolomics studies often use volatile buffers such as ammonium acetate/formate, acetic acid and formic acid [154-156]. Over the past few years, the use of 10% acetic acid gained interest for the efficient profiling of cationic metabolites by CE-MS [157-159]. This BGE is relatively easy to prepare and does not require pH adjustment. Moreover, in comparison to formic acid based BGE, it generates low CE currents, making it more suitable for the sheathless interface and also interesting for anionic metabolic profiling due to its slightly higher pH [160, 161]. Though it is not a strict buffer solution, this BGE can be used the whole day for analyses. Although the replenishment of the BGE is recommended before each analysis batch [162], it has been shown that 10% acetic acid can be conserved over an extended period, giving consistent μ_{eff} [152]. Therefore, the present work adopted 10% acetic acid (pH ~ 2.2) as the BGE, which was prepared independently in each participating laboratory.

For maximal impact, this interlaboratory study involved 20 different CE-MS platforms across 17 different laboratories. Each laboratory was provided with a set of 7 samples, including one cationic mix of 23 compounds in water (21 cationic metabolites and 2 internal standards, **Table S1**), and six samples prepared by mixing the standard solution with different levels of matrices extracted from human plasma and urine subjected to null, 5- and 10-fold dilution, separately. All the samples included paracetamol and procaine as markers for EOF time. To facilitate adoptability, our goal was to minimize or avoid modifications to routine practices of each lab by providing a freedom in experimental conditions and CE-MS instruments used, thereby making this study design unique in comparison to previous ring trials [163-165].

Because μ_{eff} is only dependent of the BGE composition, its preparation and the hydrodynamic injection mode without stacking were imposed to every participant. Stacking in CE is performed by changing the sample and/or separation conditions to induce a change in analyte velocity. In this study, all participants have been requested to use CE-MS separation conditions without implementing a stacking procedure, including the use of electrokinetic injection (the latter being selective to high-mobility compounds). Other parameters, such as capillary length, capillary diameter, voltage, pressure or ESI source parameters, were left to the discretion of each group. For minimal statistical treatment, every sample was analyzed in technical triplicates, and the injection order was at the discretion of the participants. The nature of each sample (i.e. matrix composition and a neutral marker for EOF time) was communicated to the different groups to guide procedures in capillary rinsing between runs and total acquisition time.

Sample solutions

Stock solutions of the analyte standards were prepared in water at a concentration of 1 mg/mL. Paracetamol was included at 3 mg/mL to aid signal detection for marking the EOF time. The stock solutions were stored at -20°C until usage. The standards were prepared by diluting the stock solutions in water to 20 µg/mL for every compound, except paracetamol (30 µg/mL). Pooled human plasma was obtained from Sanquin (Amsterdam, The Netherlands). In 2 mL Eppendorf tubes with 500 µL plasma, 1000 µL of ACN was added for protein precipitation, assisted by shaking for 5 min at 12,000 g on an orbital shaking table. After centrifugation at 12,000 g for 5 min at 4°C, 1800 µL of supernatant was later collected from each tube and combined together. The combined supernatant was then split into aliquots of 1800 µL, which were evaporated to dryness using a SpeedVac and reconstituted with 600 µL of the standard solution. 400 µL of dichloromethane was mixed with 500 µL of the reconstituted samples. After agitation for 5 min, 450 µL

of the aqueous phase was collected and filtered through centrifugal ultrafilters with a 3 kDa cutoff membrane from Millipore (Milford, MA, USA) at 12,000 g for 2 h at 4°C. The filtrates from different tubes were combined together. The resulting contaminants concentrations (excluding lipids and proteins) of this solution were supposed to be identical to the raw plasma. Five- and 10-time dilutions of this filtrate were prepared via dilution using the water-based standard solution. Finally, the three samples containing different levels of extracted plasma matrix were split into 100 µL aliquots and stored at -80 °C until shipment over dry ice.

The pooled urine samples were obtained from a group of healthy volunteers. In order to produce a sufficient number of samples, multiples aliquots were prepared in parallel. Briefly, 1000 µL of pooled urine was evaporated to dryness with a SpeedVac and then reconstituted with the standard solution in water. The reconstituted samples were ultra-filtrated through a 3 kDa cutoff membrane at 12,000 g for 2 hours at 4°C, followed by the merging of all the filtered solutions. The resulted solution contains the same contaminant content as raw urine. Five- and 10-times dilutions were prepared similarly as plasma-based samples via dilution in the standard solution. Finally, the three urine-based samples were split into 100 µL aliquots and stored at -80 °C until shipment in dry ice.

BGE preparation

Every participating laboratory used 10% acetic acid as the BGE, prepared following the same protocol to aid reproducibility. Briefly, approximately 80 mL of MS grade water was first added in a 100 mL volumetric flask, followed by the addition of 10 mL acetic acid (MS grade).The BGE preparation was completed by further adding water till the gauge line.

Instrumentation and procedures

Seventeen laboratories, with 20 different CE-MS platforms in total, were involved in this interlaboratory study, of which 11 systems employed a sheath-liquid (SL) interface and 9 a sheathless interface for coupling CE to MS. Among them, 10 platforms used a sheath-liquid interface based on a coaxial tube interface from Agilent Technologies. Except for one platform using CESI 8000 plus CE system with a sheathless CE-ESI interface, all the other platforms with a coaxial sheath-flow ESI interface used a custom-built CE-ESI platform or the Agilent 7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany). The SL interface was compatible with large variety of operational conditions, therefore a variety of protocols were used during this study. Fused silica capillary with internal diameters of 40 or 50 µm and different lengths (from 60 to 105 cm) were used with the sheath-liquid interface across the laboratories. During this study, each lab

used their own (preferred) sheath-liquid composition and flow-rate. The sheath-liquid-based CE systems were coupled to various types of mass spectrometers from different manufacturers, namely, the 6510, 6540, 6550, 6560 QTOF, 6230 TOF, and 6490 QqQ from Agilent (Santa Clara, CA) as well as the Impact HD and maXis-3G QTOFMS from Bruker (Bremen, Germany). The other CE-MS system consisted of a homemade CE system and a custom-built low-flow sheath-liquid interface coupled to a Bruker Impact HD QTOF (Bremen, Germany). On this platform a fused silica capillary of 40 µm internal diameter and 105 cm length was used [166, 167], and the flow-rate of the sheath-liquid was 0.6 µL/min, whereas in commercial sheath-liquid interfaces, the sheath-liquid is typically provided at a flow rate in the range from 3 to 10 µL/min.

The remaining 9 platforms made use of a sheathless interface with a porous tip, which was coupled to MS via a Nanospray source. This interface obtained from AB Sciex (Brea, CA), employed a silica capillary with 30 µm internal diameter and 91 cm length and required a CESI 8000 plus CE system also provided by AB Sciex. The CESI interface is compatible with a large variety of nanoESI sources. Consequently, in addition to AB Sciex mass spectrometers (TripleTOF 6600, 5600, QTrap 6500, 6500 plus QQQ), the CESI interface has been used during this study on some other MS brands, such as Bruker (MicrOTOF-Q II and Impact qTOF) and Thermo Fisher (Q-Exactive HF). Whereas the sheath-liquid interfaces were used with short ramp up of voltage (from 1 to 0.5 min), due to its original design, the sheathless interface required longer voltages ramp for sake of current stability (from 1 to 2 min). Apart from the large variety of instrumentation used, each laboratory used their own capillary conditioning and washing procedure. Both capillary and room temperatures used during analyses by different labs ranged from 20 to 25 °C. More details about the experimental conditions used with each platform are given in Table S2.

Data processing

Initially, 17 groups were involved in this Metabo-ring trial. One data set was triaged as peak widths about 5 min at baseline were obtained which is unusual for capillary electrophoretic separations of small molecules. Therefore, this data set was excluded from further data processing. The primary MS data were received and centralized as manufacturer raw data and processed using Skyline [168]. The data files from Bruker instruments were converted into mzML format using MSConvert [169] prior to their import into Skyline.

For robust and transposable results from one laboratory to another, a two-marker conversion was used for μ_{eff} determination using an in-house software. Paracetamol (μ_{eff} = 0 $mm^2\ kV^{-1}\ min^{-2}$) and procaine (μ_{eff} = 1559 $mm^2\ kV^{-1}\ min^{-1}$) were

used as references for μ_{eff} calculation [160, 170]. For accurate μ_{eff} measurements, the migration time has to be considered from the start of voltage application. Therefore, when necessary, the migration time was corrected to synchronize the MS acquisition with the voltage start. For the same purpose, the voltage ramps have also been considered during the conversion into mobility [170]. RMTs were calculated using procaine as reference compound. Bias represents the deviation of a measured value (X_m) (here RMT or μ_{eff}) to a reference (X_r). For this purpose, the average μ_{eff} and RMT (**Table S1**) values measured from standard solution were used as reference or baseline value for bias calculation (**equation 1**).

$$Bias\ (\%) = \frac{X_m - X_r}{X_r} \quad (1)$$

When possible, leucine and isoleucine were processed as two distinct peaks, otherwise the same values were attributed to both compounds.

Results and discussions

Relevance of standardization of BGE preparation

The present work adopted 10% acetic acid (pH ~ 2.2) as the BGE, which was prepared independently in each participating laboratory. **Figure 1** shows an electropherogram obtained for the analysis of a standard mixture of cationic metabolites by sheath-liquid CE-MS using 10% acetic acid as BGE, clearly indicating that a high separation efficiency is provided by the use of this BGE.

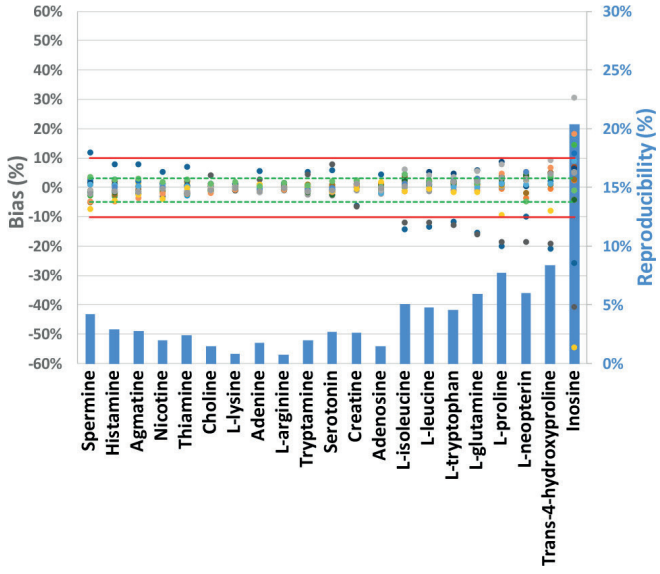

Figure 1. Typical profile obtained for the analysis of a standard mixture of cationic metabolites by CE-MS (platform 19, see **Table S2**) using 10% acetic acid as BGE.

Repeatability and reproducibility were quantified based on the data collected from 19 CE-MS platforms (**Figure 2**).The intra-laboratory repeatability of the measurements of both μ_{eff} and RMT was typically below 1% for the test compounds in water for three consecutive analyses.

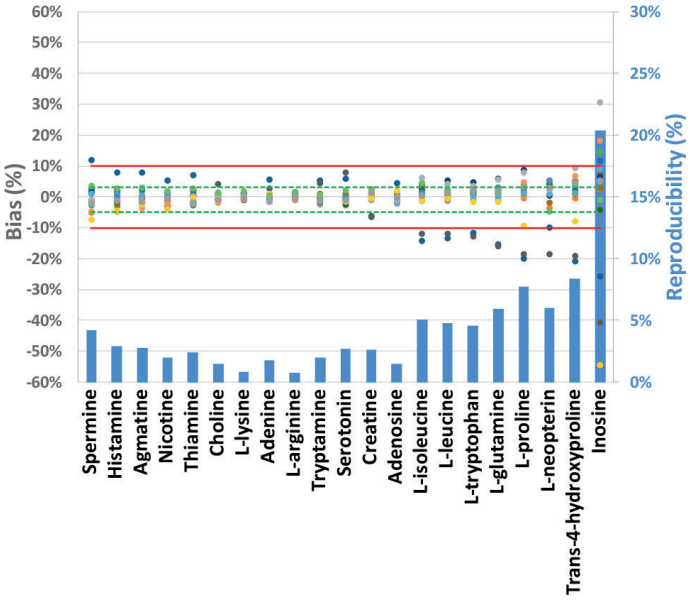

Figure 2. Individual bias to the average and interlaboratory reproducibility of μeff as obtained for the analysis of cationic metabolite standards by 19 CE-MS platforms. Green and red lines mark bias thresholds at ±5 and ±10%, respectively. Metabolites are presented in decreasing mobility order from left to right.

The average reproducibility of these parameters was about 4.3% and 5.7%, respectively, with a maximum observed for inosine in both cases. Therefore, these findings clearly indicate the presence of an significant contribution of the interlaboratory variability, leading to higher values of relative standard deviation (RSD) for reproducibility. To find the main source for the observed variation, we determined the bias of each compound as measured by each individual analytical platform. **Figure 2** reveals a relatively high degree of variation, especially for the slower-migrating compounds (longer migration times). This is expected since for a constant measurement uncertainty, the relative error increases as the reference values get closer to 0, as it is the case in the μ_{eff} scale (**Table S1**).

Interestingly, three data sets presented a lower μ_{eff} for the late-migrating compounds (i.e., L-isoleucine, L-leucine, L-tryptophan, L-glutamine, L-proline, L-neopterin, trans-4-hydroxyproline and inosine) as compared to the data obtained by the other 16 CE-MS platforms (**Figure 2**). Since μ_{eff} is theoretically influenced by the physicochemical properties of the BGE only, this phenomenon was explained by

variation in BGE preparation. Indeed, slow-migrating cationic compounds usually present a low electrical charge due to their basic functions with low pKa or due to presence of an acidic function with high pKa. Consequently, their μ_{eff} is greatly influenced by any slight variation of the acidity of the BGE. Therefore, with a pKa of 2.74, inosine was the slowest compound and also the compound with a relatively high μ_{eff} variability. Consequently, results from these 3 platforms were triaged from further data analysis in the rest of the study (**Table S2**).

Effective electrophoretic mobility *vs* relative migration time

It is relatively feasible to mitigate variations in migration time resulting from the BGE with added control over buffer composition. While it is possible to control suction effect from the ionization source, it may be difficult to tackle the adsorption of matrix components onto the inner capillary wall [152, 171, 172]. To correct for these sources of variability, the most popular method is the use of RMT [164, 173, 174]. This approach consists of comparing the velocity of the analyte ($v_{analyte}$) to that of an internal standard (v_{IS}). Both velocities are explained by the electrophoretic movement of BGE and their electrophoretic movement (μ_{EOF} and $\mu_{eff,}$ respectively) under an electric field (E) as well as the hydrodynamic movement of the BGE ($v_{hydrodynamic}$), such siphoning between the position of the inlet and the outlet of the CE capillary tip, the sheath flow surrounding of the CE capillary in the ESI source, the close-by vacuum of the atmospheric interface of the mass spectrometer, and pressure that may be applied during the separation. As expressed in **equation 2**, the hydrodynamic phenomena exert an additive effect instead of a proportional one and, therefore, they cannot be accurately corrected by a ratio approach, making the RMT approach intrinsically incorrect.

$$RMT = \frac{v_{analyte}}{v_{IS}} = \frac{E \times (\mu_{eff_{analyte}} + \mu_{EOF}) + v_{hydrodynamic}}{E \times (\mu_{eff_{IS}} + \mu_{EOF}) + v_{hydrodynamic}} \quad (2)$$

Correction based on RMT can be powerful for compounds migrating in proximity to the internal standard as they are subject to the same variation of the hydrodynamic phenomenon. However, this approach has limited ability to correct the migration of compounds far from the internal standard [152]. On the other hand, μ_{eff} depends on the hydrodynamic radius (r), the ionic charge of the compounds (q) and the viscosity of the BGE (η) (**equation 3**).

$$\mu_{eff} = \frac{q}{6\pi \cdot \eta \cdot r} \quad (3)$$

Therefore, μ_{eff} is independent of BGE velocity and is only influenced by its physicochemical properties. This parameter is measurable using a neutral marker combined with the experimental conditions.

In this study, we have chosen for a conversion based on two markers (c.a. procaine and paracetamol) in order to obtain μ_{eff} values which are independent of the operational conditions across the laboratories, such as capillary length, electric field and temperature [170, 175].

Indeed, our measurements confirmed the viability of using μ_{eff} as depicted in **Figure 3A**, the μ_{eff} presented a bias below 3.1% for all compounds except for inosine, while for the same set of metabolites, RMT showed bias up to 11%. For compounds such as tryptophan, μ_{eff} presented a variation up to 5-times lower than the corresponding RMT. Interestingly, while the reproducibility of μ_{eff} remained stable in a range from 0.8 to 3.1% for compounds with high and medium μ_{eff}, RMTs trended with migration time. Indeed, as shown in **Figure 3B**, minimal dispersion of the RMT values was observed for the compounds with migration time similar to that of the IS, whereas metabolites with extreme RMT presented with a high interlaboratory variability.

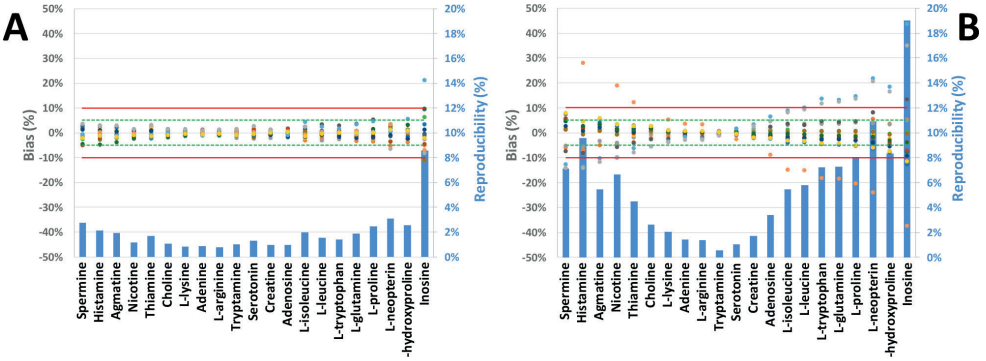

Figure 3. Individual bias and interlaboratory variability of μeff (A) and RMT (B) as determined for the analysis of standard metabolite mixture by 16 CE-MS platforms. The green and red lines denote a 5% and 10% threshold, respectively. Metabolites are presented in decreasing mobility order from left to right.

As the approach using the in-house metabolite library is based on the relative error to a reference value (i.e. the average μ_{eff}), it is important to observe the bias of each parameter. As shown in **Figure 3A**, excluding inosine, 98% of the μ_{eff} measurements are within a 5% bias limit, while 6 platforms presented RMT outside those limits (**Figure 3B**), with the maximal bias up to 28%. These results confirm the theoretical normalization power of the μ_{eff} conversion of MT in comparison to RMT, especially in the context of creating an interlaboratory database.

Influence of sample composition

High chemical complexity in plasma and urine are known to challenge molecular identifications and quantification due to matrix effects as well as instrument performance and longevity. In CZE, it is well known that the ionic strength in the sample zone influences the migration speed of the compounds at the start of the

separation. Therefore, high salt concentrations in such complex biological samples can lead to lower signal-to-noise ratios due to peak broadening and distortions in peak shapes and shifts in migration indices (migration time, RMT, and μ_{eff}).

While a "dilute and shoot" approach is viable for urine, deproteinization is usually necessary for plasma performed before analysis as preventive measures. Deproteinization is mostly performed in organic solvents (e.g., methanol or acetonitrile), followed by rigorous vortex shaking and an evaporation-to-dryness of the supernatant after which the dried extract is reconstituted into an appropriate solvent and volume for the follow-up instrumental analysis [176]. However, these purification steps do not adequately address high salt content in urine and plasma.

To evaluate the influence of sample conductivity, samples with different dilutions of urine and plasma matrices were analyzed. These concentrations represented an equivalent of undiluted, 5- and 10-times dilution of the biological fluids. As shown in **Figure 4**, the μ_{eff} reproducibility remained globally unaffected irrespective of the type and concentration of the biological matrix. However, some trends of bias can be observed when platforms are considered individually for each metabolite. Fast-migrating compounds (e.g., spermine, histamine, agmatine and nicotine) presented a bias up to 18% in both undiluted plasma and urine samples (**Figures 4A and B**). As highlighted in **Figures 4C, E** and **D, F** for μ_{eff} and RMT, respectively, these deviations to the baseline decrease with concentration of the matrix (see dilution factor).

We explain these variations by deformations of the peak for the 4 fastest-migrating metabolites. With migration in a close proximity to the sodium peak, these compounds experience peak broadening. In some extreme cases, the ion suppression induced by sodium can suppress a part of the peak leading to an incorrect detection of its apex (**Figure S1**). As shown in **Figure 4C and E**, with the exception of inosine, 15 platforms presented a bias below 5% and one with a bias below 7% in 5-times diluted plasma. Further dilution of the samples (10-times) led to bias below 5% for all platform and compounds, except for inosine due to slight variation in BGE composition as discussed above. Concerning urine, results were highly similar with only one CE platform presenting a bias above 5% from samples diluted 5-fold, excluding inosine. However, as shown in **Figure 4F,** one platform presented higher deviation for spermine compared to more concentrated samples (**Figure 4D**). No explanation has been found so far to explain this deviation in the 10-fold diluted samples while no drift was observed in the 5-fold dilution of urine.

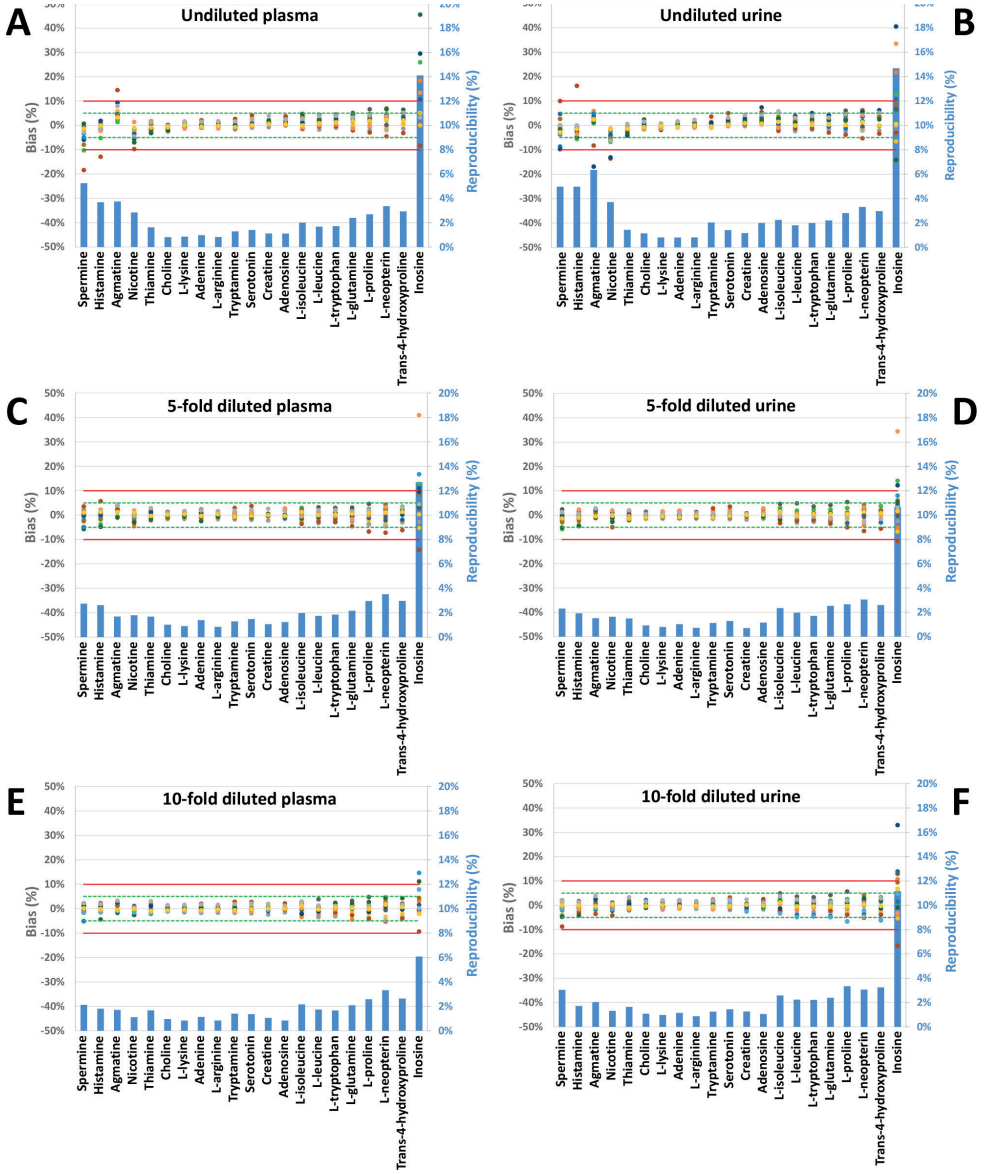

Figure 4. Individual bias and interlaboratory variability of μeff for cationic metabolites in complex biological matrixes: plasma (A, C, and E) and urine (B, D, and F) at three different concentrations (undiluted: A and B; 5-times dilution: C and D; 10-times dilution: E and F) by 16 CE-MS platforms. Metabolites are presented in decreasing mobility order from left to right.

Proposed: metabolite identification guidelines based on a common library

This work demonstrated the utility in of an interlaboratory CE-MS database to enhance metabolomics. Using standardized BGE conditions (10% acetic acid here), conversion from migration times to μ_{eff} was found to efficiently normalize separation between 13 different laboratories with interlaboratory variability below 3.1% for 20 of the 21 compounds considered.

For sake of comparison with gold standard in feature annotation in metabolomics, collisional cross section are among the most reproducible parameters with interlaboratory variation in the range of 2% [160, 165, 177] and 5% is usually as threshold for identification confirmation [130, 178]. Although HILIC is a powerful method to separate polar compounds, deviation of the retention time up to 10% to in-house database are usually considered for feature annotation [130].

As a first approach, we propose that such a scoring system can be based on different ranges of bias between μ_{eff} measurements and μ_{eff} values in databases (Table 1). Such databases should be built on a reliable manner, taking into consideration multiple technical replicates, performed on multiple laboratories, and involving different instruments. Using 10% acetic acid as the BGE, the deviation to the consensus value below 10% was easily achieved for most metabolites in complex biological matrices. By using a system suitability test based on a short panel of compounds with different migration speeds, we envision it possible to test the BGE and assess whether it is performing conformed to the database. For example, our data here exemplified μ_{eff} measurements with a bias below 5%, thus provided an added piece of information to credential compounds and aid identifications (**Table 1**). Further, credentialing may consider electrophoretic migration for added fidelity.

Table 1. Proposition of Confidence Levels for Annotation of Features Using μeff by CE-MS

Confidence level	>100 mm^2 kV^{-1} min^{-1}	<100 mm^2 kV^{-1} min^{-1}
High	< 5%	< 10% or ± 10
Medium	5% < x < 10%	10% < x < 20% or ± 20
Low (rejected hypothesis)	>10%	>20% or > ±20

As the mobility of slow-migrating compounds is more dependent on the differences of physicochemical properties of the BGE, conversion to μ_{eff} overestimates relative bias and reproducibility. For instance, in the case of inosine, μ_{eff} presented a

considerably low absolute deviation on all platforms with a maximum deviation about ± 10 mm^2 kV^{-1} min^{-1} (**Table S1**). Therefore, the use of absolute thresholds in μ_{eff} [153] may enhance identification confidence for slow-migrating metabolites. In the presence of standardized experimental conditions, the normalization to and absolute use of μ_{eff} appears to provide an added piece of compound-dependent information to aid the identification of metabolites.

Next to the μ_{eff}, this study also highlights the annotation capability of RMT, comparable to retention time in HILIC [130]. Although the use of RMT shows interlaboratory variability and bias below 10% for a very large majority of the compounds and platforms, it has to be used cautiously since few platforms presented very large deviations (up to 30%).

Conclusions and perspectives

This study encompassing 13 laboratories, 16 platforms, spanning 11 countries, and 2 continents, found CE-MS a robust and reproducible technology for metabolomics. Despite major variations in experimental conditions, CE instruments and methods, CE-ESI ion sources, and even users, conversion of migration times into μ_{eff} reduced variability from 10.9% on RMT to 3.1% in μ_{eff} scale using the same BGE composition. Tabulating μ_{eff} under specific BGE compositions into universal database adds another compound-dependent and reliable value, thus complementing traditional MS-MS/MS databases in metabolomics (e.g., HMDB[179] and Metlin[180]). The use of μ_{eff} requires only a limited number of internal standards (e.g., two different compounds used here) while substantially enhancing compound identification in metabolomics via a targeted or non-targeted approach. Based on these results, we propose here guidelines by using a scoring approach based on different μ_{eff} criteria to support feature annotation in metabolomics. Although this study focused on cationic small molecules only, we anticipate that this approach also is easily extendable to anionic metabolites, including but not limited to small organic acids, nucleotides, and sugar phosphates [153]. As LC-based separations are often challenged for such compounds, we anticipate this Metabo-ring trial to invigorate the use of CE-MS with the μ_{eff}-based approach in metabolomics and other fields.

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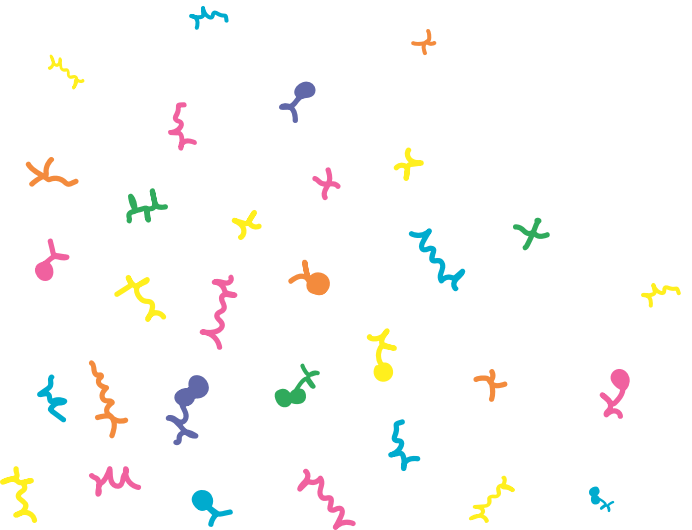
Supporting information

Additional information is available as stated in the text. This information is available free of charge via https://pubs.acs.org/doi/full/10.1021/acs.analchem.0c03129



Chapter 4

Direct profiling of endogenous metabolites in rat brain microdialysis samples by capillary electrophoresis-mass spectrometry with on-line preconcentration

Marlien van Mever, Karen Segers, Nicolas Drouin, Faisa Guled, Yvan Vander Heyden, Ann van Eeckhaut, Thomas Hankemeier, Rawi Ramautar

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Abstract

Metabolic profiling of body fluids from small animal models is often used in (translational) biological studies in order to obtain insight into the underlying molecular mechanisms of (complex) diseases. An example is the use of brain microdialysis samples from rats to study neurological disorders by means of a metabolomics approach. From an analytical point of view, the profiling of (endogenous) metabolites in rat brain microdialysates is challenging because of the limited sample volume for both sample preparation and injection, notably in longitudinal studies. In this work, we have assessed the analytical performance of capillary electrophoresis-mass spectrometry (CE-MS) for the direct profiling of endogenous metabolites in rat brain microdialysates, i.e. without using any sample preparation or derivatization. An on-line preconcentration procedure with sample stacking, which was fully compatible with the high-salt concentration in microdialysates, was used to significantly improve the detection sensitivity of the CE-MS method for metabolic profiling. A response surface methodology, applying a Box-Behnken design, was considered to determine the optimal conditions for preconcentration. A linear response (R^2>0.99) for selected metabolites in the concentration range from 0.05 to 10 μM was obtained in perfusate samples. Interday relative standard deviation (% RSD) values for peak area and migration time were 2.6-19% and below 3.8%, respectively. Limits of detection ranged from 11 to 284 nM when employing an injection volume of about 291 nL, corresponding to 17% of the total capillary volume. The utility of the CE-MS approach was demonstrated by the direct profiling of endogenous metabolites in rat brain microdialysates. At least 48 compounds could be analyzed of which 25 were provisionally identified and quantified.

Introduction

Metabolomics, i.e. the global profiling in biological samples of (endogenous) molecules with a molecular mass below 1500 Dalton, represents one of the most recent introduced 'omics' techniques. Elucidating the structure and dynamics of metabolic pathways provides essential information regarding the underlying mechanisms of the biological system concerned [181]. In the central nervous system (CNS), the exact pathological mechanisms for many neurological diseases have not yet been completely elucidated. In addition to genomics and transcriptomics, brain metabolomics offers an alternative tool for unveiling new insights into the complex structure and function of the brain and thereby plays an important role in the discovery of new therapeutics and treatments for neurological diseases [20, 21, 47].

Brain microdialysis is a widely used approach in neuroscience for the dynamic monitoring of brain neurochemistry via the collection of both endogenous and exogenous small molecules in freely moving animals. Rodents, like mice or rats, are often used for this purpose [182]. Until now, reversed-phase liquid chromatography (RPLC) in combination with classical detection methods or mass spectrometry (MS), has been most often used for the analysis of compounds in rat or mouse brain microdialysates. The analysis of low-molecular mass biomolecules in microdialysates by standard RPLC-based methods has shown to be analytically challenging, mainly due to the intrinsically low sample volumes of rat and mouse brain microdialysis samples, but also because the low concentrations of bioactive compounds in the CNS. Therefore, the collection times of microdialysis samples often have to be prolonged in order to produce enough sample material for analysis by the conventional analytical techniques, resulting in poor temporal resolution of microdialysate sampling [53, 183]. Instead of conventional RPLC columns, miniaturized (nano, capillary or microbore) RPLC-based approaches in combination with fluorescence or electrochemical detection can be considered for the selective analysis of neurotransmitters in volume-limited samples. However, when using RPLC-based approaches, a derivatization step is generally needed in order to obtain sufficient retention for polar and charged metabolites, in particular for amino acids and monoamine neurotransmitters [52, 94, 184-187].

Capillary zone electrophoresis (CE) is a very strong analytical tool for the highly efficient analysis of polar and charged compounds in biological samples without using any derivatization, as the separation mechanism of CE is based on charge-to-size ratios. Another advantage of CE is that only small sample amounts are required for both the injection (nL-range) and for the sample vial (i.e., <10 µL is sufficient to perform multiple injections), which makes it an attractive tool for the

analysis of volume-limited or scarcely available samples, such as microdialysates from animal models. Especially, CE employing laser-induced-fluorescence (LIF) detection has greatly improved the possibility for high-temporal resolution monitoring of neurotransmitters in microdialysates [188-190]. For example, CE-LIF has shown to be a very useful tool for on-line high-speed separations of amino acids and amines in rat brain microdialysates [115, 191]. However, when employing CE-LIF, derivatization is required in order to introduce a fluorophore moiety to the compounds, an approach which is not amenable to all metabolites. Moreover, when it comes to identification of compounds, the selectivity of LIF is limited.

In this work, the aim was to assess for the first time the performance of CE coupled to MS via a sheath-liquid interface for the profiling of endogenous metabolites in rat brain microdialysis samples without performing any derivatization or sample preparation. In order to enable the detection of as many endogenous metabolites as possible in rat brain microdialysis samples, an on-line preconcentration procedure was applied. For this purpose, various sample preconcentration techniques can be used, such as pH-mediated stacking, dynamic pH junction, transient-isotachophoresis, counterflow focusing and sweeping [192,187]. Especially, dynamic pH junction has shown to be very effective for the preconcentration of zwitter-ionic and basic compounds, such as amino acids and amines, in human urine and cerebrospinal fluid (CSF) using minimal sample preparation, allowing their analysis in the nanomolar range by CE-MS, thereby showing an about 10-fold sensitivity improvement [193-195]. Therefore, this strategy was also considered in this study and further optimized employing a Box-Behnken Design (BBD). The final CE-MS method allowed the reliable quantification of endogenous metabolites in rat brain microdialysate using direct sample injection. Moreover, the developed approach permits multiple injections from the same volume-restricted microdialysis sample, thereby allowing repeatability studies with scarcely available samples.

Experimental section

Chemicals and reagents

All chemicals used were of analytical grade or higher purity. Acetic acid (99-100% *m/m*), methanol and isopropanol were purchased from Biosolve (Valkenswaard, The Netherlands). Hydrochloric acid (37% *m/m*) was from Thermo Fisher Scientific (Waltham, MA, USA). Ammonium hydroxide (28–30%) was acquired from Acros Organics (Amsterdam, the Netherlands). Amino acids standards, such as glycine, serine, leucine, isoleucine, glutamine, aspartic acid, glutamic acid, methionine, phenylalanine, arginine, tyrosine and gamma-amino butyric acid (GABA), were purchased from Sigma-Aldrich (Steinheim, Germany). ^{13}C and/or ^{15}N stable-isotope labeled (SIL) internal standards were purchased from Cambridge Isotope

Laboratories (Apeldoorn, the Netherlands). A Milli-Q Advantage A10 water purification system (Merck, Darmstadt, Germany) was used to obtain pure water. Background electrolyte (BGE) solution was prepared by diluting acetic acid in water (10% *v*/*v*). As sheath-liquid, a mixture of water and isopropanol (50:50, *v*/v), containing 0.03% acetic acid was used [196]. Ammonium hydroxide (28–30%) was diluted in water to the desired concentrations (5%, 12.5% and 20%).

Sample solutions

Metabolite standards (10 mM) were dissolved in a mixture of water:acetonitrile (95:5, *v*/*v*) containing 0.5% formic acid. Standard metabolite mixtures of 27 compounds (**S-1**) were prepared in water as 50 µM stock solutions, aliquoted and stored at -80 °C. Internal standards (glutamine-C^{13}, lysine-C^{13}, aspartic acid-C^{13}, valine-C^{13}, glutamic acid-C^{13}) were prepared in BGE as 50 µM stock solutions, aliquoted and stored at -80°C. An internal standard stock aliquot was thawed the day of use, and diluted in BGE to the desired concentration (10 µM). Before injection, samples in water, perfusate or microdialysate were diluted at a 1:1 (*v*/*v*) ratio in BGE containing internal standards. The final concentration of internal standards was 5 µM.

Microdialysis sample collection

Intracerebral baseline samples from male albino Wistar rats (Charles River Laboratories, L'Arbresle, France) were collected for 2 hours. The perfusion fluid was composed of modified Ringer's solution (147 mM NaCl, 2.3 mM $CaCl_2$, 4 mM KCl) at a flow rate of 2 µL/min. Samples were collected with a temporal resolution of 20 min and split into two aliquots of 15 µl. All rats had a weight between 250-300 g. The animals were acclimatized for at least 1 week to the animal facility before implanting the microdialysis probe (CMA12/1 mm) in the nucleus tractus solitarii, as described previously [197]. Their housing was at a constant temperature (24°C), 12-hr light-dark cycle and with free access to food and water. All procedures are according to the National and European guidelines for animal experimental research and were approved by the Ethical committee for Animal Experiments of the Vrije Universiteit Brussel, Belgium (project 174-213-9: 01/02/2014-01/02/2018 and project 18-213-1: 01/02/2018-01/02/2020) [197-199].

Instrumentation and procedures

The CE-MS experiments were carried out on a 7100-capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany) hyphenated with a 6230 TOF, which was also from Agilent (Santa Clara, CA, USA). Fused-silica capillaries with an internal diameter of 50 µm were purchased from BGB Analytik (Harderwijk, The Netherlands). They had a total length of 90 cm for analysis. New capillaries

were conditioned by subsequently rinsing, at 5 bar for 1 minute, with methanol, water, sodium hydroxide 1M, water, hydrochloric acid 1M, water, hydrochloric acid 0.1 M, water and background electrolyte (BGE). Injections were performed hydrodynamically. Injected volumes were calculated with Zeecalc v1.0b (https://epgl.unige.ch/labs/fanal/zeecalc). A small plug of ammonium hydroxide was first injected using an injection volume of ~12 nL (13 s 50 mbar), followed by a sample injection volume of ~291 nL (327 s 50 mbar). CE-MS coupling was realized via a co-axial sheath-liquid ESI interface equipped with a standard triple-tube sprayer. Sheath-liquid was delivered at a flow rate of 3 µL/min by an Agilent 1260 Infinity Isocratic Pump (Agilent Technologies) with a flow splitter that splits the sheath liquid in the ratio 1:100. MS experiments were acquired in positive mode between *m/z* 65 and 1000 with an acquisition rate of 1.5 spectra/s. The nebulizer gas was set to 0 psi, while the sheath gas flow rate and temperature were set at 11 L/min and 100 °C, respectively and both were nitrogen. The ESI capillary voltage was adjusted to 5500 V. Fragmentor and skimmer voltages were set at 150 V and 50 V, respectively. MassHunter version B.06.00 (Agilent, Santa Clara, CA, USA) was used for data acquisition, instrument control and data treatment [196, 200]. From the sheath-liquid, isopropanol ($C_3H_8OH^+$) and its clusters ($[(C_3H_8O)_2+H]^+$ and $([(C_3H_8O)_3+H]^+$) with corresponding *m/z* values of 61.0647, 121.1223 and 181.1798, respectively, were used as lock masses. Data treatment was performed using MassHunter Workstation Quantitative Analysis. Peak extraction was performed with a mass error of 20 ppm and peak integrations were visually inspected to ensure correct integration.

Determination of analytical performance characteristics

The response function of the measured peak area for 28 metabolites was examined using 3 replicates at 7 different concentrations over a concentration range from 0.05 to 10 µM. As internal standard, SIL [$^{13}C_2$]-glutamine (5 µM) was used. The limits of detection (LOD) and quantification (LOQ) were determined by the standard deviation of y-residuals (s) and the slope (*b*) of the calibration curve as 3s/*b* and 10s/*b*, respectively [201]. Repeatability (expressed as percentage relative standard deviation, % RSD) for peak area and migration time (with and without normalization to an internal standard) were determined by analyzing in triplicate amino acids standard mixtures and perfusate samples spiked with amino acids. The last experiment has been repeated on three consecutive days to determine interday precision. Matrix effects were assessed using the standard addition method, by comparing the peak areas of amino acids standards prepared in modified Ringer's solution to amino acid standards prepared in water [202].

Experimental design

The on-line preconcentration procedure was optimized using response surface methodology (RSM). Preconcentration was based on a pre-injection of ammonium hydroxide prior to the sample injection. Therefore, sample injection volume (A), ammonium hydroxide pre-injection volume (B) and ammonium hydroxide concentration (C) were investigated at three levels (-1, 0, +1) using a BBD (Table 1). A total of 15 experiments, i.e. 13 design experiments with three replicate runs at the center point, were executed to estimate the response surface using Design Expert Software (S-2) (version 12, Stat Ease Inc., MN, USA). The experiments were carried out in a random sequence to minimize bias and to reduce the outcomes of unpredicted variability in the responses. The responses considered signal intensity (y_1), peak width (y_2) migration time (y_3) and resolution between isoleucine and leucine (y_4) were fitted into second order polynomial **equation 1**, given below:

$$y = \beta_0 + \sum_{i=1}^{f} \beta_i x_i + \sum_{1 \le i \le j}^{f} \beta_{ij} x_i x_j + \sum_{i=1}^{f} \beta_{ii} x_i^2 \quad (1)$$

where y is the response; β_o, β_i, β_{ij} and β_{ii} are the regression coefficients of the intercept, linear, two factor and quadratic terms, respectively; x_i and x_j represent the independent variables and f the number of factors.

Interpretation of the effects was performed by both statistical and graphical evaluation using the Design Expert software. The significance of the model equations, individual factors and factor interactions were evaluated by the analysis of variance (ANOVA) at a confidence interval (CI) of 95% in order to determine the most important factors for sample preconcentration and determine potential interaction effects between factors. Graphical evaluation of the developed quadratic models was obtained by construction of two-dimensional (2D) contour plots and three-dimensional (3D) response surface plots. The optimal values of the variables for sample stacking using ammonium hydroxide were obtained using Derringer's desirability (D) function shown in **equation 2**. Responses signal intensity (y_1), peak width (y_2), migration time (y_3) and resolution between isoleucine and leucine (y_4) were transformed by a linear transformation onto a desirability scale (between 0 and 1); y_1 and y_4 had to be maximized, while y_2 and y_3 had to be minimized. The global desirability value was determined using the Design-Expert software by modelling D in a polynomial model resulting in representative 2D contour plots.

$$D = (d_1 \cdot d_2 \cdot d_3 \cdot d_4)^{1/4} \quad (2)$$

Results and discussion

The analysis of low-volume biological samples, such as body fluids from small animal models, may provide valuable insight into complex diseases. CE-MS has shown to be a very useful technique for the profiling of metabolites in volume-restricted biological samples, such as urine from mice and sweat from infants requiring minimal sample preparation [203, 204]. Therefore, in this work, our aim was to assess for the first time the performance of CE-MS with a sheath-liquid interface for the efficient and sensitive profiling of endogenous metabolites in rat brain microdialysis samples without employing any sample preparation or derivatization.

CE-MS method development

For direct metabolic profiling of rat brain microdialysates by CE-MS, an on-line preconcentration procedure would be highly advantageous as many metabolites could be present at very low concentrations, often depending on the perfusion flowrate and sampling time employed for microdialysis. Dynamic pH junction showed to be an effective way for the preconcentration of amino acids and related compounds in urine samples [194, 205, 206]. Therefore, this on-line preconcentration technique was optimized in a systematic way in this work using a multivariate optimization approach based on BBD results. BBD was specifically selected since it requires fewer experiments than a central composite design (CCD) (i.e. 13 different experiments for BBD vs 15 for CCD to examine 3 factors), and it does not include runs where all factors are at their extreme settings [207].

Earlier studies that evaluated this type of on-line sample stacking in CE for the analysis of high-salt biological matrices revealed that three experimental parameters have a significant effect on the efficiency of the sample preconcentration, i.e., sample injection volume, ammonium hydroxide pre-injection volume and ammonium hydroxide concentration [195, 205, 206]. Therefore, these parameters were selected as factors in the BBD. Factor values, stated as levels, as listed in **Table 1**, were also selected in accordance to these previous studies.

Table 1. Factors and their factor values (stated as levels) in Box-Behnken Design

Independent factors	Levels		
	-1	0	+1
A: Sample injection volume (nL)	89	265	442
B: Ammonium hydroxide pre-injection volume (nL)	0.5	10.5	20.5
C: Ammonium hydroxide concentration (%)	5	12.5	20

As a starting point, aspartic acid and GABA were selected, as they are a slow and fast migrating compound, respectively, under the employed CE separation conditions. The structural isomers isoleucine and leucine were also selected in order to determine whether their electrophoretic separation could be maintained. Since the goal was to develop a rather fast CE-MS method with high detection sensitivity, without losing separation efficiency, selected responses were signal intensity, peak width and migration time. Additionally, the separation efficiency of the method was evaluated by determining the resolution between isoleucine and leucine.

Earlier studies revealed that the amount of ammonium hydroxide necessary for efficient sample preconcentration differed between water-dissolved samples and spiked urine samples [205]. This is most probably due to the difference in salt concentration (i.e. conductivity) and pH between the two sample types. Therefore, the effect of the on-line preconcentration parameters was examined for two sample types: 1) amino acids standards in water, and 2) amino acids spiked into microdialysis matrix. As microdialysis matrix, perfusate, or (modified) Ringers solution, was used, mainly because of the scarce availability of the valuable/ precious rat brain microdialysis samples. Before injection, samples in water or perfusate were diluted in BGE containing internal standards at a 1:1 *(v/v)* ratio. To check whether the amino acid profiles were comparable between the two sample types, CE-MS results of three BBD runs (3, 5 and 12) were evaluated (**Figure S-3**). The effect of sample preconcentration was comparable in terms of peak shape and migration order for amino acids either dissolved in water or spiked into perfusate. However, sample preconcentration was somewhat more efficient for perfusate, as the resolution between isoleucine and leucine was 5-70% higher and the separation window was 23-72% shorter, which could be due to the high salt concentration in perfusate, potentially resulting in salt-induced transient isotachophoresis in the sample zone [208] and a slightly more effective dynamic pH junction as the pH of perfusate is higher than that of water. Overall, comparable amino acid profiles were obtained in both cases and, consequently, for simplicity, amino acids standards in water were used for the BBD analyses

BBD experiments were carried out and experimental data is listed in **S-4**. Model coefficients for all responses were determined by regression analysis and shown as equations 3–9 (**S-5**). ANOVA results (**S-6**) show that the ammonium hydroxide pre-injection volume significantly affects all responses, and thus can be considered as the most important factor for the efficiency of sample stacking. In previous research, Tak *et al.* [29] observed a dependence of sample injection volume on ammonium hydroxide volume for the peak shape and separation efficiency of different amino acids. When using multi-factor optimization such as response surface modelling,

possible interaction effects are taken into account [209, 210]. The 3D surface plots (**Figure 1**) show interaction effects for signal intensity of A) GABA and B) aspartic acid and of C) resolution between isoleucine and leucine. Two-factor interaction effects can be assumed from these plots. Two-factor interaction effects can be seen in response surface plots if the behavior (effect) of the first factor is different at the low and high levels of the second (and vice versa). For instance, in the first two GABA plots this clearly can be observed. 3D surface plots for peak width and migration time are shown in **S-7A** and **S7-B**, respectively. As can be seen in both the ANOVA results and the 3D surface plots, indeed interactions seem to play a role for peak shape and separation efficiency, which is in agreement with earlier findings.

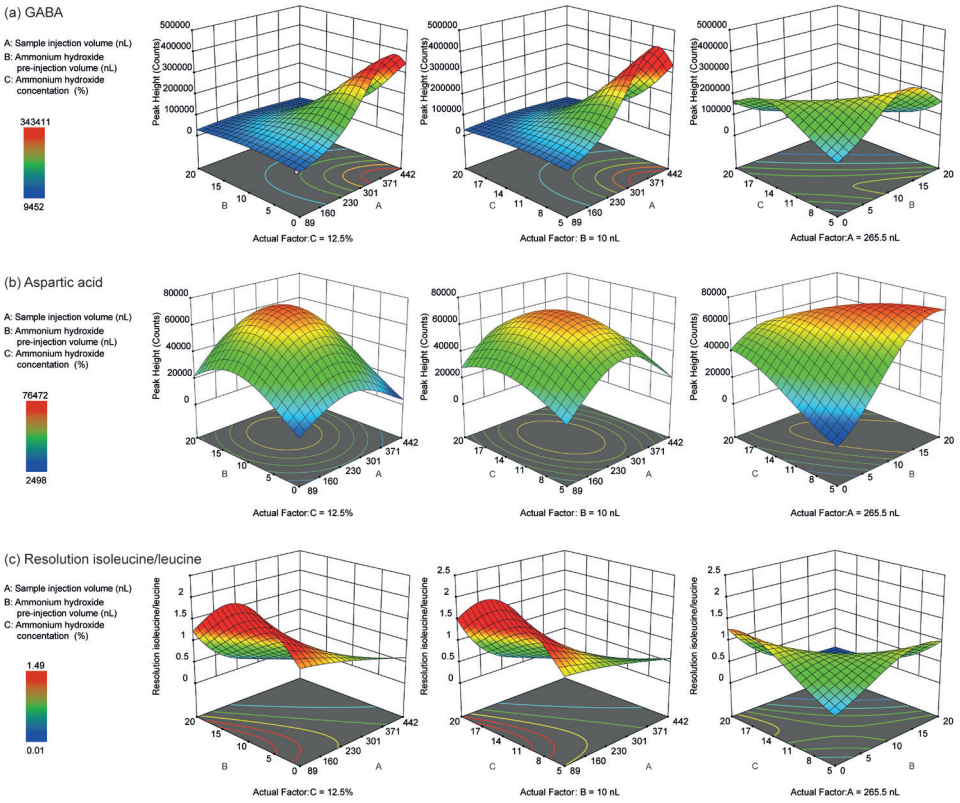

Figure 1. 3D response surface plots showing the signal intensity as determined by CE-MS for A) GABA, B) aspartic acid and C) the resolution of the isomers isoleucine/leucine as a function of (left) sample injection volume (A), and ammonium hydroxide pre-injection volume (B), (middle) sample injection volume (A) and ammonium hydroxide concentration (C) and (right) ammonium hydroxide concentration (C) and ammonium hydroxide pre-injection volume (B). The third factor is kept constant at the value given below the plot.

To identify the optimal conditions for sample stacking, Derringer`s desirability multi-criteria decision making was applied (as shown in equation 2) [211]. The goal of this optimization was to find a set of conditions with the best compromise

between the individual goals, i.e. maximize signal intensity, minimize peak width, minimize migration time and maximize resolution. Desirability plots combining these goals are shown in **S-8**, where a higher D-value denotes a more favorable option. An optimal response was achieved with a sample injection volume of 270-300 nL, an ammonium hydroxide pre-injection volume of 12-18 nL, and an ammonium hydroxide concentration of about 5%.

Two optimal parameter settings (option 1. sample injection volume: 291 nL, ammonium hydroxide pre-injection volume: 12 nL, ammonium hydroxide percentage: 5%; and option 2. sample injection volume: 291 nL, ammonium hydroxide pre-injection volume: 16 nL, ammonium hydroxide percentage: 5%) were tested for the amino acid test mixtures (5 µM) comprised of 12 amino acids (including GABA, glycine, arginine, valine, aspartic acid, serine, glutamine, phenylalanine, methionine, glutamic acid, isoleucine and leucine) with different physico-chemical properties. Only the ammonium hydroxide volume was varied, since this factor should have the largest impact on stacking performance as was determined earlier. The analysis was performed for both amino acids standard mixtures and perfusate samples spiked with amino acids, and the corresponding extracted ion electropherograms (EIEs) are shown in **S-9** for options 1 and 2. When looking at the amino acid profiles, it can be stated that for both sample types a larger pre-injection volume (solution 2) shows slightly higher signal intensities (about 0.2-14%) in perfusate. However, the resolution between isoleucine and leucine is 43% lower compared to a lower pre-injection volume (i.e., 0.9 vs. 1.4). For this reason, solution 1 was considered as best and thus used for follow-up studies.

Analytical performance evaluation

After determining the optimal conditions for on-line preconcentration with sample stacking, the analytical performance of the CE-MS method was further evaluated using amino acids standard mixtures and perfusate samples spiked with amino acids (10-5-1-0.5-0.1-0.05 µM) by establishing calibration curves in order to assess the linearity range, LODs, LOQs in the different matrices, as well as migration time and peak area precision. A metabolite mixture composed of 28 compounds was spiked into perfusate and analyzed (**S-1**). SIL glutamine (5µM) was used as internal standard.

A linear response (and with R^2>0.99) for selected metabolites in the concentration range from 0.05 to 10 µM was obtained in perfusate samples. Linearity was evaluated by a lack-of-fit (LOF) test [212, 213]. The method yields linearity for all test compounds. Detection limits ranged from 6.2 to 70 nM for amino acid standards dissolved in water and from 11 to 284 nM for amino acids spiked

into perfusate (**Table 2**). In general, higher LODs and LOQs were obtained for the compounds spiked into perfusate samples, which is due to matrix effects as explained in the final part of this section. Precision of the CE-MS method for direct profiling of metabolites was assessed based on the repeated analyses of perfusate samples spiked with amino acids at one concentration level (5 µM). Intraday RSD values (*n*=3) for peak areas and migration times of all analytes were better than 22% and 1.3%, respectively, while interday RSDs (*n*=9) were below 19% and 3.8%, respectively (**Table 3**). By using relative migration times (RMT), thereby using internal standard procaine as the migration time marker, instead of migration times, interday RSD values for RMTs were below 1.5% (**S-10**). Given that perfusate was analyzed directly by CE-MS, the obtained **Figure**s of merits for repeatability could be considered as highly favorable and acceptable for comparative metabolic profiling studies. **Figure 2** clearly shows that all compounds in perfusate could be analyzed with a high separation efficiency (i.e., plate numbers per meter were above 300,000 for all compounds) and a good detection sensitivity. Under these conditions, the structural isomers isoleucine and leucine were separated with a resolution of 1.4.

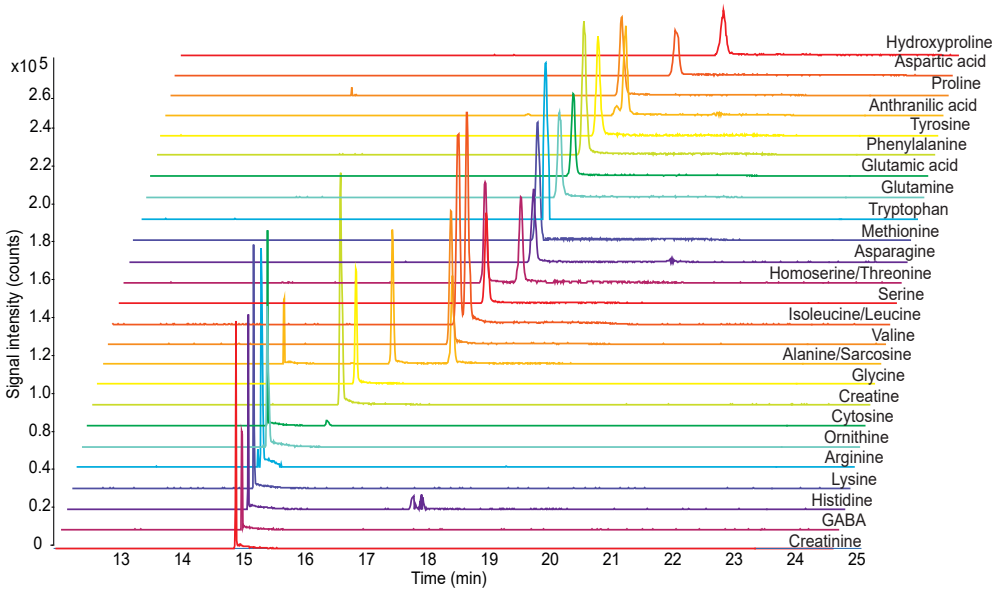

Figure 2. Extracted-ion electropherograms obtained by CE-MS from the analysis of 28 metabolites (5 µM) spiked in perfusate. Separation conditions: BGE, 10% acetic acid; sample injection volume 291 nL; ammonium hydroxide (concentration: 5%) pre-injection volume, 12 nL.

Table 2. Summary of linear range, the F-value of the Lack-of-fit (LOF) test (Fcrit, 95% = 3.26), limit of detection (LOD) and limit of quantification (LOQ), of a six-point calibration for metabolite standards and metabolites spiked in perfusate as obtained by CE-MS. See section 2 for experimental conditions.

Analyte	Water				Perfusate		
	Range (nM)	*LOF*	*LOD (nM)*	*LOQ (nM)*	*LOF*	*LOD (nM)*	*LOQ (nM)*
Glycine	50-10000	0.242	11	37	0.151	42	142
Alanine	50-10000	0.113	22	75	0.445	35	116
Sarcosine	50-10000	0.760	16	53	0.372	19	63
GABA	50-10000	0.142	19	64	0.120	49	164
Serine	50-10000	0.103	15	51	0.267	33	109
Cytosine	50-10000	0.167	25	85	0.001	27	89
Creatinine	50-10000	0.098	22	72	0.042	35	116
Proline	50-10000	0.607	70	234	0.352	24	79
Valine	50-10000	0.159	49	162	0.900	15	51
Threonine	100-10000	1.066	31	103	0.464	87	290
Homoserine	50-10000	0.510	42	139	0.247	29	97
Hydroxyproline	50-10000	0.325	44	148	0.167	11	37
Creatine	50-10000	0.035	20	66	0.188	18	59
Isoleucine*	50-10000	0.002	33	110	0.906	29	96
Leucine*	50-10000	0.034	28	92	0.438	25	82
Asparagine	50-10000	0.953	26	87	0.589	50	166
Ornithine	50-10000	0.078	25	83	0.049	45	149
Aspartic acid	100-10000	0.986	56	188	0.267	61	202
Anthranilic acid	100-10000	0.074	35	117	0.621	77	256
Glutamine	50-10000	1.176	18	58	1.924	14	46
Lysine	100-10000	1.384	15	51	0.127	84	282
Glutamic acid	50-10000	2.044	16	54	0.298	20	67
Methionine	50-10000	0.310	7.5	25	0.009	17	58
Histidine	50-10000	0.012	8.9	30	0.047	12	39
Phenylalanine	50-10000	0.233	20	68	0.513	16	54
Arginine	50-10000	0.166	6.2	21	0.158	55	184
Tyrosine	50-10000	2.962	16	54	0.256	36	120
Tryptophan	500-10000	0.481	33	110	0.057	284	946

* isoleucine and leucine were only partially separated

Subsequently, the effect of matrix interferences was evaluated. Especially, when injecting biological sample without using any sample pretreatment, ion-suppression can occur when matrix substances co-migrate with the analytes of interest. In order to assess matrix effects, the standard addition method was applied. The amino acid standards spiked into perfusate were analyzed and the results were compared to the standard solutions in water. Results for 12 amino acids with varying physico-chemical properties are shown in **Figure 3**. Both the calibration curves of amino acids in standard solutions in water and in perfusate were linear in the concentration range up to 10 µM. The slopes were different for all compounds, except for aspartic acid. The slopes in perfusate are lower than in water, resulting in higher LOD and LOQ values.

Table 3. Intra- and interday precision (expressed as %RSD values) of migration time (MT) and peak area (Area) obtained for metabolite standards and for metabolites spiked in perfusate (5µM).

Analyte	Water				Perfusate			
	RSD % MT		*RSD % Area*		*RSD % MT*		*RSD % Area*	
	Intraday (n=3)	Interday (n=3)	Intraday (n=3)	Interday (n=3)	Intraday (n=3	Interday (n=3)	Intraday (n=3)	Interday (n=3)
Glycine	2.9	3.3	5.4	9.6	0.91	2.8	4.7	5.3
Alanine	3.0	3.4	4.3	6.0	0.83	2.9	3.3	5.4
Sarcosine	3.2	3.6	2.7	5.1	0.73	3.1	2.8	6.5
GABA	2.5	3.2	2.8	3.3	1.3	2.6	22	17
Serine	3.2	3.6	3.7	6.7	0.67	3.2	3.2	4.4
Cytosine	2.4	3.1	4.6	4.4	1.3	2.6	13	14
Creatinine	2.5	3.2	5.7	4.6	1.2	2.6	13	11
Proline	3.4	3.8	2.4	5.8	0.58	3.6	1.2	6.5
Valine	3.2	3.6	4.5	6.0	0.76	3.1	0.9	6.6
Threonine	3.3	3.6	3.2	6.1	0.65	3.3	1.2	3.9
Homoserine	3.2	3.5	1.8	6.1	0.68	3.2	1.7	4.5
Hydroxyproline	2.8	3.2	5.3	6.4	0.89	2.7	3.0	2.6
Creatine	2.8	3.2	6.4	7.0	0.89	2.7	3.0	3.4
Isoleucine*	3.1	3.5	5.8	6.1	0.71	3.1	3.2	9.7
Leucine*	3.1	3.5	5.2	6.2	0.71	3.1	4.5	9.9
Asparagine	3.3	3.7	2.5	6.0	0.59	3.3	2.0	4.1
Ornithine	2.5	3.1	5.3	5.8	1.2	2.7	11	16
Aspartic acid	3.5	3.9	2.5	6.0	0.52	3.8	8.8	8.1
Anthranilic acid	3.3	3.7	4.1	3.7	0.52	3.5	1.1	8.1
Glutamine	3.3	3.7	2.8	7.2	0.61	3.4	2.3	4.5
Lysine	2.5	3.1	2.8	5.3	1.3	2.6	5.5	15
Glutamic acid	3.3	3.7	5.6	7.0	0.61	3.4	2.1	4.7
Methionine	3.3	3.7	3.8	4.9	0.62	3.4	1.3	6.4
Histidine	2.5	3.1	2.1	5.3	1.8	2.6	7.5	19
Phenylalanine	3.3	3.7	3.4	4.8	0.58	3.4	2.3	8.6
Arginine	2.5	3.1	2.0	4.2	1.2	2.6	8.5	12
Tyrosine	3.3	3.7	2.3	4.9	0.59	3.5	2.0	4.7
Tryptophan	3.2	3.6	1.3	3.8	0.59	3.3	0.68	5.1

* isoleucine and leucine were only partially separated

This clearly indicates that all metabolites (apart from aspartic acid) experience a matrix effect, which was more pronounced for fast migrating compounds as they migrate close to the salt plug (MT~14.0-15.5 min), which can be seen in S-11, where a TIE is shown. Therefore, in order to allow a reliable quantification of metabolites in microdialysis samples, calibration curves need to be constructed in perfusate to account for matrix effects.

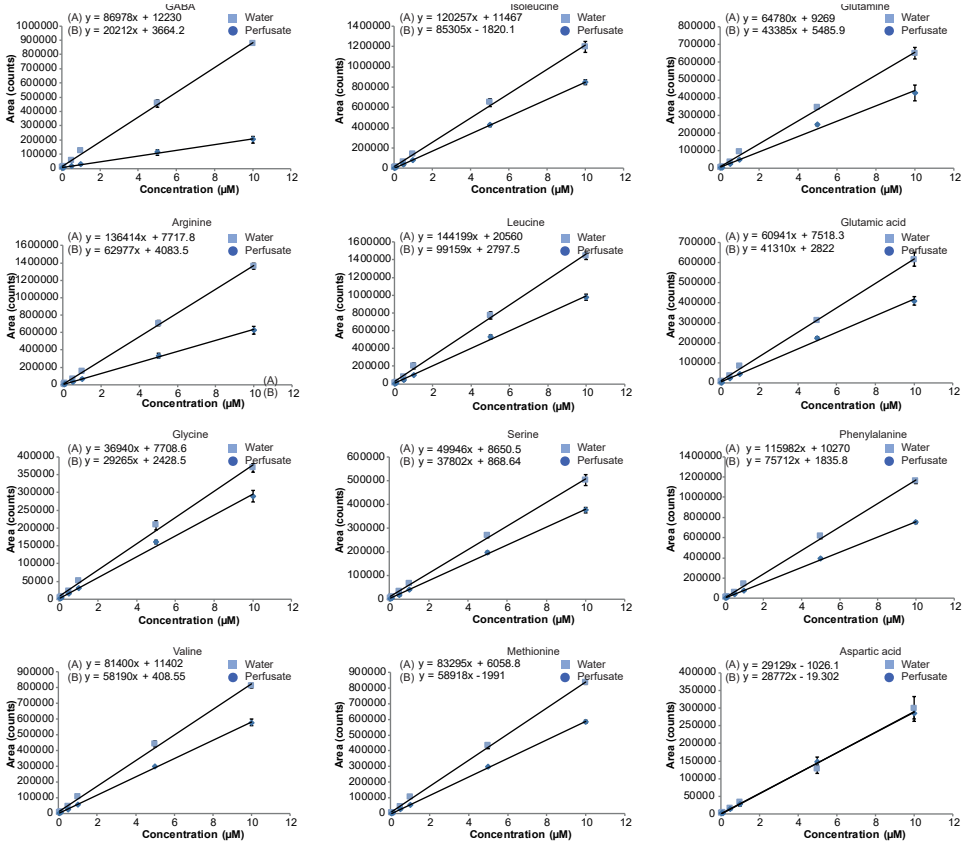

Figure 3. Calibration curves for (A) amino acid standards in water and (B) amino acids spiked in perfusate. The standard concerned is indicated above the **Figure**. Separation conditions: BGE, 10% acetic acid; sample injection volume 291 nL; ammonium hydroxide (concentration: 5%) pre-injection volume, 12 nL.

Metabolic profiling of rat brain microdialysates

The utility of the CE-MS method for the direct profiling of endogenous metabolites in rat brain microdialysates was demonstrated by the analysis of a basal rat brain microdialysis sample of about 10 µL, which was diluted with BGE (1:1, *v/v*) and stable isotope labeled (SIL) internal standards were added (lysine-C^{13}, aspartic acid-C^{13}, valine-C^{13}, glutamine-C^{13}, glutamic acid-C^{13}). The total sample volume was divided in three equal portions and each portion was analyzed 4 times, where injection from the same sample vial was performed. Before and after the microdialysis sample analysis, a calibration curve in perfusate was run for quantification purposes and also to check the performance of the CE-MS method.

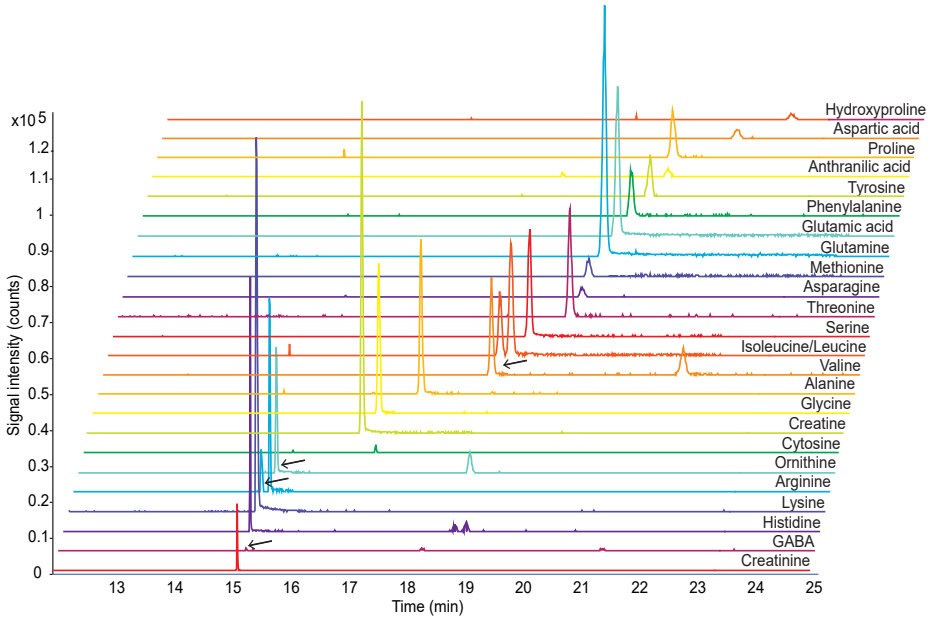

Figure 4. Extracted-ion electropherograms obtained by CE-MS from the analysis of endogenous metabolites in basal rat brain microdialysate. Separation conditions: BGE, 10% acetic acid; sample injection volume 291 nL; ammonium hydroxide (concentration: 5%) pre-injection volume, 12 nL.

The number of detected compounds was manually determined in the *m/z* range from 65 to 1000, where only peaks with a detection response above 500 counts were included (except for GABA and cytosine). In total, 48 compounds were detected in rat brain microdialysis (**Table 4**). Peak identification in microdialysis samples was carried out by comparing migration times and *m/z* values with those of the metabolite standards, and 25 compounds could be identified (**Figure 4**). The repeatability was determined for all identified compounds in microdialysis samples using twelve repeated analyses. For all analytes, RSD values for peak areas (not corrected by an internal standard) and migration times were better than 20% and 1.4%, respectively.

Comparing concentrations of metabolites determined in microdialysis samples with literature values is difficult as (small) variations in the microdialysis process between labs may have an impact on the actual metabolite concentrations present. Still, for the compounds GABA and glutamate we could perform such a comparison with one previous study in which a microdialysis probe was employed for the collection of sample from the same brain region [21].

Table 4. An overview of compounds detected by CE-MS in a rat brain microdialysis sample. Separation conditions: BGE, 10% acetic acid; sample injection volume, 291 nL; ammonium hydroxide (5%) pre-injection: 12 nL.

Identified compounds				
Detected *m/z*	**Analyte**	**RSD % MT**	**RSD % Area**	**Average concentration ± %RSD (nM) (n=12)**
76.0393	Glycine	1.1	8.0	2984 ± 8
90.0550	Alanine	1.1	16	1837 ± 15
104.0706	GABA	1.3	26	59 ± 14*
106.0499	Serine	1.0	3.8	2693 ± 5
112.0505	Cytosine	1.2	26	28 ± 10*
114.0662	Creatinine	1.4	20	351 ± 24
116.0706	Proline	1.0	3.2	1257 ± 4
118.0863	Valine	1.1	2.9	1422 ± 3
120.0655	Threonine	1.1	4.5	2151 ± 4
132.0655	Hydroxyproline	1.1	4.4	277 ± 7
132.0768	Creatine	1.1	7.5	28 ± 10
132.1019	Isoleucine	1.1	4.2	620 ± 5
132.1019	Leucine	1.1	4.3	976 ± 5
133.0608	Asparagine	1.1	6.7	228 ± 10
133.0972	Ornithine	1.3	12	1618 ± 13
134.0448	Aspartic acid	1.1	9.9	430 ± 4
138.0550	Anthranilic acid	1.0	8.6	153 ± 11
147.0764	Glutamine	1.1	4.0	5744 ± 2
147.1128	Lysine	1.3	13	2817 ± 3
148.0604	Glutamic acid	1.1	2.8	449 ± 2
150.0583	Methionine	1.1	17	285 ± 15
156.0768	Histidine	1.3	8.3	967 ± 10
166.0863	Phenylalanine	1.1	3.2	635 ± 5
175.1190	Arginine	1.3	7.4	927 ± 8
182.0812	Tyrosine	1.0	5.3	757 ± 5
Unidentified compounds				
Detected *m/z*				
89.107	*130.086*	*169.057*	*178.065*	*209.092*
106.086	*134.101*	*170.092*	*180.027*	*228.098*
118.061	*136.063*	*170.121*	*184.073*	*423.166*
118.085	*139.050*	*171.063*	*186.016*	
130.050	*154.086*	*176.102*	*194.156*	

Asterisks (*) denote that value is below limit of quantification, but above limit of detection

This study provided a concentration of about 300 nM for glutamate and 12 nM for GABA in rat brain microdialysis sample, whereas in our study we have found a concentration of about 449 nM for glutamate and 59 nM for GABA. Though, rather comparable results were obtained, we still decided to test whether our CE-MS

method can be used for the reliable quantification of metabolites in microdialysis samples. For this purpose both perfusate and actual microdialysis samples were spiked with isotope-labeled amino acids (5 µM) and the peak areas obtained by CE-MS were compared. The results of this experiment (**S-12**) show that comparable peak areas were obtained for SIL glutamine, valine and aspartic acid (student's t-test at 95% CI, paired). For SIL glutamic acid and lysine, peak areas were 9% lower and 15% higher, respectively (**S-12**), which we could attribute to the analytical variation. On the basis of these findings, we are confident that our CE-MS method can be used for the reliable quantification of metabolites in microdialysis samples when employing standard curves of metabolites prepared in perfusate. **Table 4** gives an overview of the compounds detected and quantified and also of compounds detected but not identified and quantified in a rat brain microdialysis sample by CE–MS. For quantification, isotope-labeled aspartic acid, glutamic acid, glutamine, lysine and valine were used as internal standards for their corresponding amino acids, whereas isotope-labeled glutamine was used as internal standard for the other compounds, as this internal standard gave the most repeatable relative peak area values.

Conclusions and perspectives

In this work, we have evaluated for the first time the analytical performance of CE-MS for the direct analysis of endogenous metabolites in rat brain microdialysis samples. This method allows to obtain LODs for amino acids in rat brain microdialysates in the range which is rather comparable to LC-MS approaches [94], with the main differences that in CE only an injection volume of about 300 nL is utilized, whereas typically 5000 nL is injected in LC-MS, and no derivatization is needed. Therefore, the proposed CE-MS method is highly suitable for metabolic profiling of volume-limited biological samples. Even though lower detection limits can be obtained using electrokinetic injections [112], an advantage of our method is that it allows to perform multiple injections from the same sample, whereas only a single injection can be performed with electrokinetic injection. Another strong point of our method is that highly repeatable migration times are obtained for metabolites in rat brain microdialysis samples without using any sample pretreatment. This approach offers potential for comparative metabolomics studies using microdialysis samples, that may potentially lead to the discovery of novel metabolic markers for neurological diseases. It is anticipated that a further improvement in detection sensitivity could be achieved by employing a sheathless instead of a sheath-liquid interface for coupling CE to MS, potentially enabling the evaluation of biochemical changes in the brain at a very short temporal resolution. Moreover, such approach could be further extended to mouse brain microdialysis studies, where volumes are even smaller.

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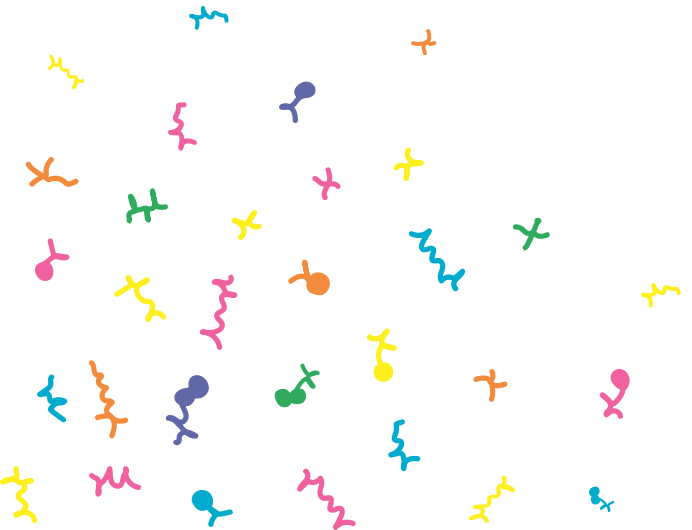
Supporting information

Additional information is available as stated in the text. This information is available free of charge via https://www.sciencedirect.com/science/article/pii/S0026265X2030552X



Chapter 5A

CE-MS for anionic metabolic profiling: an overview of methodological developments

Marlien van Mever, Thomas Hankemeier, Rawi Ramautar

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Abstract

The efficient profiling of highly polar and charged metabolites in biological samples remains a huge analytical challenge in metabolomics. Over the last decade, new analytical techniques have been developed for the selective and sensitive analysis of polar ionogenic compounds in various matrices. Still, the analysis of such compounds, notably for acidic ionogenic metabolites, remains a challenging endeavor, even more when the available sample size becomes an issue for the total analytical workflow. In this paper, we give an overview of the possibilities of capillary electrophoresis-mass spectrometry (CE-MS) for anionic metabolic profiling by focusing on main methodological developments. Attention is paid to the development of improved separation conditions and new interfacing designs in CE-MS for anionic metabolic profiling. A complete overview of all CE-MS-based methods developed for this purpose is provided in table format (Table 1) which includes information on sample type, separation conditions, mass analyzer and limits of detection (LODs). Selected applications are discussed to show the utility of CE-MS for anionic metabolic profiling, especially for small-volume biological samples. On the basis of the examination of the reported literature in this specific field, we conclude that there is still room for the design of a highly sensitive and reliable CE-MS method for anionic metabolic profiling. A rigorous validation and the availability of standard operating procedures would be highly favorable in order to make CE-MS an alternative, viable analytical technique for metabolomics.

Introduction

Metabolomics is considered within the field of analytical chemistry a well-accepted analytical approach for the global profiling of metabolites, i.e. small (endogenous) molecules with a molecular weight below 1500 Da. Currently, the Human Metabolome Database contains more than 100,000 metabolite entries with a wide dynamic concentration range, i.e. from mM to the pM-level [214]. A major part of these metabolite entries consists of lipids and exogenous compounds derived from nutrients and drugs. The metabolome is affected by both internal and external factors/stimuli, and therefore directly reflects the underlying biochemical activity and status of the biological system in question. Metabolic profiles may provide a wealth of information which can be used for disease prediction, disease progression, and treatment outcome [215, 216].

Nowadays, analytical techniques such as NMR spectroscopy, liquid chromatography-mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) are generally used for metabolic profiling studies [217-219]. CE-MS has emerged as a strong analytical tool for the profiling of polar and charged metabolites, such as phosphorylated sugars, organic acids, amino acids, and nucleotides, since its separation mechanism is based on charge-to-size ratios, thereby providing complementary information to other separation techniques [220]. Like CE-MS, hydrophilic interaction liquid chromatography (HILIC)-MS also emerged as a powerful analytical tool for the profiling of (highly) polar metabolites. Recent studies have indicated that both analytical techniques provide complementary metabolic information and in that context the use of both approaches is preferably required in order to get a full picture of the polar and charged compounds present in a given biological sample [196, 221, 222]. It would also be interesting to compare CE-MS with zwitter-ionic HILIC columns and recently developed ion-exchange LC systems for metabolic profiling studies. Ion-pair reversed-phase LC-MS has also been employed for the profiling of polar and charged metabolites [223, 224]. However, the use of ion-pair agents in LC-MS may result in severe ion suppression and may contaminate the ion source and ion optics. In addition, ion-pair agents may contribute to column instability and increased re-equilibration time.

The first research where CE-MS was used for global metabolic profiling of biological samples was performed by Soga and co-workers [36]. In this work, a bare fused-silica capillary and acidic separation conditions were used for the analysis of cationic metabolites, performing CE-MS in positive ionization mode. In general, this CE-MS method has been used now by various research groups as it provides acceptable performance metrics for the profiling of cationic metabolites [225-231]. However, in order to attain full coverage of ionogenic metabolites, both cationic and

anionic metabolites need to be analyzed by CE-MS. However, for anionic metabolic profiling the number of studies with CE-MS reported in the literature is far less than the number of studies involving cationic metabolic profiling. Soga *et al*. studied the anionic metabolic profile of extracts from *Bacillus subtilis* cells by CE-MS using a cationic polymer-coated capillary and weakly alkaline ammonia buffers, employing reversed CE polarity and negative ionization mode [37, 232]. Büscher *et al*. performed a cross-platform study for metabolic profiling of yeast extracts, and indicated CE as the least suitable platform for analyzing biological samples as it lacked the required robustness [233]. On the other hand, the CE-MS method at low-pH separation conditions employing a fused-silica capillary could be used in a robust way for cationic metabolic profiling of a yeast extract, and observed matrix effects were significantly lower as compared to the other chromatographic methods evaluated in this work. In agreement with the work of Büscher *et al*., Soga *et al*. demonstrated in another work that the long-term stability of the CE-MS method using a cationic-coated capillary for anionic metabolic profiling was relatively poor [234]. Authors found that the stability issue with the CE-MS method for anionic metabolic profiling appeared to be caused by corrosion of the stainless steel ESI needle when employing reversed CE polarity and negative ionization mode conditions. To overcome this issue, a platinum sprayer needle was used for CE-MS analysis in reversed polarity mode [234], although the platinum sprayer is not necessary for anionic metabolic profiling when applying normal CE polarity at high-pH separation conditions. Because of these stability issues together with lower sensitivity using negative ionization mode detection, the perception had risen that CE-MS was not suitable for global metabolic profiling, especially when compared to other chromatographic techniques such as LC-MS and GC-MS.

It is clear that the development of a robust and sensitive CE-MS approach for anionic metabolic profiling requires special attention. The improvement of CE-MS separation conditions and recent developments in interfacing designs, such as the sheathless porous-tip interface and the use of modified sheath-liquid (SL) interfaces, show great potential for sensitivity enhancement of profiling anionic metabolites. Therefore, in this review an overview of CE-MS approaches for anionic metabolic profiling is provided, covering the literature published between May 2002 and December 2018. In that context, the current work can be regarded as an important (complementary) addition to our previous CE-MS-based metabolomics reviews [235-240], which we provide bi-annually for Electrophoresis and in which the usefulness and developments of CE-MS approaches for anionic metabolic profiling were not considered in detail so far. Major technological developments that led to the improvement of the robustness and sensitivity of CE-MS for anionic metabolic profiling are considered and representative examples in various application fields are highlighted.

Technological developments

In this section, we will pay attention to CE-MS approaches that have been developed for the profiling of anionic metabolites, with a main focus on improvement of detection sensitivity (i.e. metabolic coverage) and robustness. A comprehensive overview of CE-MS methods developed for the profiling of anionic metabolites in the time period from May 2002 until December 2018 is provided in Table 1. Only those studies are included in Table 1 which reports the development of new CE-MS approaches for anionic metabolic profiling. For a complete overview of classical CE-MS employing a standard co-axial SL interface at high-pH separation conditions and MS detection in negative ion mode, i.e. the approach also used by the company Human Metabolome Technologies, we refer to especially the tables of our previous reviews [235-240].

Improving sensitivity/metabolic coverage

A major issue observed in CE-MS using negative ionization mode are the relatively low metabolite responses in comparison to those in positive ionization mode. Kok *et al*. evaluated different background electrolyte (BGE) compositions and SL additives in order to enhance metabolite responses in CE-MS in negative ionization mode [241]. It was found that the inclusion of triethylamine (TEA; pH 11.7) in the BGE and SL provided lower limits of detection and greater metabolome coverage than common negative ionization CE-MS methods for metabolic profiling where ammonia containing buffers are used. However, when using the same method in positive ion mode, TEA could lead to ion suppression issues.

A few years ago, a novel technique called paired ion electrospray ionization (PIESI) was developed by Armstrong and co-workers [242]. PIESI employs specially synthesized multifunctional cationic ion-pair reagents (IPRs) to form positively charged adducts with the anions to be analyzed. The adducts are detected in the positive ion mode and at higher *m/z* ratios providing improved signal-to-noise ratios and LODs that often are orders of magnitude better than those obtained with native anions in the negative ion mode. Recently, Lee *et al*. developed a CE-PIESI-MS method to analyze fatty acids (FAs) [243]. In this study, di-cationic IPRs were continuously introduced into the SL interface to generate positively charged adducts for anionic metabolites after electrophoretic separation. A preliminary study has shown that the addition of IPR prior to or during separation showed less effective complex formation and thus less improvement in terms of sensitivity compared to negative detection (**Figure 1**). An optimized concentration of 250 µM IPR was added to the SL, as this ensured sufficient complexation without contaminating the ion source due to excess IPR. The developed method (BGE: 30 mM ammonium formate in 40% *v/v* acetonitrile (CAN), pH 10) provided LODs

which ranged from 0.13 to 2.88 µg/mL for 15 FAs in a test mixture. The CE-PIESI-MS method was used to determine FAs in cheddar cheese and powdered coffee samples and showed that regarding sample preparation, in contrast to GC-MS and LC-MS methods, only a simple sample extraction step was needed to measure the FA concentration in the samples without signal suppression.

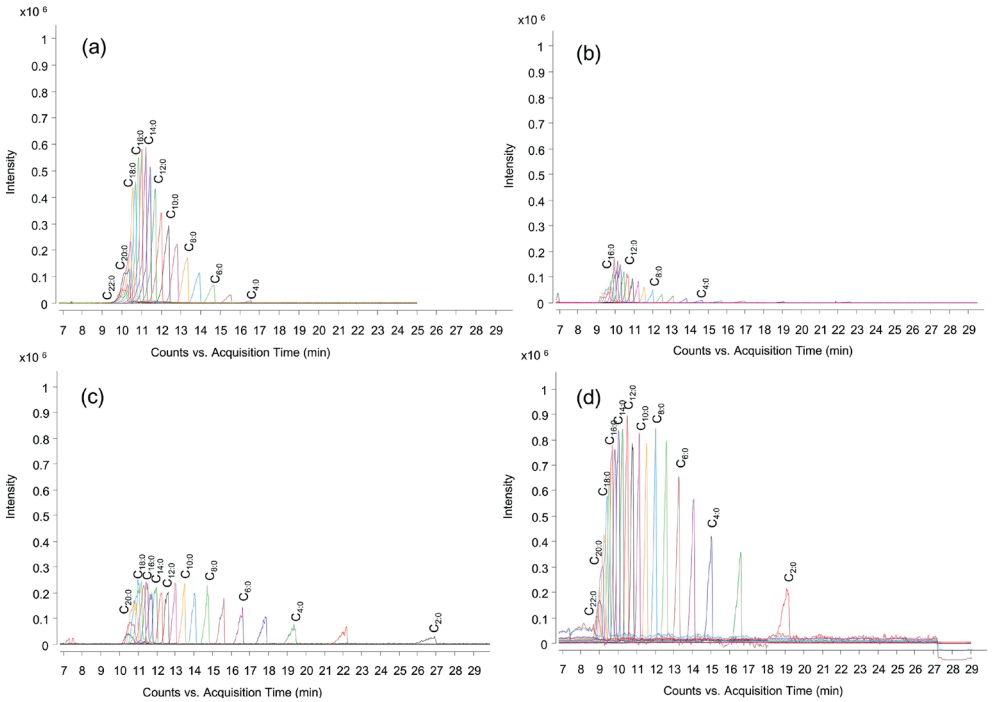

Figure 1. Extracted ion electropherograms of 21 linear FAs (C2¤C22:0, 100 µg/mL) obtained A) in negative ESI mode by CE-MS using a BGE of 40% acetonitrile in 30 mM ammonium formate at pH 10 B) in positive ESI mode with a pre-column technique and adding 250 µL ion pair reagent 1 (IPR1) in the FA standard mixtures, C) in positive ESI mode with an on-column technique and adding 250 µL IPR1 into the BEG solution, and D) in positive ESI mode with a post-column technique using a SL containing 250 µL IPR1 in 50% IPA solution. Reproduced from [243] with permission

In order to achieve coverage for a broad range of metabolite classes, analytical approaches are needed that can be used for the profiling of both cationic and anionic metabolites, ideally using the same separation conditions (i.e. capillary, BGE and SL), and in some cases the same CE polarity and/or MS detection mode, as described by the following examples. In 2010, Wakayama *et al*. created a separation method for the analysis of amino acids and carboxylic acids, where detection of both species was achieved in a single CE-MS run by changing the polarity of the ESI-MS during the time difference of the detection time between cationic and anionic metabolites [244]. When analyzing solely in positive ion mode, amino acids were mainly detected in their protonated form: $[M+H]^+$, whereas the anionic carboxylic acids were mostly detected as adduct peaks: $[M+NH_4]^+$.

Subsequently, when analyzing the same compound mixture in negative ionization mode without adjusting the main parameters, several amino acids were detected in their deprotonated form: $[M-H]^-$. In the optimized CE-MS method, the ESI-MS polarity was changed from positive to negative after the detection of amino acids. A relatively high sheath-gas pressure (20-25 psi vs. 5 to 10 psi in conventional CE-MS experiments) was required for stable spray formation, especially in negative ion mode. The CE-MS method was used to study the Crassulacean acid metabolism in pineapple leaf extracts, and showed effective determination of both anionic and cationic metabolites in a single run. The results confirmed the diurnal change in malate, aspartate, asparagine and citrate concentration in the pineapple leaves, and showed an opposite pattern for succinate and glutamine.

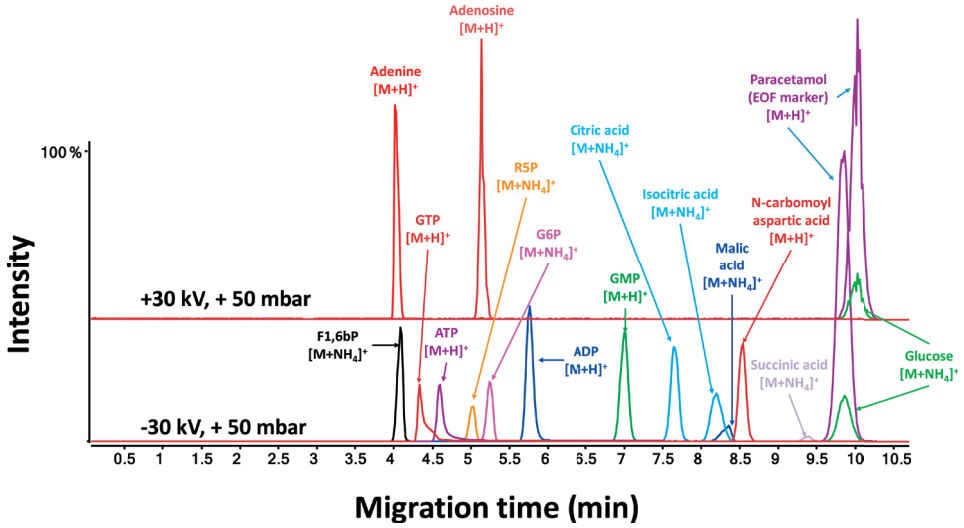

Figure 2. Extracted ion electropherograms obtained for a metabolite test mixture by CE-MS in positive ionization mode. Electrophoretic separation performed at low-pH separation conditions using 10% acetic acid as BGE. Reproduced from [196] with permission.

Drouin *et al*. developed a CE-MS method for the analysis of both cationic and anionic metabolites in one single run by measuring solely in positive ion mode [196]. A two-step approach was conducted, where cationic metabolites were measured in normal CE polarity, and reverse CE polarity was used to measure anionic metabolites. Equally to the study by Wakayama *et al*. [244], adduct formation was observed when applying positive ion mode. Moreover, it was found that the formed ammonium adducts for compounds (cations and anions) without amino groups showed higher analyte responses compared to their deprotonated species when detected in negative ionization mode. The sensitivity was further enhanced by adjusting some MS source parameters (higher capillary voltage: 5500 V, higher sheath-gas: 11 L/min) and turning off the nebulizing gas. The CE-MS method could be used for the analysis of both cationic and anionic species in

positive ion mode, as shown in **Figure 2** for a test mixture of metabolites. The method was applied to analyze a commercially available metabolomics library, where more than 76% of the 596 compounds could be observed. The obtained findings revealed that CE-MS was especially well-suited for the analysis of polar and charged metabolites, thereby showing a strong complementarity in comparison to chromatographic-based techniques. However, it should be noted that this CE-MS method employed a relatively high fragmentor voltage (380 V), which might induce in-source fragmentation due to energetic collisions that occur in the ESI source. This effect was earlier studied by Godzien *et al.*, who evaluated the impact of the enhancement of fragmentor voltage (150-230 V) on in-source fragmentation [245].

Alternative to the conventional SL interface, new low-flow SL interfacing designs have been recently developed for CE-MS-based metabolomics studies. Liu *et al.* developed a modified co-axial SL nanosprayer to analyze nucleotides in single neuron cell extracts [246]. The lab-fabricated nanosprayer has a smaller diameter capillary outlet [231], which is 40-µm instead of a typical 50 or 75-µm internal diameter, allowing lower SL flow rates (<1 µL/min) and no nebulizer gas is needed. These adjustments reduced sample dilution, improved repeatability and detection limits. In order to withstand corrosion, a platinum alloy emitter was used. Analysis was performed using a BGE of 20 mM ammonium bicarbonate (pH 10) and a SL of isopropanol and water (1:1, *v*/*v*) containing 0.2 mM ammonium bicarbonate, and was delivered at 600 nL/min. The method allowed the analysis of nucleotides in extracts of individual *Aplysia californica* sensory neurons with LODs of 2 to 22 nM. The implementation of an on-line pre-concentration method, i.e. large-volume sample stacking, further improved the detection limits of the method.

Recently, Portero *et al.* developed a method that allows sequential cationic and anionic analysis of metabolites in a single cell off a live vertebrate embryo of the South African frog *Xenopus laevis* [247]. The low-flow SL CE-MS interface was custom-built and supplemented with a nitrogen gas filled chamber to minimize electrical discharges and produce a stable ESI spray in both positive and negative ion mode. The design of the interface and how the behavior of the electrospray at the ESI emitter tip was evaluated, including a comparison of the stable Taylor-cone in positive ion mode and the non-axial emission and electrical discharge in negative ion mode without the nitrogen gas filled chamber, is shown in **Figure 3A**. Additionally, **Figure 3B** illustrates the stabilization of the total ion electropherogram signal measured in negative ion mode when including the nitrogen bath gas. The sample was collected via *in situ* capillary micro-sampling [248], where circa 10 nL of the cell content (i.e. 5% of the total cell volume) was aspirated. Following, a one-pot metabolite extraction was applied by ejecting the collected sample into 4 µL of 40% ACN containing 40% methanol (MeOH). The resulting cell extract was analyzed

with CE-MS on the same set-up, applying both cationic and anionic conditions using different BGEs, e.g. 1% *v/v* formic acid (pH 2.8) for cationic analysis and 20 mM ammonium bicarbonate (pH 8.2) for anionic analysis. Cationic and anionic analysis provided complementary information in terms of metabolite identification, and the separation performances were comparable to other CE-MS interfaces, such as the sheathless porous tip interface [249]. Overall, the proposed approach is very promising for single cell analysis, however, a single cell with relatively large dimensions (circa 200 nL content) was used in this work. It would be of great interest to develop a CE-MS workflow for single cell mammalian metabolomics, as the content of such cells often range in the low pL-range (e.g., content of single HepG2 cells corresponds to roughly 3 pL). Clearly, this work is still an enormous analytical challenge.

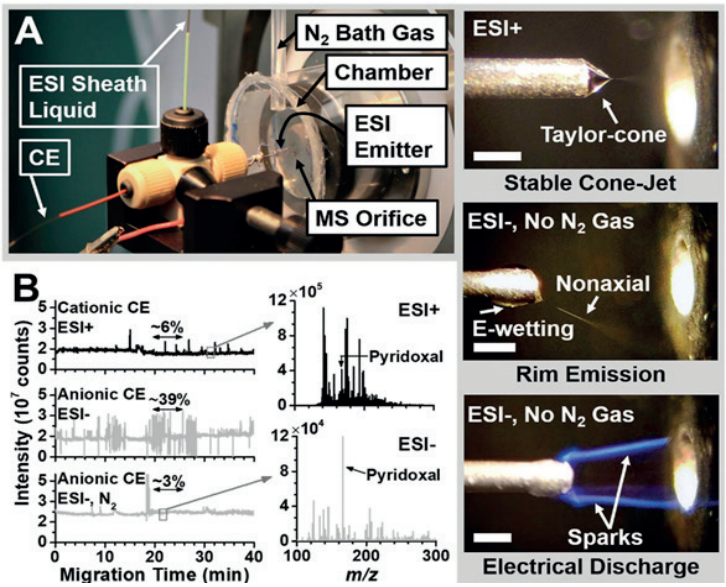

Figure 3. CE-MS for cationic and anionic metabolic profiling. A) The CE-MS interface with major components labeled. Microscopy comparison of stable Taylor-cone in ESI+ (top panel) and non-axial (rim) emission (middle panel) and electrical discharge (spark) in ESI- without nitrogen bath gas. Scale bars = 250 µm. B) Total ion chromatogram (TIC) revealing stable operation during cationic separation with ESI+ (top panel). ESI in negative ion mode for anionic separation (middle panel) was stabilized upon enclosing the electrospray emitter in a nitrogen-filled environmental chamber (bottom panel). Spray stability is quantified as percentage RSD of the total ion current. Representative mass spectra of a V1 cell extract revealing simplified spectral complexity during ESI−. Reproduced from [247] with permission.

Lin *et al*. demonstrated a novel electrokinetically pumped SL ESI interface to analyze heparin oligosaccharides [250]. In this method, 10 mM ammonium acetate containing 80% *v/v* MeOH (pH 7.5) was used as BGE and SL, the separation was conducted in reverse CE polarity and negative ionization mode. The capillary end was capped with a protein-coated spray emitter sheath capillary, allowing the addition of SL to the electroosmotic flow (EOF) and creating a stable electrospray

(**Figure 4**). The optimized CE-MS method showed to be applicable for disaccharide compositional analysis, bottom-up analysis, and top-down analysis. For the top-down analysis performed with CE-MS, a lower level of ammonium adduct formation was observed than when using HILIC-MS, resulting in less false positives [251].

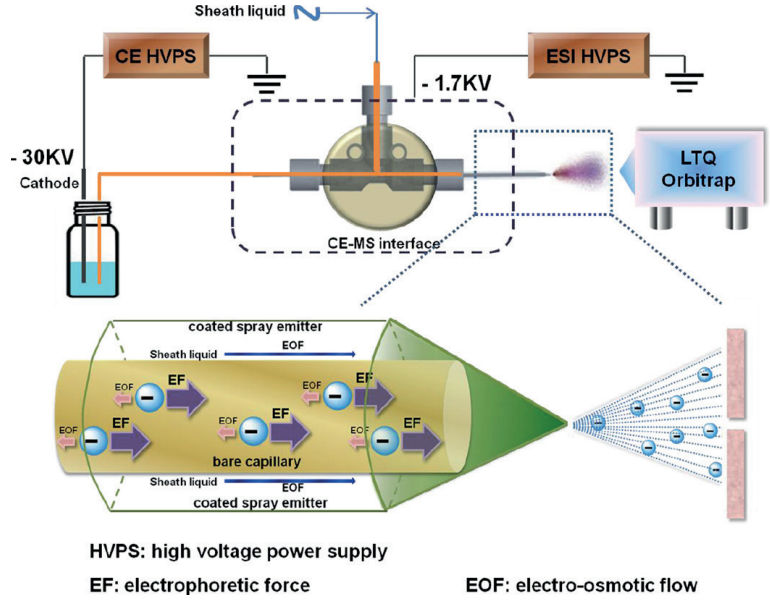

Figure 4. Schematic representation of the negative mode CE-MS system. A reverse polarity separation under a dominant electrophoretic force and low EOF is used to move analytes down a bare separation capillary. The cathode end of the separation capillary is capped with a protein-coated spray emitter sheath capillary with SL pumped by EOF, mixing with separation flow and affording a stable electrospray of negatively charged analytes that is introduced into an LTQ Orbitrap for MS analysis. Reproduced from [250] with permission.

CE-MS-based metabolomics studies are typically performed with a SL interface. However, the addition of SL will cause dilution of the CE effluent, thereby limiting the detection sensitivity. Therefore, new interfacing designs such as the sheathless porous tip interface [252] are gaining interest for metabolomics studies [228, 253, 254]. Bonvin *et al*. developed a non-aqueous CE-MS (NACE-MS) method for the analysis of acidic compounds using a sheathless interface [255]. In NACE, instead of an aqueous BGE, an organic solvent is used as BGE. In this NACE-MS method, the BGE was composed of 5 mM acetic acid in an acetonitrile-methanol mixture (80:20, *v*/*v*). The sheathless NACE-MS method improved the sensitivity by 2- to 50-fold for the determination of glucuronides in human urine compared to results obtained with a SL interface.

Gulersonmez *et al*. evaluated the performance of CE-MS using a sheathless porous tip interface for the analysis of anionic metabolites in biological samples by employing the same experimental conditions as for the profiling of cationic metabolites, only switching the CE separation polarity [249]. The method comprised a BGE of 10%

acetic acid (pH 2.2), and anionic metabolites from glioblastoma cell extracts were detected in negative ion mode detection. The injection volume was approximately 20 nL and LODs between 10 and 200 nM were obtained for test compounds, showing a significant improvement when compared to LOD values obtained with SL CE-MS systems. It should be noted that this approach can only be applied for the separation of acidic metabolites with a pKa value below 4.2. The limited durability of using a single porous tip capillary (typically between 100 and 200 runs) in CE-MS prevents its use for the analysis of large sets of biological samples [249, 256]. A proper sample preparation is critical when using these capillaries. Still, CE-MS with a sheathless interface has shown to have high potential for volume-restricted metabolomics.

Improving reproducibility

One of the main reasons for the under-use of (standardized) CE-MS methods for metabolic profiling of anionic species is the concern of its lack in reproducibility. In a recent study performed by Acunha *et al.*, a fused-silica capillary was coated with poly-(N,N,N',N')-tetraethyldiethy-lenetriamine,N-(2-hydroxypropyl) methacryl amide (PTH), and its utility for the profiling of anionic metabolites was evaluated in orange juice and wine samples [257]. The polymeric dynamic coating could be generated in an automatic procedure and the CE-MS method provided an acceptable repeatability for anionic metabolic profiling, i.e. RSDs (n=3) for migration times and peak areas for selected metabolite standards were below 0.2 and 2.1%, respectively. Additionally, adenosine triphosphate (ATP) was detected when employing the PTH coating, while it was not detected using an uncoated capillary. The PTH coating showed to be an interesting strategy for the analysis of anionic metabolites with the CE polarity reversed, while the method is still suitable for MS detection. Overall, the CE-MS method allowed the detection of 87 metabolites in orange juice and 142 metabolites in red wine, demonstrating the utility of this approach for the characterization of food products.

Yamamoto et al. has demonstrated that the use of alkaline aqueous ammonia solutions (pH > 9) as BGE leads to chemical degradation of the outer polyimide capillary coating, causing incidental capillary fractures [258]. This effect is depicted in **Figure 5A**, where images of polyimide coated fused-silica capillaries are shown after an exposure of 70 days to different aqueous alkaline solutions. Long-term exposure of the capillary to an ammonium bicarbonate solution (pH 10) resulted in degradation of the outer polyimide coating. This was also observed when using other ammonium containing buffers such as ammonium acetate or ammonium hydroxide at elevated pH conditions. When exposing the capillary to aqueous alkaline buffers free of ammonia such as borate (pH 10), or to a primary/

secondary amine buffer such as ethylamine (pH 10), no weakening of the capillary coating was observed. Additionally, when using a weakly alkaline ammonium bicarbonate buffer (pH 8.5), there was no capillary degradation. These findings were confirmed when the fracture resistance of fused-silica capillaries was compared after a bending force (90° angle) was applied manually, where each capillary was exposed to a different solution over a 26-day period (**Figure 5B**).

Subsequently, they tested the long-term performance of the polyimide coated capillaries when using compatible alkaline BGEs, i.e. ammonium bicarbonate (pH 8.5), ammonium acetate (pH 8.5), ethylamine (pH 10.0), diethylamine (pH 11.0), and pyrrolidine (pH 11.3), for anionic metabolite profiling of biological samples. Pooled urine samples were measured in 34 runs (or 238 repeated sample injections) under alkaline conditions using multi-segment injection (MSI)-CE-MS with negative ion mode detection [227]. In MSI, multiple samples can be injected serially in a single capillary, where the sample segments are spatially positioned between BGE zones. A throughput of <5 min per sample was achieved. Hence, aminolysis of the outer polyimide capillary coating can effectively be prevented by using less alkaline ammonium buffers (< pH 8.5) or substituting the BGE by less nucleophilic or alkaline buffers without ammonia. The findings of this work have important consequences as various CE-MS methods described in this paper employed BGEs with a pH above 9.0 and often it was not clear from these papers whether the polyimide coating was removed. Therefore, it is crucial to consider this work in the design of CE-MS methods for anionic metabolic profiling at high-pH separation condition.

Large-scale metabolomics is a promising approach to identify novel biomarkers. Recently, Harada *et al*. assessed the long-term performance of CE-MS for metabolic profiling of more than 8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52-month period [140].

Using two separate CE-MS systems for anionic and cationic metabolic profiling, respectively, more than 150 polar metabolites could be identified. The study provided an absolute quantification for 94 polar metabolites in plasma with a similar or better reproducibility (i.e., in quality control (QC) samples RSD for peak area was below 20% for 64 metabolites and less than 30% for 80 metabolites out of the 94 metabolites) when compared to other MS-based analytical platforms employed for large-scale metabolomics studies.

The overall metabolic coverage of the employed CE-MS methods was limited in comparison to other established LC-MS platforms, as CE-MS was not able to detect most of the apolar metabolites in plasma. Azab *et al*. developed a standardized

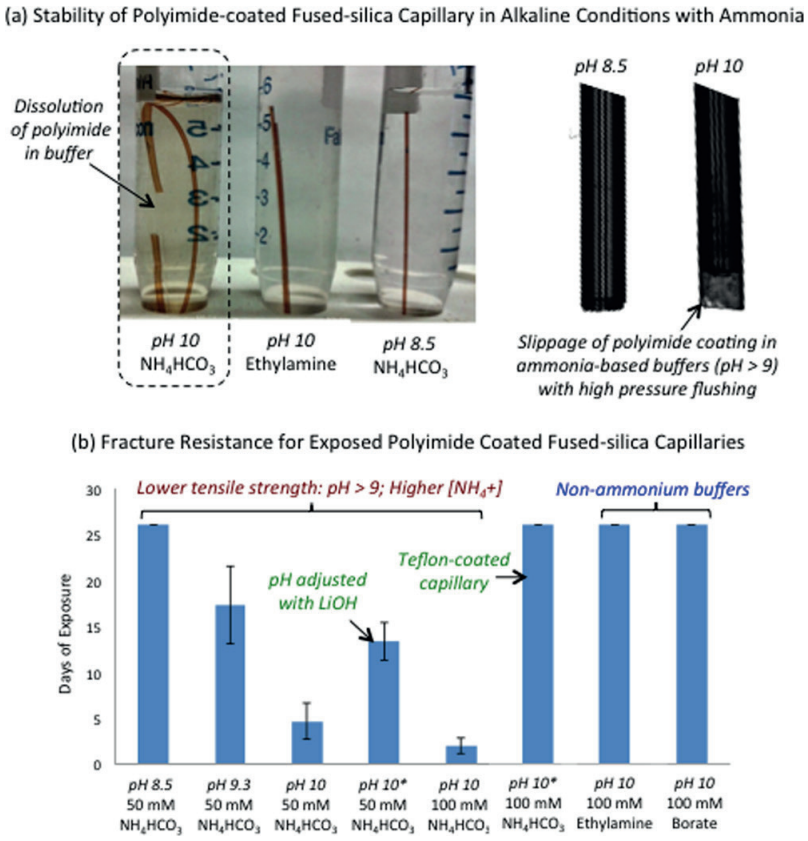

Figure 5. A) Images showing the impact of prolonged exposure (70 days) of polyimide coated fused-silica capillaries in aqueous alkaline ammonium buffers that result in softening/deformation of the outer coating and polymer dissolution. High-pressure (90 kPa) flushing with 50 mM ammonium bicarbonate buffers for 24 h demonstrate elongation of the outer polymer coating beyond the fused-silica capillary tip at pH 10 that is not observed at pH 8.5. B) A comparison of changes in the tensile strength and resistance to fractures with repeated bending (90˚) to a series of fused-silica capillary segments (where error bars represent ±1 s, n=6) exposed to different aqueous alkaline solution (i.e., buffer type, pH, ionic strength), indicating that higher ammonia concentrations and increased pH conditions accelerate polyimide aminolysis shortening their average lifespan due to capillary column breakage. Reproduced from [258] with permission.

high-throughput NACE-MS method for the determination of FAs in blood specimens [259]. A MSI approach was used where a serial injection of seven independent samples (including a QC sample) within a single CE run was applied, thereby improving the throughput of the method. For the separation, a BGE of 70% ACN, 15% MeOH, 10% H_2O, and 5% isopropanol in ammonium acetate (pH 9.5), and a SL of 80% MeOH with 0.5% ammonium hydroxide was used. In order to prevent aminolysis and swelling of the outer polyamide capillary coating which is often the case when using alkaline ammonia containing BGEs (pH > 9.0) [258] and organic solvents that have long-lasting contact with the capillary [260], the outer coating was removed from the capillary terminal ends (± 7 mm). During capillary flushing

and sample injection, the nebulizer gas was turned off to prevent causing current drops caused by air plugs inside the capillary. In order to prevent corona discharge in negative ion mode, a sprayer voltage of −3.5 kV was used. In the optimized MSI-NACE-MS method, seven serum extracts were analyzed after a methyl-tert-butyl ether extraction [261] without chemical derivatization, and most FAs were detected as their deprotonated molecular ions [M–H]−. A throughput of <4 min/sample was achieved, and the method sensitivity showed to be comparable to conventional GC methods. This work clearly shows the utility for MSI-NACE-MS for lipid profiling.

Recently, Höcker *et al.* assessed the analytical performance of CE-MS for a selected group of analytes using three different interfacing designs, i.e. the co-axial SL interface, the electrokinetic-driven nanoflow SL interface and the sheathless porous-tip interface [262]. The conventional SL interface showed good robustness and flexibility, but in case an improved detection sensitivity was required, both the electrokinetic-driven SL interface and sheathless interface could be considered for this purpose, as they provided a sensitivity enhancement of an at least 100-fold. Still, in contrast to CE-MS using a standard SL interface, the long-term performance of the latter interfaces still needs to demonstrated. The durability or lifespan of a single capillary in these interfaces is also an important aspect and such data needs to be provided, preferably by multiple laboratories. It should be noted that in this study, a standard SL interface was employed. Recent studies have shown that with alteration of the MS source parameters, such as for example setting the nebulizing gas to 0 psi, a sensitivity improvement of an at least 15-fold can be achieved using a SL interface [196].

Application field	Sample matrix	BGE	Capillary	MS analyzer	LOD[a]	Notes	Ref.
Biomedical	Liver and serum extracts of AAP-treated mice	50 mM ammonium acetate (pH 8.5)	Cationic polymer: SMILE(+) capillary	TOF	ns.		[55]
	Mouse liver extracts	50 mM ammonium acetate (pH 8.5)	Cationic polymer: COSMO(+) capillary	TOF	0.03-0.87 µM	Platinum ESI needle; internal standards for quantification	[24]
	Human urine, rat urine	25 mM triethylamine (pH 11.7)	Fused-silica capillary	(Q-)TOF	0.7-9.1 µM		[31, 56]
	Human urine	50 mM ammonium bicarbonate (pH 9.5)	Fused-silica capillary	TOF	0.4 µM		[57]
	Human serum	5 mM ammonium acetate (pH 10.8)	Fused-silica capillary	QqQ	0.05-0.81 µM	Home-made sheathless interface	[58]
	Human Urine	5 mM ammonium acetate in ACN-MeOH 80:20 (v/v)	Fused-silica capillary with a porous tip	Quadrupole	5 ng/mL	Non-aqueous capillary electrophoresis (NACE); porous tip sheathless interface	[45]

Application field	Sample matrix	BGE	Capillary	MS analyzer	LOD[a]	Notes	Ref.
	Glioblastoma cells	10% acetic acid (pH 2.2)	Fused-silica capillary with a porous tip	TOF	10-200 nM	Low pH BGE for anionic metabolic profiling; porous tip sheathless interface	[39]
	Human urine	50 mM ammonium bicarbonate (pH 8.5)	Fused-silica capillary	TOF	ns.	Multi-segment injection (MSI)-CE-MS; hydrodynamic pressure gradient applied during separation	[48]
	Arixtra® and LMWH (Lovenox®)	10 mM ammonium acetate in 80% aqueous methanol (pH 7.5)	Fused-silica capillary	LTQ Orbitrap	ns.		[40]
	HEK 294T cells	10% acetic acid (pH 2.2)	Fused-silica capillary	TOF	ns.	Low pH BGE for anionic metabolic profiling; anionic metabolites detected in positive ion mode; no nebulizing gas applied; pressure of 30 mbar at the CE inlet	[10]
	Human plasma	50 mM ammonium acetate (pH 8.5)	Fused-silica capillary	TOF	ns.	Internal standards for quantification	[59]

Application field	Sample matrix	BGE	Capillary	MS analyzer	LOD[a]	Notes	Ref.
	Human plasma and serum	70% acetonitrile, 15% methanol, 10% H2O, and 5% isopropanol in ammonium acetate (pH 9.5)	Fused-silica capillary	TOF	0.70 µM	Multi-segment injection non-aqueous capillary electrophoresis-mass spectrometry; Internal standards for quantification	[50]
	Aplysia californica cells	20 mM ammonium bicarbonate (pH 8.2)	Fused-silica capillary	Q-TOF	5.5 nM	Custom-built co-axial sheath-flow CE-ESI interface; CE-ESI emitter tip emerged in a N_2 bath gas chamber	[37]
Microbial/plant	*Bacillus subtilis* cell extract	50 mM ammonium acetate (pH 9.0)	Cationic polymer: SMILE(+) capillary	IT	0.3-6.7 µM		[22]
	Bacillus subtilis cell extract	50 mM ammonium acetate (pH 7.5)	Neutral polymer: DB-1 capillary	IT	0.4-3.7 µM	Pressure assisted CE (PACE)	[21]
	Anionic standards	50 mM trimethylamine acetate (pH 10.0)	Fused-silica capillary	IT	ns.	Pressure assisted CE (PACE)	[60]

Application field	Sample matrix	BGE	Capillary	MS analyzer	LOD[a]	Notes	Ref.
	Human plasma and serum	70% acetonitrile, 15% methanol, 10% H2O, and 5% isopropanol in ammonium acetate (pH 9.5)	Fused-silica capillary	TOF	0.70 μM	Multi-segment injection non-aqueous capillary electrophoresis-mass spectrometry; Internal standards for quantification	[50]
	Aplysia californica cells	20 mM ammonium bicarbonate (pH 8.2)	Fused-silica capillary	Q-TOF	5.5 nM	Custom-built co-axial sheath-flow CE-ESI interface; CE-ESI emitter tip emerged in a N_2 bath gas chamber	[37]
Microbial/plant	*Bacillus subtilis* cell extract	50 mM ammonium acetate (pH 9.0)	Cationic polymer: SMILE(+) capillary	IT	0.3–6.7 μM		[22]
	Bacillus subtilis cell extract	50 mM ammonium acetate (pH 7.5)	Neutral polymer: DB-1 capillary	IT	0.4–3.7 μM	Pressure assisted CE (PACE)	[21]
	Anionic standards	50 mM trimethylamine acetate (pH 10.0)	Fused-silica capillary	IT	ns.	Pressure assisted CE (PACE)	[60]

Application field	Sample matrix	BGE	Capillary	MS analyzer	LOD[a]	Notes	Ref.
	Transgenic rice plants	20 mm ammonium acetate (pH 6.8)	Neutral polymer: DB-WAX capillary	Quadrupole	ns.	Internal standards for quantification	[67]
	Pineapple leaves	1 M formic acid (pH 1.8)	Fused-silica capillary	QqQ	0.5–10 μM	High-speed sheath gas flow applied	[34]
	Moss extract (*Physcomitrella patens*)	50 mM ammonium formate (pH 8.0); 50 mM ammonium acetate (pH 10.0) containing 50% methanol	Fused-silica capillary	IT	0.13–17 μM	Pressure assisted CE (PACE); Internal standards for quantification	[68]
Food/ environmental	Ale	2 mM TMA and 5 mM Tris (pH 8.5)	Fused-silica capillary	Quadrupole	0.05–0.1 μg/mL		[69]
	Apples, grapes, oranges, tomatoes	32 mM ammonium formate (pH 3.1)	Fused-silica capillary	Quadrupole	0.1–3 μg/mL		[70]
	Apple juice	20 mM ammonium formate (pH 10)	Fused-silica capillary	IT	1.1–3.5 μg/mL		[71]

Application field	Sample matrix	BGE	Capillary	MS analyzer	LOD[a]	Notes	Ref.
	Orange juice and red wine	1 M formic acid, pH 2.4	PTH coated capillary	TOF	0.1-16.4 ppm		[47]
	Cheese and coffee samples	ammonium formate buffer containing 40% acetonitrile modifier	Fused-silica capillary	TOF	0.13 to 2.88 mg/mL	Addition of ion pairing reagents as addition to the sheath liquid to detect anionic metabolites/ion pair complexes in positive ion mode	[33]

[a]LOD = limit of detection (S/N = 3); ns, not specified in paper.

Conclusions and perspectives

CE-MS employing a standard SL interface performs well for cationic metabolic profiling as shown by various research groups. However, concerning anionic metabolic profiling further development is needed in order to obtain a reliable approach that can preferably be easily used by multiple groups. One of the main challenges is the search for the most optimal CE-MS interfacing design when it comes to robustness, sensitivity and user-friendliness. Overall, on the basis of our assessment of the reported literature in this specific field in the given time period, the development of a highly sensitive and reliable CE-MS method for anionic metabolic profiling will remain an active area of research. In our opinion, the profiling of anionic metabolites in limited amounts of mammalian cells will be an important application field of CE-MS. The implementation of rigorous validation and the availability of standard operating procedures would be highly favorable in order to make CE-MS an alternative, viable analytical technique for metabolomics. Therefore, our intention is to set-up an inter-laboratory CE-MS study using both the standard SL and the sheathless porous tip interface for metabolic profiling. Such data is urgently needed to actually show the suitability of CE-MS for long-term anionic metabolic profiling. The availability of open access peer-reviewed protocols would be very helpful for this purpose, and in that context the developments are going into the right direction [248, 263].

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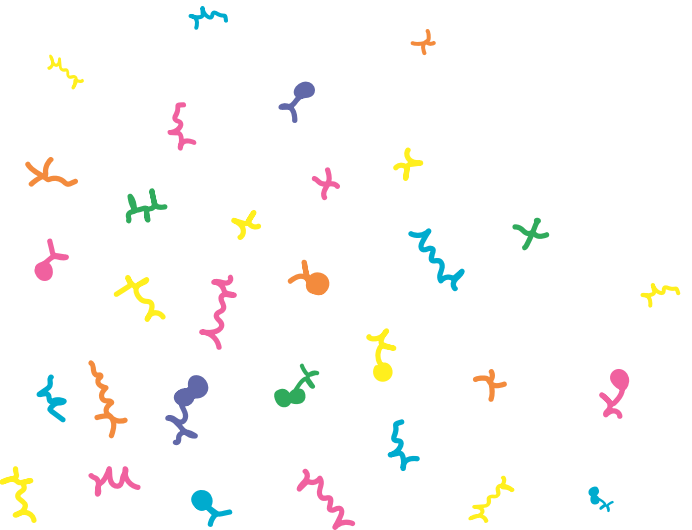

Chapter 5B

Profiling acidic metabolites by capillary electrophoresis-mass spectrometry in low numbers of mammalian cells using a novel chemical derivatization approach

Marlien van Mever*, Cornelius C. W. Willacey*, Wei Zhang, Nicolas Drouin, Alphert E. Christina, Peter W. Lindenburg, Jaco P. D. van Veldhoven, Daan van der Es, Amy C. Harms, Thomas Hankemeier[$], Rawi Ramautar[$]

* Authors contributed equally

[$] Authors contributed equally

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Abstract

The simultaneous analysis of a broad range of polar ionogenic metabolites using capillary electrophoresis - mass spectrometry (CE-MS) can be challenging, as two different analytical methods are often required, i.e. one for cations and one for anions. Even though CE-MS has shown to be an effective method for cationic metabolite profiling, the analysis of small anionic metabolites often results in relatively low sensitivity and poor repeatability. In this work, a novel derivatization strategy based on trimethylmethaneaminophenacetyl bromide was developed to enable CE-MS analysis of carboxylic acid metabolites using normal CE polarity (i.e. cathode in the outlet) and detection by mass spectrometry in positive ionization mode. Optimization of derivatization conditions was performed using a response surface methodology after which the optimized method (incubation time: 50 minutes, temperature: 90°C and pH: 10) was used for the analysis of carboxylic acid metabolites in extracts from HepG2 cells. For selected metabolites, detection limits were down to 8.2 nM, and intraday relative standard deviation values for replicates (n=3) for peak areas were below 21.5%. Metabolites related to glycolysis, tricarboxylic acid cycle and anaerobic respiration pathways were quantified in 250,000 cell lysates, and could still be detected in extracts from only 25,000 HepG2 cell lysates (~70 cell lysates injected).

Introduction

The metabolome consists of a vast number of components, including a wide variety of compound classes, such as amino acids, organic acids, nucleotides, fatty acids, etc. Profiling of small (endogenous) molecules in biological matrices, coined as metabolomics, is of fundamental importance to elucidate the cellular metabolism under pathophysiological conditions. The diversity in chemical and physical properties of the metabolites, as well as the broad concentration range in which they are present in different biological matrices, makes their (simultaneous) analyses challenging. Analytical techniques that are often used for metabolomics studies include nuclear magnetic resonance (NMR) spectroscopy [219], and liquid chromatography (LC) [264] or gas chromatography (GC) [265] coupled to mass spectrometry (MS) [266]. In recent years, capillary electrophoresis coupled to mass spectrometry (CE-MS) gained attraction in the scientific community for metabolomics, notably for the analysis of polar ionogenic metabolites given its separation mechanism, but also for the analysis of volume-limited biological samples [111, 220, 267-269].

Within the last decade, CE-MS has been highlighted as an effective method for cationic metabolite profiling, thereby applying normal CE polarity and detection by MS in positive ionization mode [270], as exemplified by a recent study that included a large cohort of over 8000 biological samples [270], and also by the recent Metabo-ring trial [271], which both revealed that CE-MS is a viable and reproducible approach for metabolomics. On the other hand, the profiling of anionic metabolites by CE-MS has been considered a challenging endeavour [272]. Anionic metabolic profiling is usually perfomed by a method first proposed by Soga et al., [37] where a cationic polymer-coated capillary in combination with a weakly alkaline ammonia buffer is used, thereby employing reversed CE polarity and negative ionization mode. However, the long-term stability of the the cationic coated capillary proved to be relatively poor [234], which was mainly due to corrosion of the stainless steel ESI needle when employing reversed CE polarity and negative ionization mode conditions. Additionally, a cross-platform study revealed that the use of a reverse CE polarity in conjunction with MS detection in negative ionization mode for anionic metabolite profiling resulted in relatively poor metabolite responses and repeatability [233]. By replacing stainless steel for platinum as ESI spray needle greatly improved method performance and long-term stability when employing CE with cationic-coated coatings under reversed polarity for anionic metabolic profiling [234]. However, a platinum needle is not required for anionic metabolic profiling under normal CE polarity conditions as electrolytic corrosion at the anode is avoided. Recently, Yamamoto *et al*. showed that commonly used ammonium acetate or ammonium formate background electrolytes (BGEs)

with a pH above 9.0 contributed to incidental capillary fractures via irreversible aminolysis of the outer polyimide coating [258]. Prevention of polyimide aminolysis could be easily achieved by using weakly alkaline, ammonia containing buffers (pH < 9.0).

To circumvent the issues described above, new efforts have been made to increase the performance of CE-MS for anionic metabolite profiling. For example, Lee *et al*. proposed a novel strategy based on the use of ion-pair reagents for analysis of short-chain fatty acids in the positive ion mode by MS [243]. The so-called paired ion electrospray ionization (PIESI)-MS allowed for lower detection limits and higher repeatability compared to analysis in negative ionization mode. However, ion suppression could still occur due to the ion pair reagents. Zhang *et al*. developed a sheathless CE-MS method for the profiling of nucleotides in the positive ion mode and employing a BGE of ammonium acetate with pH 9.7 with electrophoretic separation in normal polarity mode [273]. Another strategy was recently proposed by Drouin *et al*., utilizing a two-step CE-MS method for the analysis of cations and anions by using the same analytical conditions [196], except only reversing the CE polarity. Some anions were detected as their ammonium adducts, and higher sensitivity was achieved than when using MS detection in negative ionization mode. Though the same MS conditions could be used, a CE polarity switch was still required.

Chemical derivatization is a sample preparation technique that subjects target compounds to chemical reactions with a derivatization reagent and provides them more favorable physicochemical properties [274], such as changes in polarity [275, 276], separation, stability, ionization and vulnerability to ion suppression. Chemical derivatization is most commonly applied to GC-MS and LC-MS [275, 277-280]. Surprisingly, there is a great minority in CE-MS applications, while the use of chemical derivatization could enhance ionization [275, 276, 278, 279, 281]. Various efforts to analyze anionic metabolites as cationic analytes by CE-MS using chemical derivatization have been developed over the past years. For example, Yang *et al*. developed a relatively time-consuming (2h) two-step derivatization approach for the analysis of carboxylic acid-containing metabolites in urine using *N*-butyl-4-aminomethylpyridinum iodide and *N*-hexyl-4-aminomethyl-pyridinum iodide [282]. D'Agostino *et al*. used the reagent maleimide to label thiols in plasma. However, the derivatization using maleimide was restricted to the analysis of thiols only [283]. More recently, Huang et al. proposed a strategy for the derivatization of amines, hydroxyls and carboxylates using a two stage derivatization using 3-(Diethylamino) propionyl chloride and N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b] pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide [284].

In this study, a novel derivatization strategy based on trimethylmethane-aminophenacetyl bromide (TmAmPBr) was developed to enable CE-MS analysis of carboxylic acid-containing metabolites using normal CE polarity and MS detection in positive ionization mode. The use of TmAmPBr, which contains a quaternary amine group, provides derivatives with a permanent positive charge, which is beneficial in terms of ionization efficiency. The mechanism of action of this reagent is similar to the methods published by Willacey *et al.* (2019) [275, 279] and Guo *et al.* (2010) [285]; in which dimethylaminophenacyl bromide (DmPABr) was used for the labelling of carboxylic acids. Though, the reaction mechanism follows the same S_n2 reaction kinetics as phenacyl bromide and DmPABr (**Figure 1**), further optimization of the derivatization conditions using TmAmPBr has been carried out to ensure that reliable derivatization could be achieved in the presence of the quaternary amine using a response surface methodology (RSM). As a first step, the derivatization for metabolites containing a carboxylic acid group was investigated, since these metabolites are currently the most challenging to analyse using CE-MS. For method development, 11 acidic metabolites associated with glycolysis, tricarboxylic acid (TCA) cycle and anaerobic respiration were selected and the analytical performance was assessed by considering aspects like linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ). The applicability of the overall method was demonstrated for the analysis of acidic metabolites in extracts from low numbers of HepG2 cells.

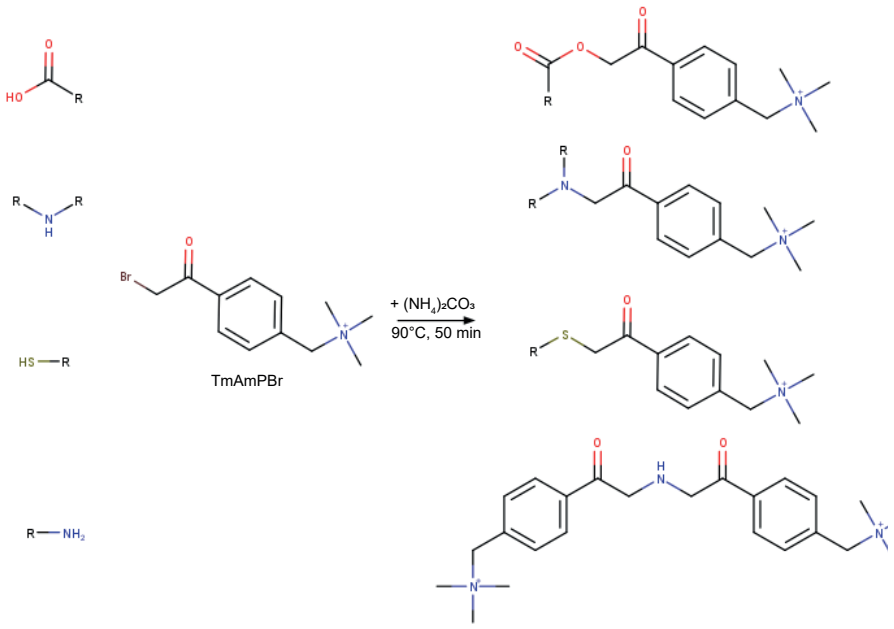

Figure 1. SN2 reaction scheme of TmAmPBr with the carboxylic acid, thiol, secondary amine, and primary amine, respectively.

Experimental section

Chemicals and reagents

All used chemicals were of analytical grade or higher. Paracetamol and oxaloacetic acid were acquired from Fluka (Buchs, Switzerland). Ammonium carbonate was purchased from Merck (Darmstadt, Germany). Glutaric acid, procaine, malonic acid, pyruvic acid, α-ketoglutaric acid, fumaric acid, isocitric acid, succinic acid, malic acid, citric acid, lactid acid, triethanolamine (TEOA) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (Steinheim, Germany). Deuterated stable-isotope labeled (SIL) standard succinic acid-d6 was from Cambridge Isotope Laboratories (Apeldoorn, the Netherlands). Hydrochloric acid (37% *m*/*m*) was acquired from Thermo Fisher Scientific (Waltham, MA, USA). Acetic acid and isopropanol were from Biosolve (Valkenswaard, The Netherlands). Acetonitrile (ACN) and dimethylformamide (DMF) were purchased from Actu-All Chemicals (Oss, The Netherlands) and Honeywell (Rosmalen, The Netherlands), respectively. The procedure for synthesis of TmAmPBr is described in **S-1**.

A Milli-Q Advantage A10 water purification system (Merck, Darmstadt, Germany) was used to obtain pure water. Metabolite standard solutions (1 mg/mL) were prepared in DMF/DMSO (50/50 *v*/*v*). Working standard mixtures of 12 compounds (500 µM) were prepared by dilution with DMF/DMSO, aliquoted and stored at -80 °C. Internal standard succinic acid-D_6 was prepared in DMF/DMSO (125 µM), and internal standards paracetamol and procaine were prepared in BGE (50 µM). Internal standards were aliquoted and stored at -80°C, and internal standard stock aliquots were thawed on the day of use. Ammonium carbonate was prepared in water to the desired concentrations. When necessary, the pH was adjusted using a 1M sodium hydroxide solution. As BGE solution, 10% (*v*/*v*) acetic acid (1.7 M) in water was used. Sheath-liquid consists of water and isopropanol (50:50, *v*/*v*), supplemented with 0.03% (*v*/*v*) acetic acid.

Derivatization optimization by RSM

The derivatization procedure was optimized using a RSM. As a first step, a suitable alkaline solution was selected based on compatibility with CE-MS analysis, thereby comparing triethanolamine and ammonium carbonate. Subsequently, a screening design was used to determine the main variables affecting the derivatization procedure. A two-level (2^{5-1}) fractional factorial design was built with five variables ammonium carbonate pH (X1), ammonium carbonate concentration (X2), incubation temperature (X3), incubation time (X4) and shaking speed (X5), resulting in an experimental design of 16 trials at low (-1) and high (+1) levels (**S-2**). The screening design was used to evaluate the relative magnitude of the statistical significance of the effects by pareto charts. Thereafter, a Central

Composite Design (CCD) was built for optimization of the derivatization procedure. Each factor was set to five levels: plus and minus α (axial points), plus and minus 1 (factorial points) and the center point, resulting in a total of 50 experiments, i.e. 42 design experiments with 8 replicate runs at the center point (**S-3**). The experiments were carried out in a random sequence to minimize bias and to reduce the outcomes of unpredicted variability in the responses. The responses were peak area ratios (corrected for the internal standard) of derivatised fumaric acid (Y1), oxaloacetic acid (Y2), α-ketoglutaric acid (Y3), pyruvic acid (Y4). The statistical and graphical interpretation of the effects was performed by using the Design Expert Software (version 12, Stat Ease Inc., MN, USA). Optimal values of the variables for derivatization were obtained using Derringer's desirability (D).

Mammalian cell lysate sample collection and preparation

Human liver cancer cells (HepG2) cells were cultured, harvested and collected in-house as described previously in [273]. First, the harvested cells were counted using a TC10 Automated Cell Counter (Bio-Rad Laboratories), and the live cell density was at 7.4×10^6 cells/mL. Pre-heated (37°C) culture medium (5 mL) was added to the Petri dishes (60 mm, n = 3), and after dispersion of the cell mixture, 135 µL of the cell mixture (~10^6 live cells) was added to the medium. The Petri dishes were gently shaken to distribute the cells evenly before incubation (37°C in 95% air/5% CO_2). After the cells adhered to the bottom of the Petri dishes (after ~7.5 h), the dishes were taken out of the incubator, the medium was aspirated and pre-heated (37°C) PBS (6 mL) was used to wash away residual culture medium, after which the PBS was removed. To quench intracellular enzymatic reactions, ice-cold methanol/H_2O (80:20, *v*/*v*) mixture (1 mL) was added into every Petri dish. The dishes were placed on ice, and scraped in order to get all the cells off the surface. The HepG2 cells were collected in aliquots of 2×10^6 cells per vial in Eppendorf vials and stored at -80°C until sample preparation was performed. The cell supernatant was aliquoted into new Eppendorf vials equivalent to 5×10^5, 2.5×10^5, 1×10^5, 5×10^4, 2.5×10^4, 1×10^4 and 5×10^3 cells. Sample clean-up to remove lipid and protein contents was performed by a liquid-liquid extraction procedure using ice-cold water/methanol/chloroform (1/1/1, *v*/*v*/*v*). The methanol/water phase was collected, evaporated and then reconstituted in 10 µL DMF/DMSO which was used for derivatization.

Derivatization and quantification of mammalian cells for sensitivity analysis

The described derivatization parameters were optimized using RSM (section 2.2). HepG2 cell supernatants were dried using Labconco SpeedVac (MO, United States). Dried residues were reconstituted in 10 µL DMF/DMSO (50/50 *v*/*v*). Then

subsequent additions were made of 10 µL ammonium carbonate (65 mM, pH 10), 5 µL internal standard solution (125 µM) and 20 µL TmAmPBr (40 mg/mL), with vortexing after each addition. Subsequently, the Eppendorf vial was placed into a shaking incubator at maximum speed (900 RPM) for 50 min at 90 °C to ensure thorough derivatization. After incubation, 10 µL acetic acid (10% *v*/*v*, 1.7 M) was added to the vial to quench the reaction, followed by an additional 20 min of incubation under the same conditions.

Instrumentation and procedures

CE-MS analyses were performed on a 7100-CE system from Agilent Technologies (Waldbronn, Germany), hyphenated with an Agilent 6230 TOF (Santa Clara, CA, USA). A co-axial sheath-liquid ESI interface coupled via a triple-tube sprayer was used. Sheath-liquid was delivered at a flow rate of 3 µL/min by an Agilent 1260 Infinity Isocratic Pump (Agilent Technologies) with a flow splitter (ratio 1:100). Fused silica capillaries (internal diameter: 50 µm) were purchased from BGB Analytik (Harderwijk, The Netherlands), and cut manually to a length of 70 cm. Conditioning of new capillaries was done by subsequently rinsing with MeOH, water, sodium hydroxide 1 M, water, hydrochloric acid 1 M, H_2O, hydrochloric acid 0.1 M, H_2O and BGE, each at 5 bar for 1 minute. Injections were performed hydrodynamically for 24 s at 50 mbar (27.4 nL), and volumes were calculated with Zeecalc v1.0b (https://epgl.unige.ch/labs/fanal/zeecalc). The separation voltage was 30 kV and additional pressure was applied (40 mbar) during separation.

CE-MS experiments were acquired in positive ionization mode, between *m*/*z* 65 and 1000 with an acquisition rate of 1.5 spectra/s. The following MS settings were used; nebulizer gas: 0 psi, sheath gas (nitrogen) flow rate: 11 L/min, sheath gas temperature: 100 °C, ESI capillary voltage: 5500 V, fragmentor voltage: 150 V, skimmer voltage: 50 V, as adapted from previous work [111, 286]. From the sheath-liquid, isopropanol $[C_3H_8O+H]^+$ and its clusters $[(C_3H_8O)_2+H]^+$ and $[(C_3H_8O)_3+H]^+$ with corresponding *m*/*z* values of 61.06479, 121.12231 and 181.17982, respectively, were used as lock masses. For data acquisition and treatment, MassHunter version B.06.00 (Agilent, Santa Clara, CA, USA) was used. Peak extraction was performed with a mass error of 20 ppm and peak integrations were visually inspected to ensure correct integration.

Analytical performance evaluation

The optimized derivatization procedure was evaluated in terms of calibration curves, repeatability, limits of quantification (LOQ), and limit of detection (LOD) as well as matrix effect. The response function of the measured peak area for 11 carboxylic acid metabolites (**S-4**) was examined using 3 replicates at 8 different

concentration points over a concentration range from 0.7 to 90 µM. As internal standard, SIL succinic acid-D_6 (25 µM) was used. Intraday repeatability (expressed as percentage relative standard deviation, % RSD) was evaluated for replicates at the same day (n=3). The LOD and LOQ were estimated as 3 σ/slope and 10 σ/slope, respectively (σ = standard deviation of the lowest calibration point).

To evaluate matrix effect regarding TmAmPBr derivatization and CE-MS analysis, the matrix effect factor (MEF) was determined for SIL succinic acid-D_6, which was spiked into the sample after extraction, but before derivatization. MEF was calculated as follows (**equation** 1):

$$Matrix\ effect\ factor\ (MEF) = \frac{AUC_{water} - AUC_{cells}}{AUC_{water}} * 100 \quad (1)$$

Where AUC_{water} is the averaged area of the SIL-internal standard in standards and is the peak area of the SIL-internal standard in a cell sample. MEF was calculated for all calibration points.

Results & Discussion

Optimization of reaction conditions

Initially, the optimized reaction conditions for derivatization with DmPABr were adapted for TmAmPBr derivatization (incubation time: 60 min, incubation temperature: 65°C) [279]. Four carboxylic acid metabolites with different physicochemical properties, namely, pyruvic acid, oxaloacetic acid, α-ketoglutaric acid and fumaric acid (**S-4**), were selected as test analytes for optimization of the reaction conditions. Two types of derivatives, singly derivatized and doubly derivatized acids (**S-5**), were observed for oxaloacetic acid, α-ketoglutaric acid and fumaric acid, since there are two carboxylic acid groups present on these compounds, whereas for pyruvic acid only the single derivatized acid was detected, as pyruvic acid only contains one carboxylic acid group. Since the doubly derivatized acids contain two positive charges, they migrated faster and appeared at the front in the electropherogram, followed by singly derivatized acids. Probably, singly derivatized acids are still observed for oxaloacetic acid, α-ketoglutaric acid and fumaric acid, due to incomplete derivatization, or because of sterical hindrance during the derivatization reaction. Additionaly, peak intensity and shapes were more desirable for doubly derivatised metabolites, and therefore, for oxaloacetic acid, α-ketoglutaric acid and fumaric acid, the doubly derivatized forms were selected for further optimization experiments, whereas for pyruvic acid, the singly derivatized variant was evaluated. The presence of singly derivatized and doubly derivatized acids could be detrimental for detection sensitivity of diprotic carboxylic acids, as the signal for a certain analyte is splitted. This issue was further investigated under

the optimized conditions. Preliminary analysis showed that derivatization was achieved adapting the protocol that was developed for derivatization with DmPABr, however, with undesirable peak shapes and signal intensities, and a large distortion in the total ion electropherogram TIE signal was observed. A closer inspection of the recorded mass spectra revealed ion suppression presumably attributed to the use of TEOA (*m/z* 150.112) as base catalyst. Additionally, the high conductivity of TEOA could cause a destacking effect during CE-MS analysis, thereby causing poor peak shapes. Therefore, ammonium carbonate was proposed as base catalyst due to its compatibility with ESI-MS, relatively high pH buffering range and lower conductivity. More favourable peak shapes and an increase of signal intensity were observed for the selected compounds when ammonium carbonate (100 mM, pH 9.2) was used, as shown by an increase in peak area by 89-99% (S.6). When using the water-soluble base ammonium carbonate as base catalyst for the reaction, the total elimination of water content is no longer realised. However, previous research has shown that a water content of <40% does not significantly change the derivatization efficiency for derivatization with DmPABr [275], and is also not essential for the derivatization of carboxylic acids. Due to the similarity of DmPABr and TmAmPBr, it was expected that the water content (~22%) does not significantly impact the reaction efficiency, as confirmed experimentally (**S-7**). Pyruvic acid is shown to be more impacted by the presence of water when compared to the other organic acids. This may be due to the higher reaction energy required between ketoacids and TmAmPBr. Other than this, the potential small decrease in reaction efficiency is a reasonable compromise for the large reduction of ion suppression.

Thereafter, a multivariate screening design (two-level fractional factorial design, see **S-2** for factors and factor levels) was performed to determine significant parameters for the derivatization procedure. Earlier studies that evaluated derivatization strategies revealed that various experimental parameters have an effect on the efficiency of derivatization, such as pH of the reaction solution, reaction duration and reaction temperature [94, 275, 284, 287, 288]. Therefore, these parameters were selected as factors for the screening design. Additionally, the stirring speed during the reaction was added as a factor [289]. As previously mentioned, the water content in the reaction mixture was kept constant (~22%). The amount of derivatization reagent was also kept constant at 40 mg/mL to ensure an excess in reagent and thus complete derivatization [290]. The screening design experiments were performed, and the main effects of the five studied variables are presented in the form of Pareto charts (**S-8**). The magnitude of the effects is displayed on the bar charts, and the vertical axis shows the accompanying t-values. The plots indicate that all five investigated factors are above the t-value limit, and are therefore possibly important.

Therefore, for the optimization experiments, all factors were considered. Factor levels, as listed in S.3, were selected in accordance to previous studies [275].

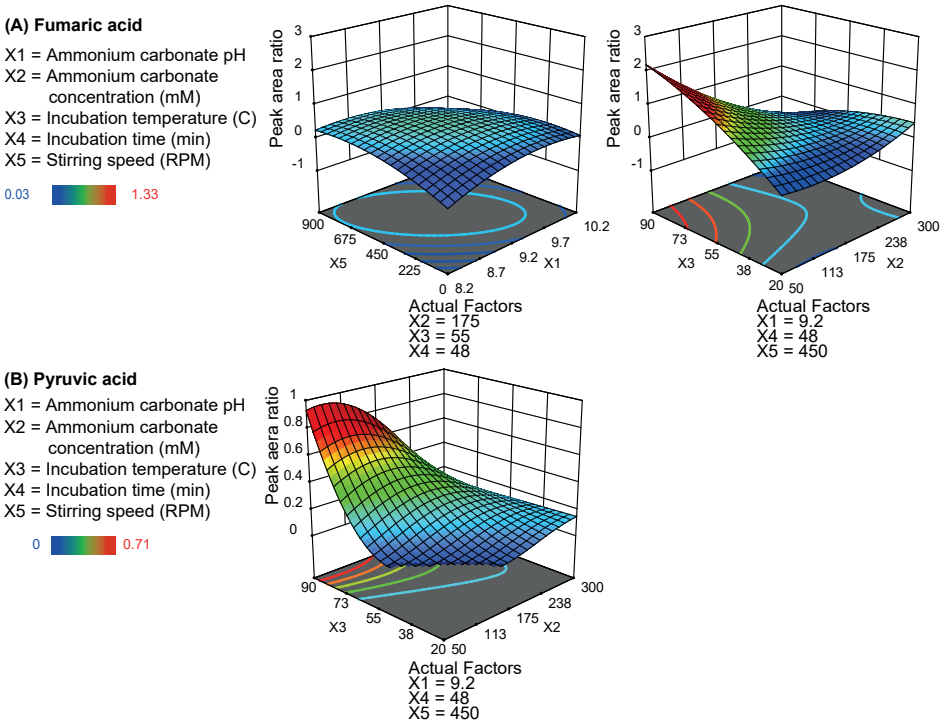

Figure 2. Optimization design 3D surface plots for A) fumaric acid and B) pyruvic acid.

For further optimization of the reaction conditions for derivatization of carboxylic acid metabolites using TmAmPBr. CCD experiments were conducted. Regression analysis was performed to fit the response function, and non-significant terms were eliminated to simplify the models, leading to reduced models for all responses (**S-9**). ANOVA results showed that factor X3 (incubation temperature) and interaction factor between factors X2 and X3 (ammonium carbonate concentration X incubation temperature) were significant for all responses ($p<0.05$) and thus can be considered as the most important factors for the derivatization process. According to analysis of variance (ANOVA) results and 3D response surface plots (**Figure 2**), two-factor interaction effects were assumed. Therefore, a multi-factor optimization is desirable for the optimization for this derivatization procedure. Factor X5 (stirring speed) showed no significant effect for the responses, so was fixed at maximum speed (900 RPM). According to 3D response surface plots (**Figure 2**), a high incubation temperature (>80°C) and incubation time of at least 40 minutes are necessary for formation of derivatized acids. To determine the optimal parameters for the derivatization process, Derringer's desirability multi-criteria decision making was applied [211]. The goal of this optimization was to find a set of conditions with the best compromise between the individual goals, *i.e.* the highest

peak area for all four tested metabolites. Desirability plots combining these goals are shown in **S-10**, where a higher D-value denotes a more favorable option. The optimal response was achieved with ammonium carbonate pH > 9.4, ammonium carbonate concentration between 50 and 100 mM, incubation temperature > 80 °C, incubation time > 40 min, and stirring speed of 900 RPM. Five optimal parameter settings were tested in order to verify the predicted responses and determine the optimal method (option 1: ammonium carbonate pH 10, ammonium carbonate concentration of 65 mM, incubation temperature 90 °C, incubation time of 50 min, and stirring speed of 900 RPM, option 2: ammonium carbonate pH 9.8, ammonium carbonate concentration of 85 mM, incubation temperature 90 °C, incubation time of 75 min, and stirring speed of 900 RPM, option 3: ammonium carbonate pH 9.6, ammonium carbonate concentration of 70 mM, incubation temperature 80 °C, incubation time of 40 min, and stirring speed of 900 RPM, option 4: ammonium carbonate pH 9.4, ammonium carbonate concentration of 55 mM, incubation temperature 90 °C, incubation time of 45 min, and stirring speed of 900 RPM, option 5: ammonium carbonate pH 9.2, ammonium carbonate concentration of 60 mM, incubation temperature 90 °C, incubation time of 40 min, and stirring speed of 900 RPM) for organic acid test mixtures (500 µM) comprised of 11 organic acids (**S-4 and S-5**) with different physicochemical properties. The highest signal intensities for target metabolites were obtained with option 1: ammonium carbonate pH 10, ammonium carbonate concentration of 65 mM, incubation temperature 90 °C, incubation time of 50 min, and stirring speed of 900 RPM. For this reason, these conditions were used for follow-up studies.

Analytical performance evaluation

After determining the optimal conditions for the derivatization of carboxylic acid metabolites with TmAmPBr, the analytical performance of the method (including the derivatization procedure and CE-MS method) was evaluated by preparing dilution series, LODs, LOQs and peak area repeatability for a mixture of test compounds.

Figure 3 shows extracted ion electropherograms obtained for the analysis of derivatized metabolites by CE-MS. As already stated above, two types of derivatives, singly derivatized (mono) and doubly derivatized (di) acids (**S-5**), were observed for most organic acids including fumaric acid, succinic acid, malic acid and glutaric acid, due to the presence of two carboxylic acid groups. On the other hand, for pyruvic acid and lactic acid only the single derivatized acid was detected, as these compounds only contain one carboxylic acid group. For malonic acid, oxaloacetic acid and α-ketoglutaric acid, the mono-form was not detected under the current conditions, or showed a very low response (< 500 counts), and were therefore

excluded from **Figure 3**. For citric acid and isocitric acid, only the single and double derivatized species were detected, whereas theoretically, three labels could be added due to three carboxylic acid groups. The triple derivatized species is not formed, which is possibly due to steric hinderance. In addition, a repulsion effect may occur due to the high density of positive charge caused by the addition of the quaternary amines on TmAmPBr. Interestingly, for compounds malic acid and citric/isocitric acid, multiple peaks were observed for the singly derivatized species. This is possibly due to the asymmetry of the molecules, therefore showing different labelled species. This could be further examined by for instance using MS/MS or ion mobility MS. For the acids which show both single and double derivatized species, the ratio between the mono- and di-form was investigated, and showed to be stable across the linear range 0.7-90 µM (at concentrations <5.7 µM, the mono-form was not detected), e.g 1:3 for fumaric acid and 1:4 for glutaric acid 1:5 for malic acid and 1:4 for succinic acid, for singly:doubly derivatized species, respectively.

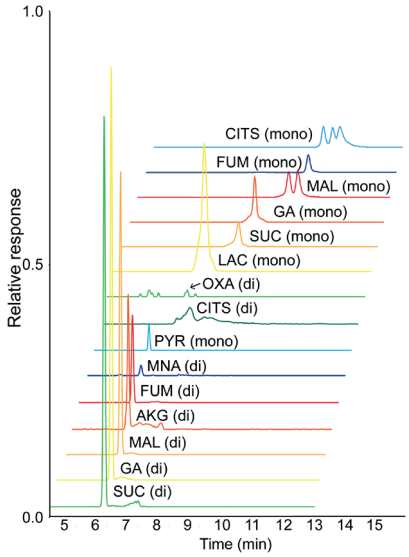

Figure 3. Extracted-ion electropherogram obtained by CE-MS from the analysis of 12 metabolites (90 µM) after derivatization with TmAmPBr. Separation conditions: BGE, 10% (v/v) acetic acid (1.7 M); sample injection volume 27 nL.

For low molecular mass compounds, the CE separation is driven by the difference in ionization in solution. Since the derivatization blocks the ionizable function and brings a permanent charge, the separation is only mediated by the difference in hydrodynamic radius. This explains why all compounds with the same degree of derivatization are migrating in a very narrow time range, e.g. fumaric acid (migration time (MT) 6.64 min), succinic acid (MT 6.63 min) and malic acid (MT 6.65 min). As TOF-MS was used for detection, it was still possible to make a

selective distinction between the closely migrating metabolites. Citric acid and isocitric acid were not completely baseline resolved (resolution = 1.1). However, in this work, the emphasis was on optimization of the derivatization procedure and on its value for the profiling of acidic metabolites in positive ion mode by CE-MS.

The stability of the derivatives was examined by analysing derivatized calibration standards (90 µM) which were stored for 24 hours in the autosampler tray (22 °C), and also for one month at -18 °C and at -80 °C. The samples were then compared against freshly derivatized samples. No difference was observed for peak areas (student's t-test at 95% CI, paired), as expected according to previous research [285].

Table 1. Summary of linear range, R2 value, the F-value of the Lack-of-fit (LOF) test (Fcrit, 95% => 2.74), limit of detection (LOD) and limit of quantification (LOQ), of 8-point calibration lines for metabolite standards. Repeatability was determined for the highest and lowest calibration points. See section 2 for experimental conditions.

Compound	Range (µM)	LOF	LOD (nM)	Repeatability at highest Concentration (n=3) (% RSD)	Repeatability at lowest concentration (n=3) (%RSD)
Pyruvic acid (mono)	0.7-90	0.9	28.7	10.0	7.4
Fumaric acid (di)	0.7-90	1.4	8.2	13.8	5.8
Glutaric acid (di)	0.7-90	0.6	16.4	10.9	14.0
Citric acids (di)*	11.4-90	1.6	52.3	10.7	34.8
Malic acid (di)	0.7-90	1.8	8.8	12.2	10.5
Succinic acid (di)	0.7-90	0.5	89.5	9.1	4.8
Lactic acid (mono)	0.7-90	1.8	72.9	7.1	3.1
Oxaloacetic acid (di)	5.7-90	0.03	610	13.1	16.5
Malonic acid (di)	1.4-90	0.4	56.9	17.0	1.2
α-Ketoglutaric acid (di)	1.4-90	0.1	49.7	21.5	6.4

*isocitric and citric acid (di) were not baseline separated and integrated as one peak

Eight-point dilution series, including replicates in order to include variations in sample preparation, were established for standards, and SIL succinic acid-d_6 (25 µM) was used as internal standard. A linear response (and with R^2>0.961) for model compounds was obtained in the concentration range from 0.7 to 90 µM, and linearity was further evaluated by a lack-of-fit (LOF) test [212], and the method yielded linearity for all test compounds. Repeatability of the derivatization procedure for standards was assessed for replicates based on the CE-MS analyses of standards at two concentration levels (highest and lowest calibration points) (**Table 1**). The repeatability of the derivatization was determined by performing the derivatization procedure in parallel (n=3). Intraday RSD values (n=3) for internal standard corrected peak areas of all analytes were better than 21.5% for the highest calibration point, and below 16.5% for the lowest calibration point, except for the citric acids (36.2%), that were integrated as one peak. Considering

this is the repeatability for both the experimental and analytical variation, the obtained numbers are acceptable.

Detection limits were calculated estimated on the diluton series as 3 σ/slope and 10 σ/slope, respectively (σ = standard deviation of the lowest calibration point), and were ranging from 8.2 to 610 nM for carboxylic acid standards (**Table 1**). Compared to other CE-MS studies detecting carboxylic acids [37, 234], we observed an improvement of 10-100 times in estimated detection limits by using TmAmPBr derivatization. This improvement is probably due to the switch in MS ionization polarity from negative to positive mode, thereby allowing for the use of low pH BGE, bare-fused silica capillaries and a stainless steel ESI needle, creating stable electrospray.

Applicability to mammalian cells

The applicability of the optimized method for the analysis of acidic metabolites in biological samples was evaluated using HepG2 cells as a model system. For this, a dilution series of cell lysate with a methanol/water (8:2, *v*/*v*) mixture was performed, yielding a sample range from 250,000 to 5,000 HepG2 cell lysates per 10 µL. Samples were prepared using the optimized conditions for TmAmPBr derivatization, after which derivatives were analysed by CE-MS. In **Figure 4**, extracted ion electropherograms obtained by CE-MS analysis of carboxylic acid metabolites after derivatization of an extract with a metabolite content equivalent to 25,000 HepG2 cell lysates is shown. Nine out of the eleven model compounds could still be detected in a cell extract with an amount of 25,000 cell lysates, where only the content that corresponds to ~70 cell lysates was injected into the capillary. **Figure 5** shows that a linear response was obtained for the endogenous carboxylic acid concentrations when increasing cell numbers from 5,000 to 250,000, except for citric acids and oxaloacetic acid, which could only be detected in 250,000 and 100,000 HepG2 cell lysates. Repeatability of the derivatization procedure for carboxylic acid metabolites was assessed for replicates based on the CE-MS analyses of extracts at two concentration levels (250,000 cell lysates and 25,000 cell lysates). Intraday RSD values (n=3) for peak areas of all analytes were better than 26.4% and 26.9% for 25.000 cell lysates and 250.000 cell lysates, respectively. For the acids which show both single and double derivatized species, the ratio between the mono- and di-form was also investigated in matrix (250.000 cell lysates), and showed to be stable.

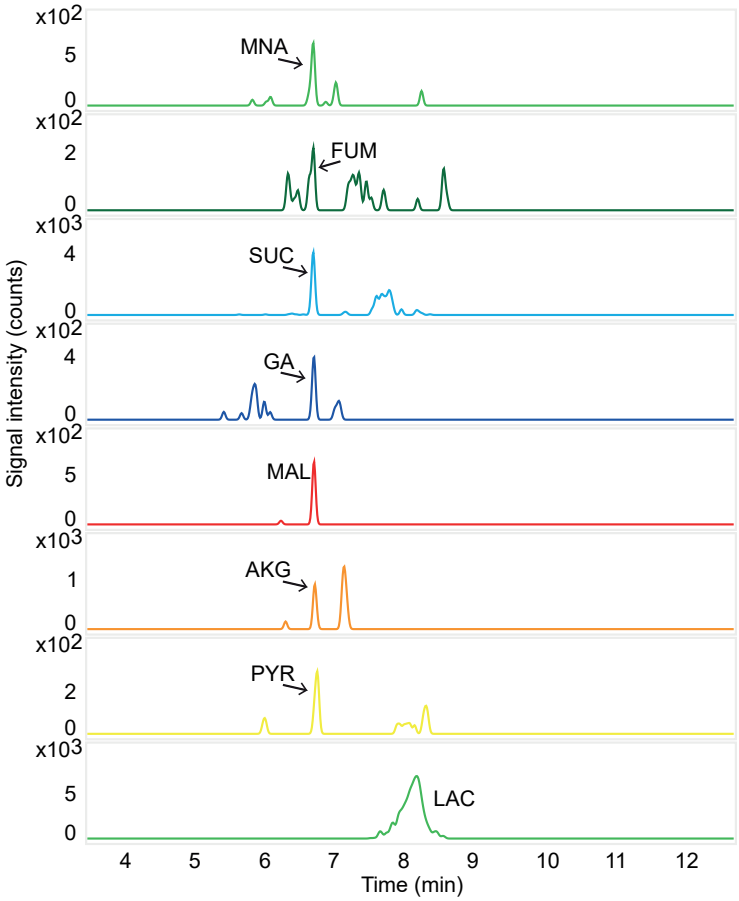

Figure 4. Extracted-ion electropherograms obtained by CE-MS from the analysis of 25,000 HepG2 cell lysates after derivatization with TmAmPBr. Separation conditions: BGE, 10% (v/v) acetic acid (1.7 M); sample injection volume 27 nL.

Matrix effect was evaluated by comparing the response of SIL-internal standard succinic acid-d6 in standard solution to its response in a derivatized matrix. MEF was calculated and used to quantitatively evaluate the matrix effect for each cell calibration sample (ranging from 5000 – 250,000 cell lysates) [291], and was between -0.4% and -0.7%. Table 2 gives an overview of the compounds detected in a HepG2 cell extract sample equivalent to 250,000 cell lysates (corresponding to ~700 cell lysates injected) by CE-MS.

It was investigated whether TmAmPBr could be used for the derivatization of other compounds besides carboxylic acid containing metabolites by extracting additional features using the data obtained for 250,000 HepG2 cell lysates as starting material. The features were provisionally annotated using theoretical *m/z* values based on expected labelling patterns [275]. For amino acids such as asparagine, aspartic acid, gamma-aminobutyric acid, glutamine, glutamic acid, glycine, proline, serine and valine, only a signal was observed for the theoretically determined *m/z* value,

and no signal was observed for *m/z* value corresponding to the non-derivatized species. Therefore, it is expected that besides carboxylic acid groups, also other functional groups (such as amine and thiol groups) could be derivatized using TmAmPBr (**S-11**). However, verification using metabolite standards and further examination of the derivatization labelling patterns is needed in order to improve the ability to simultaneously analyze cations and anions in a single CE-MS run.

Table 2. Concentrations determined for carboxylic acid metabolites in extracts from 250.000 HepG2 cell lysates (corresponding to ~700 cell lysates injected) by CE-MS. ** denotes outlier removal from a single point (Dixon test for outliers at 99% CI, two-tailed test, ©XLSTAT).

Compound	Concentration (µM) ± std in 250.000 cells (n=3)	Repeatability (n=3) in 250.000 cells	Repeatability (n=3) in 25.000 cells
Pyruvic acid	7.4 ± 1.3	19.4	26.4
Fumaric acid	3.6 ± 0.1	11.9** (n=2)	16.6
Glutaric acid	2.8 ± 0.1	15.8	13.1
Malic acid	15± 2.0	15.4	3.8
Succinic acid	7.3 ± 0.9	20.3	12.3
Lactic acid	105 ± 20	17.5	4.9
Oxaloacetic acid	8.8 ± 6.3	25.1** (n=2)	<LLOQ
Malonic acid	55 ± 9.5	17.1	21.8
a-Ketoglutaric acid	7.8 ± 2.7	15.0** (n=2)	6.4
Citric acids*	5.1 ± 0.2	26.9	<LLOQ

*isocitric and citric acid (di) were not baseline separated and integrated as one peak

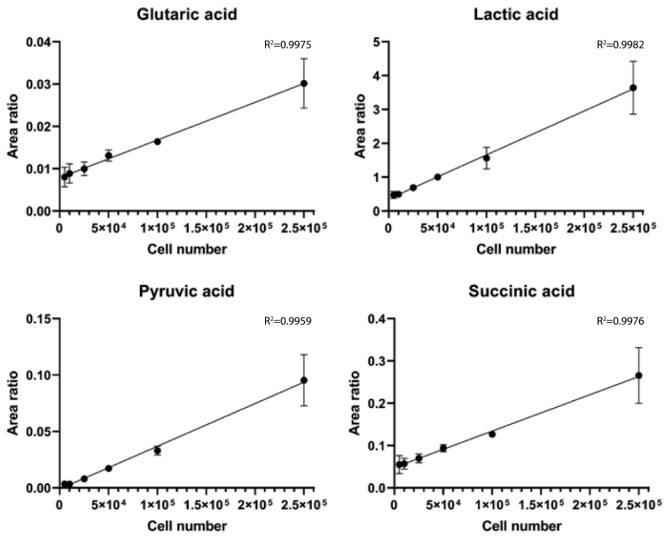

Figure 5. Glutaric acid, lactic acid, pyruvic acid and succinic acid peak area ratios in HepG2 cells (n = 3) ranging from 5000 – 250,000 cells.

Conclusions and perspectives

In this work, we have proposed a novel chemical derivatization approach which enables the analysis of acidic metabolites by CE-MS by using exactly the same conditions as employed for the analysis of basic metabolites. TmAmPBr derivatization of metabolites containing carboxylic acid groups was optimized using RSM, allowing for a multivariate and systematic optimization. The overall method could be used for the quantification of selected acidic metabolites in extracts from 250,000 HepG2 cell lysates, which corresponds to the amount of about 700 cell lysates injected into the capillary.

However, to allow the reliable quantification of a broad range of metabolites, the electrophoretic separation needs to be improved further by using a longer capillary. Moreover, we will investigate the use of SIL internal standards for every metabolite, by using an ICD approach during derivatization [25]. The novel derivatization procedure can be further expanded to a wider selection of metabolites, allowing for the analysis of both acidic and basic metabolites in a single CE-MS run using the same separation conditions. Before that, further evaluation of labelling patterns and potential competition between functional groups (such as amine and thiol groups) when present in the sample matrix is essential. As the focus in the current work was mainly on assessing the utility of the new derivatization agent for analysis of acidic metabolites, limited attention has been paid on improving the resolution of the electrophoretic separation of the acidic metabolites. Resolution could be improved by the use of longer capillaries (obviously resulting in longer separation times) and/or by the use of CE coupled to ion mobility MS (CE-IM-MS) to improve separation between analytes with very similar electrophoretic mobilities [28]. Such aspects will be considered before applying the overall method to the study of biomedical/clinical questions. Moreover, mobility markers procaine and paracetamol will be added after quenching the derivatization procedure in order to be able to determine the electrophoretic mobility, which could significantly aid the identification process in metabolomics, as shown in our recent Metabo-ring study [271]. As the compounds obtain a permanent positive charge after derivatization, it would be interesting to assess the use of electrokinetic injection and also the use of electroextraction for further improving the detection limits [292].

Acknowledgements

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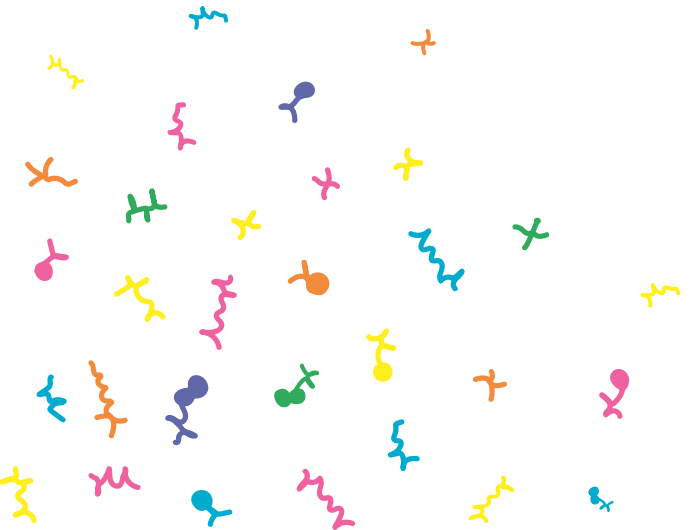
Supporting information

Additional information is available as stated in the text. This information is available free of charge via https://chemistry-europe.onlinelibrary.wiley.com/doi/full/10.1002/ansa.202100054



Chapter 6

Probing the effects of cortisol and specific receptor involvement in zebrafish larvae using a CE-MS metabolomics workflow

Marlien Admiraal-van Mever*, Maricruz Mamani-Huanca*, Erin Faught*, Ángeles López-Gonzálvez, Thomas Hankemeier, Coral Barbas, Marcel J.M. Schaaf, Rawi Ramautar

* Authors contributed equally

Manuscript in preparation

Abstract

The zebrafish (Danio rerio) is increasingly used as a model system in biomedical research. Zebrafish embryos and larvae can fill the gap between cultured cells and mammalian animal models, because they can be obtained in large numbers, are small in size and can easily be manipulated genetically. Given that zebrafish larvae are intrinsically biomass-limited samples, we have developed a capillary electrophoresis-mass spectrometry (CE-MS) workflow for the profiling of (endogenous) metabolites in extracts from individual zebrafish larvae and pools of small numbers of larvae. The developed CE-MS workflow was used to analyse the metabolite levels in extracts from pools of 1, 2, 4, 8, 12, 16, 20 and 40 zebrafish larvae, and for six selected endogenous metabolites a linear response ($R2>0.98$) was obtained in extracts from these pools. The repeatability was satisfactory, with inter-day RSD values for peak area of 9.4-17.7% for biological replicates (n=3 over three days). Furthermore, the method allowed analysis of over 70 endogenous metabolites in a pool of 12 zebrafish larvae, and 29 endogenous metabolites in only an extract from one zebrafish larva. The CE-MS workflow was used to study the role of the mineralocorticoid receptor in mediating the effects of cortisol in wild-type (WT) and ubiquitous MR-knockout (MRKO) zebrafish larvae model systems.

Introduction

Stress can trigger a cascade of stress hormones that produce physiological changes via a multitude of metabolic pathways. The exact biological response to a stressor is highly individualized, as it depends on factors such as genetic background, biological age and personality [293]. In this context, metabolomics, i.e., the analysis of (endogenous) small molecules within a biological system, could be considered a valuable approach to gain a deeper understanding of the molecular responses occurring after exposure to a stressor. The primary stress hormone in humans is cortisol, which is mediated by two nuclear receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors have different affinities for cortisol, with GR being activated at high cortisol levels, and MR being already activated at basal cortisol levels. While the role of cortisol-GR signaling during stress is well characterized [294], the role of MR on mediating the effects of cortisol is less known [295, 296].

Zebrafish (*Danio rerio*) are increasingly used as a model system in biomedical research, mainly because of their genetic and cellular homology with mammals [297]. Other advantages of this species include their rapid development (zebrafish larvae have functioning organs and a complex behavioral repertoire a few days post-fertilization) and high production of offspring (100-200 eggs), allowing for high throughput studies [298, 299]. Furthermore, zebrafish allow to fill the gap between *in vitro* studies and rodent models in predictive animal model studies of human physiology [300]. Within the last decade, zebrafish have proven to be a very effective stress-model [301, 302].

To study metabolism in zebrafish larvae, a number of analytical techniques have been considered, with chromatographic based techniques coupled to MS being the most popular, including RPLC-MS [303], HILIC-MS [304] and GC-MS [305]. Additionally, direct infusion MS [298] and NMR spectroscopy [306] have been used. Given that zebrafish larvae are intrinsically material-limited samples, capillary electrophoresis-(CE)-MS is a very suitable technique for zebrafish larvae analysis, mainly due to its microscale character, low sample consumption and complementary separation mechanism towards chromatographic techniques. A few years ago, the potential of CE-MS was already demonstrated for metabolic profiling of zebrafish larvae by our group [307], using a flow-through micro vial CE-MS interface for the analysis of an extract from a single zebrafish larva (48 h post fertilization). However, only a few metabolites could be detected with that approach and the coupling of CE to MS via this interface was quite complicated; moreover, the reproducibility of this method for metabolomics studies has not been demonstrated so far. In comparison, the use of a conventional co-axial sheath-liquid interface for coupling

CE to MS has proven to be robust and reproducible for metabolomics studies, as shown in a recent Metabo-ring study [271]. Shanmuganathan and co-workers have developed a robust sheath-liquid CE-MS-based workflow for metabolic profiling of more than 1000 serum samples from a multi-ethnic cohort of pregnant women with an acceptable precision [308]. Harada and co-workers used sheath-liquid CE–MS for the comprehensive profiling of charged and polar metabolites in more than 8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52-month period [140]. In our group we have recently demonstrated that sheath-liquid CE-MS is well-suited for the efficient and sensitive profiling of polar ionogenic metabolites in various volume-restricted biological samples, demonstrating the value of this method for probing metabolic changes in biomass-restricted model systems [68, 111].

In this study, a CE-MS-based workflow has been developed for the profiling of polar ionogenic metabolites in extracts from zebrafish larvae in order to study the involvement of MR in mediating the effects of cortisol in zebrafish larvae. For this purpose, wild-type (WT) and ubiquitous MR-knockout (MRKO) zebrafish larvae were used and exposed to exogenous cortisol treatment. So far, it has hardly been investigated what the specific role of MR is during stress on metabolite levels, and which mechanisms might be important in this association.

Experimental section

Chemicals and reagents

All chemicals used were of analytical grade or higher purity. Acetic acid (99-100% m/m), methanol and isopropanol were acquired from Biosolve (Valkenswaard, The Netherlands). Ammonium hydroxide (28–30%) was purchased from Acros Organics (Amsterdam, the Netherlands). Sodium hydroxide (98% m/m) and hydrochloric acid (37% m/m) were from Merck (Darmstadt, Germany), and Thermo Fisher Scientific (Waltham, MA, USA), respectively. Amino acids standards were from Sigma-Aldrich (Steinheim, Germany). ^{13}C and/or ^{15}N stable-isotope labelled (SIL) internal standards were purchased from Cambridge Isotope Laboratories (Apeldoorn, the Netherlands). A Milli-Q Advantage A10 water purification system (Merck, Darmstadt, Germany) was used to obtain pure water. Ascorbic acid was from Sigma-Aldrich (Steinheim, Germany).

Ascorbic acid stock solution was prepared as a 1 mg/mL stock solution in water, and through dilution with water a working solution with a final concentration of 125 µM was prepared. A fresh solution of ascorbic acid was prepared daily.

Background electrolyte (BGE) solution was prepared by diluting acetic acid in water (10% *v*/*v*). As sheath-liquid, a mixture of water and isopropanol (50:50, *v*/*v*), containing 0.03% (*v*/*v*) acetic acid was used. Prior to first use, the sheath liquid was degassed for 10 minutes.

Sample solutions

Stable-isotope-labeled (SIL) metabolite standards (1 mg/mL) were dissolved in a Milli-Q water. SIL metabolite mixtures of 8 compounds (**Table S-1**) were prepared in water as 100 µM stock solutions, aliquoted and stored at -20°C. Migration time markers procaine, paracetamol and methionine sulfone were prepared in water as 100 µM mixed solutions, aliquoted and stored at -20°C. The final concentration of migration time markers was 50 µM.

Zebrafish larvae maintenance and cortisol treatment

Adult zebrafish (*Danio rerio*; AB/Tupfel long fin (ABTL) strain) were maintained in compliance with the directives of the local animal welfare committee of Leiden University. Specifically adults were housed on a recirculating system (Fleuren & Nooijen, The Netherlands) on a 14:10 light:dark cycle. Water was maintained at 28.5 °C, pH 7.4, 300 µS conductivity, and 10% of the water was exchanged daily. Animals were fed twice daily with Gemma micro 500 diet (Skretting, Norway) in the morning and live Artemia (Great Salt Lake Brine Shrimp Cooperative, USA) in the afternoon.

A zebrafish strain (*Danio rerio*) wild type (WT) and zebrafish mutants lacking nr3c2 ($^{inr11/inr11}$; hereafter MRKO). WT and MRKO zebrafish larvae were maintained at 28.5 ºC in 20 x 100 mm plates with 1x embryo medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4 + 0.1 ppm methylene blue antifungal agent), with daily water changes until 3 days post-fertilization (dpf), according to guidelines of the Zebrafish Model Organism Database (zfin.org). At 3 dpf, larvae were transferred to 6-well plates (20 fish/well) and treated with either cortisol (5 µg/mL) or a vehicle control (0.05% ethanol). The larvae were then raised to 5 dpf, and all treatments were refreshed daily. Larvae were euthanized with an overdose of MS-222 (0.4 mg/L, Sigma). A pool of 1, 2, 4, 8, 12, 16, 20 and 40 larvae were collected, and each pool was considered a sample.

Experimental design

To study the role of MR in the metabolic response to cortisol in zebrafish larvae, a workflow using CE-MS has been developed and the following experimental design has been designed (**Figure 1**). First, homogenization and metabolite extraction were optimized, then the number of larvae per sample was set. Finally, four groups

have been used to survey the involvement of MR. For this purpose, WT zebrafish larvae and MRKO zebrafish larvae were used, both groups were stimulated with 5 µg/mL cortisol (F) (WT-F and MRKO-F) and by 0.05 % ethanol as a control-vehicle (V) (WT-V and MRKO-V). Ten samples per group divided into two batches were used, however, due to a pressure error during sample analysis, the second batch was eliminated.

Zebrafish larvae homogenization and metabolite extraction

To determine the most optimal metabolite extraction protocol, three homogenization strategies were compared for zebrafish larval samples, and were carried out using homogenization based on probe homogenization (strategy 1), homogenization using a Bullet Blender (strategy 2), and homogenization based on tip sonication (strategy 3). The conditions for each of the strategies are shown in **Table 1** and each was repeated three times. Three steps were applied in each experiment, for which groups of zebrafish larvae containing 8 zebrafish and 50 µl of methanol in a 1.5 ml Eppendorf vial were used. First, the extraction solvent was added. The second step was homogenization of the samples, and finally in the third step the samples were evaporated and reconstituted for analysis.

After homogenization, the homogenate was centrifuged (16,100 × *g*., 5 min, 4 °C), and 450 µL (strategy 1) or 90 µL (strategies 2 and 3) supernatant was pipetted into a 0.5 µL Eppendorf vial. The samples were evaporated in a SpeedVac Vacuum concentrator (Thermo Savant SC210A, Waltham, Massachusetts, United States), and reconstituted in 20 µL BGE prior to injection.

To determine the recovery of each strategy, SIL internal standards were spiked in the zebrafish larvae samples before homogenization and after sample preparation. Recovery was calculated based on **equation 1**:

$$\% \text{ Recovery} = \text{Area IS spiked before} / \text{Area IS spiked after} * 100\% \quad (1)$$

Table 1. Conditions used for each strategy to determine the best method of metabolite extraction from zebrafish larvae.

Strategy	Extraction Solvent	Sample homogenization
1	350 µL cold methanol 80 µL cold water 10 µL ascorbic acid (125 µM) 10 µL water with IS (100 µM)	Homogenizer (1000 × g, 30 s)
2	30 µL cold methanol 10 µL ascorbic acid (125 µM) 10 µL water with IS (100 µM)	Bullet blender (500 × g, 5 min)
3	30 µL cold methanol 10 µL ascorbic acid (125 µM) 10 µL water with IS (100 µM)	Tip sonication (1 x 3 s pulse, 30% amplitude)

(IS) internal standards (stable-isotope-labeled (SIL) metabolite standards and procaine, paracetamol and methionine sulfone)

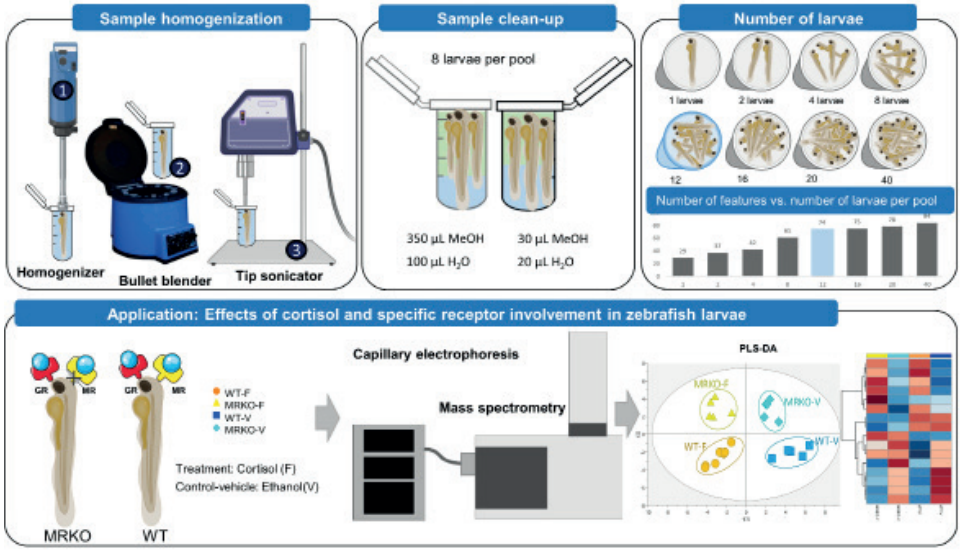


Figure 1. Workflow developed to study the effects of cortisol and involvement of specific receptors in zebrafish larvae using CE-MS.

CE-MS-based metabolic profiling of Zebrafish larvae extracts

Metabolic profiling was carried out with the 7100 CE system from Agilent Technologies (Waldbronn, Germany) which was coupled to a 6230 TOF-MS instrument from Agilent (Santa Clara, CA, USA) utilizing a co-axial sheath-liquid ESI interface equipped with a triple-tube sprayer. Sheath-liquid was delivered at a flow rate of 3 µL/min by an Agilent 1260 Infinity Isocratic Pump (Agilent Technologies) with a flow splitter that splits the sheath liquid in the ratio 1:100. Fused-silica capillaries with an internal diameter of 50 µm were purchased from BGB Analytik (Harderwijk, The Netherlands), and cut to a total length of 90 cm. New capillaries were conditioned by subsequently rinsing, at 5 bar for 1 minute, with methanol, water, sodium hydroxide 1M, water, hydrochloric acid 1M, water and background electrolyte (BGE). Injections were performed hydrodynamically for 54 s at 50 mbar (35.3 nL), and injected volumes were calculated with Zeecalc v1.0b (https://epgl.unige.ch/labs/fanal/zeecalc). For metabolite separation, a voltage of 30 kV was applied. The current observed during the experiment under these conditions was 12.9 µA. MS experiments were acquired in positive mode between 50 and m/z 1000 with an acquisition rate of 1.5 spectra/s. The nebulizer gas was set to 0 psi, and the sheath gas flow rate and temperature were set at 11 L/min and 100 °C, respectively and both were nitrogen. The ESI capillary voltage was set to 5500 V. Fragmentor and skimmer voltages were set at 100 V and 50 V, respectively. In order to obtain information about fragments, in-source fragmentation was used, applying 200 V at the fragmentor. MassHunter version B.06.00 (Agilent, Santa Clara, CA, USA) was used for data acquisition, instrument

control and data treatment. From the sheath-liquid, isopropanol ($C_3H_8¬OH^+$) and its clusters ($[(C_3H_8¬O)_2 +H]^+$ and $([(C_3H_8¬O)_3 +H]^+$) with corresponding m/z values of 61.06479, 121.12231 and 181.17982, respectively, were used as lock masses.

Data processing

Peak extraction was performed using MassHunter Quantitative Analysis (B.10.00, Agilent Technologies) with a mass error of 20 ppm and peak integrations were visually inspected to ensure correct integration. The response function of the measured peak area for 6 endogenous metabolites of which stable-isotope-labeled (SIL) internal standards were available for each was examined using 3 biological replicates for at least 8 different numbers of zebrafish larvae pools (from 40 larvae down to 1 larva). As internal standard, accompanying SIL standards (50 µM) were used. Repeatability (expressed as percentage relative standard deviation, % RSD) for peak area was determined by analyzing SIL standards spiked into a zebrafish sample (n=3). The last experiment has been repeated on three consecutive days to determine interday precision.

The data deconvolution process for the untargeted metabolomics was processed using MassHunter Profinder (B.10.00, Agilent Technologies), to obtain a data matrix in an appropriate format. Two consecutive algorithms were used in the software to perform the deconvolution. The first, performs the Molecular Features Extraction (MFE), reduces the data by removing non-specified information. The second algorithm, Find by Ion (FbI) was used for targeted feature extraction and to obtain a better accuracy of the data. Finally, abundance of the molecule, mass accuracy and migration time for each feature in all samples were obtained as matrix data [309]. Data quality was ensured by excluding background noise and unrelated ions, molecular features present in 50% of the quality control (QC) injections with a relative standard deviation (RSD) less than 30%, and present in 75% of the study samples, were maintained. Missing values were estimated using the k-nearest neighbours (kNN) algorithm.

Subsequently, the matrix was normalized by the total useful signal (TUS), then an unsupervised principal component analysis (PCA-X) model was generated in order to observe the trend of the QCs, detect outliers and observe natural clustering trends.

Statistical analysis

Multivariate data analysis (MVDA) was used to investigate the differences between the four groups. supervised models such as the partial least square discriminant analysis (PLS-DA) and orthogonal partial least square discriminant analysis (OPLS-

DA) were performed to discriminate the variation between groups. The quality of the models was evaluated by explained variance (R^2) evaluating the model by maximizing the variance given by the variables and predicted variance (Q^2) evaluating the predictive ability of the model. These parameters were provided by SIMCA-P (Version 17, Umetrics, Sartorius Stedim Biotech) software. Finally, the percentage change was calculated by comparing cases with controls. Following biomarker selection, a receiver operating characteristic (ROC) curve analysis was applied to the identified potential biomarker using the biomarker analysis module in the MetaboAnalyst 5.0 webserver. In addition, univariante ROC curve analysis was applied to calculate the area under the curve (AUC) for the characteristics identified from the comparison of MRKO-F vs WT-F, this was followed by a multivariate ROC curve analysis that was used to calculate AUCs for the combination of more than two characteristic metabolites. The ROC curves were generated by Monte-Carlo cross validation (MCCV) using balanced sub-sampling. For each MCCV at least two-thirds of samples were used to select significant features that were used to build the classification model. To validate the classification model, the remaining one-third of the samples were used to validate the classification model. The support vector machines (SVM), method was selected as the feature classification and ranking method and used the top six metabolites to generated a classification model.

Metabolite identification

Compounds were tentatively annotated based on their exact mass (maximum error of 20 ppm), isotopic distribution and adduct formation using the CEU Mass Mediator search tool [310]. Furthermore, to enhance confidence in metabolite identification, relative migration times (RMT), effective mobility values (μ_{eff}) and in-source fragments were compared with respect to standards analysed in a previous experiment [271, 311].

Results and discussion

CE-MS method development

In metabolomics studies, typically, the efficient homogenization of tissues and extraction of metabolites from tissue homogenates are the steps that are most time-consuming and error-prone [312]. Therefore, as a first step, the homogenization and metabolite extraction were evaluated for zebrafish larvae samples. For metabolite extraction, a single-phase extraction method based on protein precipitation my methanol was selected due to its effective capability to precipitate proteins [313, 314], speed and simplicity [315]. As the focus of the current study was solely on the polar metabolome, a single-phase extraction method is sufficient to extract

all the metabolites of interest. However, when the goal is to perform a global metabolomics study on zebrafish samples with interest on both polar and nonpolar metabolites of the same sample, a two-phase extraction protocol might be more beneficial (i.e., Bligh&Dyer extraction), as such an extraction protocol allows the analysis of both polar (aqueous layer) and nonpolar metabolites (chloroform layer) within the same sample, using different analytical platforms [316].

After selection of the extraction strategy, three different homogenization strategies were tested, i.e., homogenization based on standard homogenization (strategy 1), homogenization using a Bullet Blender (strategy 2) [11, 20], and homogenization based on tip sonication (strategy 3) [21]. All three strategies were applied to small pools of zebrafish larvae (8 larvae per pool) of 5 dpf. A selection of SIL standards (50 µM) (**Figure S-1**) was spiked into the zebrafish larvae sample before and after sample preparation to determine the recovery and repeatability of the different strategies. After complete homogenization, homogenates were collected. After metabolite extraction, the metabolite content of the homogenates was analyzed using CE-MS, whereafter recoveries of SIL standards were calculated. As shown in Table S-2, satisfactory recoveries were observed for all three homogenization strategies: between 84-98% for strategy 1, between 88-107% for strategy 2 and between 100-104% for strategy 3. The repeatability of peak areas of SIL standards was determined for SIL standards spiked at the beginning of the workflow for biological replicates as the relative standard deviation (%RSD), and was between 6.3-17.3% for strategy 1, between 4.5-15.0% for strategy 2 and between 3.1-8.4% for strategy 3.

Another important aspect during tissue homogenization is metabolite stability, as degradation could occur due to time duration, (high) temperatures and the applied frequency. As is it expected that catecholamines such as epinephrine and dopamine play an important role in mediating the stress response in zebrafish larvae [317], precautions were taken by adding an antioxidant at the beginning of the workflow in order to stabilize metabolites throughout the sample preparation. As antioxidant, ascorbic acid (12.5 µM) was selected due to its compatibility with MS detection [187]. Additionally, at pH 2.2, ascorbic acid will have no net charge, causing it to migrate with the EOF and thereby not disturbing the detection of cationic metabolites of interest. To evaluate the metabolite stability during sample preparation, two catecholamines epinephrine and dopamine, which are well-known metabolites that are prone to degradation, were included in the SIL standard mixture. As is shown in **Table S-2**, good recoveries for epinephrine and dopamine were observed for all homogenization strategies, with 96% and 98% for strategy 1, 88% and 96% for strategy 2 and 101% and 101% for strategy 3, which indicates there is no significant loss during sample preparation.

Furthermore, when selecting an appropriate sample preparation strategy, the time duration per sample is an important factor. Due to the size of the homogenizer tip for strategy 1, a minimal total volume of 500 µL needs to be present in the Eppendorf vial in order to perform proper homogenization, while in case of the Bullet Blender and tip homogenizer, 100 µL of total volume showed to be sufficient. After homogenization, either 450 µL (strategy 1) or 90 µL (strategies 2 and 3) of supernatant was transferred into another Eppendorf vial and dried in a Speedvac. Because the increased volume of strategy 1, the time for evaporation was more than tripled (2+ hours instead of 30 minutes). Additionally, because zebrafish larvae at 5 dpf encompass approximately 300 nL volume, it would be most beneficial to have as little dilution as possible during sample preparation. When comparing the time duration per sample for strategies 2 and 3, homogenization by the Bullet Blender takes 5 minutes per 24 samples (~13 seconds per sample), while tip sonication takes 3 seconds per sample. Taking into account the recovery and repeatability as well as the duration of the sample preparation strategy, strategy 3 (homogenization by tip sonication) was selected for further experiments.

The analytical performance of the CE-MS workflow was further evaluated for pools containing a small number of zebrafish larvae (8 fish per pool). Precision of the CE-MS workflow for profiling of endogenous metabolites in zebrafish larvae was assessed based on the repeated analyses of zebrafish samples spiked with glutamine-^{13}C as SIL internal standard (50 µM). Intraday RSD values (n=3) for corrected peak areas of all endogenous analytes for biological replicates were between 0.9-20.3%, while interday RSDs (n=3 over 3 days) were below 28.2% for 20 detected endogenous metabolites (**Table S-3**). Migration time repeatability was assessed by converting to effective mobilities (μ_{eff}), the repeatability was below 1.3% for interday analysis.

Subsequently, the response function of endogenous metabolites in decreasing numbers of zebrafish larvae per pool were investigated using SIL standards. Zebrafish pools of 40, 20, 16, 12, 8, 4, 2 and 1 zebrafish were spiked with SIL epinephrine-D_3, gamma-aminobutyric acid-D_6, glutamine-^{13}C, glutamic acid-^{13}C,^{15}N, asparagine-^{13}C,^{15}N, lysine-^{13}C and aspartic acid-^{13}C,^{15}N (50 µM), and calibration curves for endogenous metabolites corrected by their corresponding SIL analogue were prepared (in case the endogenous metabolites were detected). A linear response (and with R^2>0.98) for selected metabolites was obtained (**Figure 2A**). Thereafter, it was investigated how many features could be extracted from the data resulting from the different zebrafish pools. As can be seen in **Figure 2B**, 84 features were found in a pool of 40 zebrafish larvae, whereas only 29 features could be extracted from a single zebrafish larva. For this study, the zebrafish pools containing 12 larvae were selected for further experiments, as a lower amount of larvae showed

to result in more efficient metabolite homogenization when employing low solvent volumes during sample preparation. Furthermore, for our application it was not necessary to show inter-individual differences between the fish, therefore making it unnecessary to further scale down the amount of fish per pool.

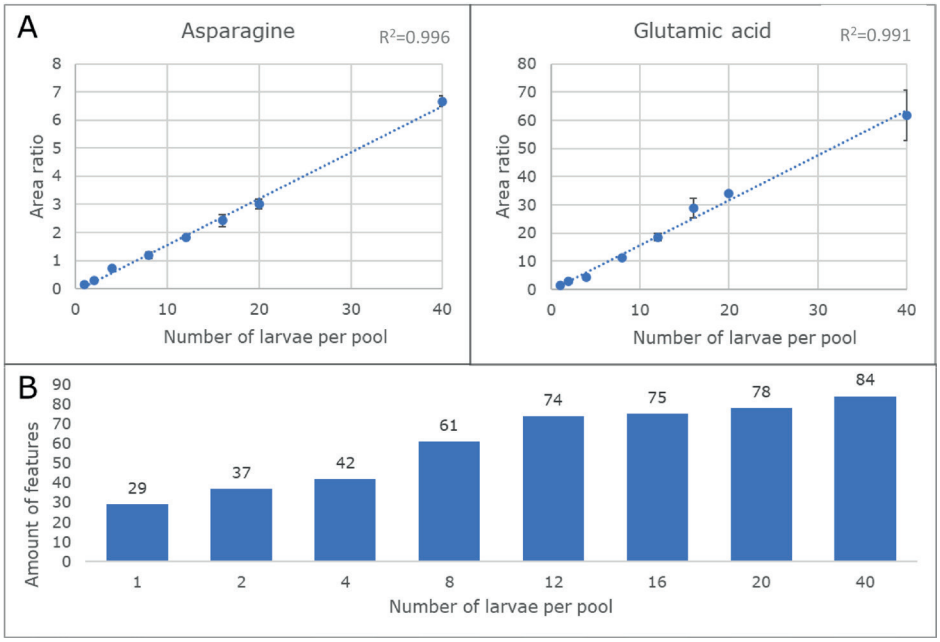


Figure 2. A) Response function for lysine, asparagine, glutamic acid, and aspartic acid in different zebrafish larvae pools as determined using CE-MS, and B) Number of features that could be extracted from CE-MS data obtained after analysis of zebrafish larvae pools.

Application of CE-MS to a zebrafish stress model

With the developed CE-MS workflow the aim was to study the role of polar ionogenic metabolites in zebrafish under a stress scenario and thus to potentially reveal the role of MR in mediating the effects of cortisol. After sample analysis, data processing was performed, which revealed 139 metabolic features. The signals were then filtered for presence in blanks, in clusters and in QC samples with a % RSD less than 30%. Then the signals belonging to adducts and fragments were removed. The final data set was reduced to 61 compounds (Table S-4). The analytical performance of CE-MS was evaluated using the unsupervised PCA-X (R2= 0.448), resulting in a clustering of quality control samples (QC's) distributed along the working list, showing high sensitivity and reproducibility throughout the analysis, indicating adequate data quality performance (**Figure 3A**).

Additionally, a supervised clustering PLS-DA model was constructed to obtain a better separation. The PLS-DA analysis score plot showed a clear separation between the four groups of cortisol-stimulated zebrafish larvae and controls.

The PLS-DA model had an R2 value of 0.611 and Q2 value of 0.487 (**Figure 3B**). After this evaluation, OPLS-DA analysis was performed to compare two groups at a time. Two comparisons were performed. First, we compared the group of Wild type zebrafish larvae stimulated with cortisol (WT-F) versus the group of Wild type zebrafish larvae stimulated with ethanol as control (WT-V), the model presented an R2 value of 0.987 and Q2 value of 0.841 (**Figure 3C**). Then, the groups of MR- Knockout zebrafish larvae stimulated with cortisol (MRKO-F) versus the group groups of MR- Knockout zebrafish larvae stimulated with ethanol (MRKO-V) as a control were compared, the model presented an R2 value of 0.998 and Q2 value of 0.736 (**Figure 3D**).

To select the metabolites responsible for the clustering and separation of the groups from the OPLS-DA models, Jack-Knife confidence intervals were calculated, variables importance in projection (VIP >1) and p(corr) >|0.5| were considered. In addition, for all OPLS-DA models, the validated cross-sectional scores (CV-scores) plots were shown, which revealed that 99% of the samples were correctly classified in their corresponding group. The results for the WT-F to WT-V and MRKO-F to MRKO-V comparisons of multivariate statistics are tabulated in Table 2.

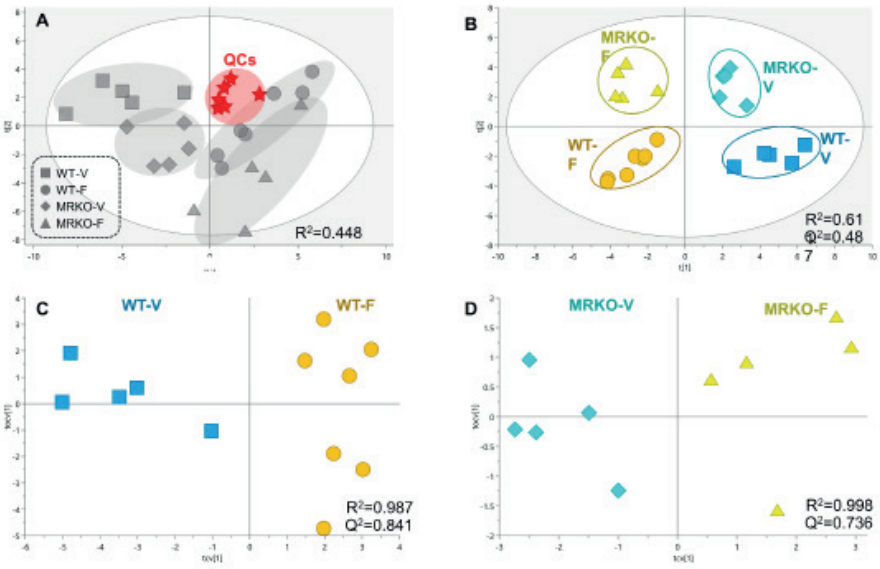


Figure 3. A) Principal component analysis (PCA-X) score plot with an explained variance R2 = 0.448, quality control (QC, red starts), B) PLS-DA model, C) CV-OPLS-DA model for WT-F (n=7) vs. WT-V (n=5) comparison, D) CV-OPLS-DA model for MRKO-F (n = 5) vs. MRKO-V (n=5) comparison.

Table 2. Differentially identified metabolite contents in the comparison of cortisol-stimulated zebrafish larval groups and control groups.

Compound	Mass (Da)	RSD (%) QC	p(corr)	VIP	JK	change (%)	p(corr)	VIP	JK	change (%)
Sarcosine	89.0475	8.0	0.9	1.6	Yes	58.8	0.9	1.8	Yes	47.5
Alanine	89.0476	2.4	0.9	1.4	Yes	23.2	0.8	1.7	Yes	12.8
Serine	105.0420	4.3	-1	1.6	Yes	-37.6	-0.8	1.7	Yes	-23.4
Cytosine	111.0427	27.6	0.7	1.3	Yes	83.9	0.5	1.1	-	47.2
Asparagine	132.0527	3.8	-0.8	1.4	Yes	-23.2	-0.6	1.1	-	-9.5
Ornithine	132.0890	7.2	-0.9	1.6	Yes	-66.6	-0.9	1.7	Yes	-49
Aspartic acid	133.0368	4.6	-0.5	1.0	-	-15.5	-0.7	1.6	Yes	-37.7
Acetylcholine	145.1091	6.0	NS	<1	-	NS	0.6	1.3	Yes	36.1
N_1-Acetylspermidine	187.1670	10.2	0.7	1.2	-	50.2	0.6	1.4	Yes	56.5
Cystathionine	222.0692	9.1	-0.6	1.2	Yes	-26.8	NS	<1	-	NS
Cytidine	243.0845	12.3	0.7	1.3	Yes	47.6	0.6	1.2	-	45
Aspartyllysine*	261.1314	20.5	-0.7	1.2	Yes	-49.9	-0.6	1.3	-	-40.6
Glutathione disulfide	612.1478	27.4	-0.8	1.4	Yes	-54.6	NS	<1	-	NS

(NS) Not significant, and (*) Contains multiple annotation options

To evaluate the regulation of MR and GR in response to cortisol stress in zebrafish larvae. The WT-F group was compared to WT-V, as a result 12 metabolites appeared to be modulated in response to stress. Increases in sarcosine, alanine, cytosine, N-acetylspermidine and cytidine levels were observed. In contrast, levels of serine, asparagine, ornithine, aspartic acid, cystathione, gutation disulfide and a dipeptide (Asp-Lys) were decreased (**Table 2**). On the other hand, MRKO-F larvae were compared with MRKO-V larvae. As a result, 11 metabolites were modulated. Increases in sarcosine, alanine, cytosine, acetylcholine, N-acetylspermidine and cytidine levels were observed.

However, decreased levels of serine, ornithine, aspartic acid, glutathione disulfide and a dipeptide (Asp-Lys) were observed (**Table 2**). Those metabolites that are altered in WT but not in MRKO were observed to highlight MR-dependent metabolites.

Interpretation of the main findings

Currently, zebrafish are used as a complement to rodent models to study neuropsychiatric disorders [31], because of their similarity to the manifestations of neuroendocrine mechanisms. So far, numerous behavioral and transcriptomic studies on zebrafish larvae in response to different stimuli have shown interesting results [32-34]. Cortisol is the main stress hormone and binds with different affinities to both GR and MR. High levels of cortisol bind to GR, whereas at basal levels of cortisol it binds to MR [35]. Even though the role of cortisol-GR signaling is well characterized, the role of MR is still less known. The metabolomic profile could help in understanding the role of both receptors under stress. Therefore, we present here an untargeted study based on CE-MS to determine the role of polar metabolites in mediating the GR and MR response to stress.

To evaluate the involvement of GR and MR in response to stress, we studied the effect of cortisol on the metabolome of both WT and MRKO zebrafish larvae. Metabolites

that were found to be commonly modulated following cortisol stimulation in both WT and MRKO zebrafish larvae compared to their respective controls were: alanine, sarcosine, serine, cytosine, asparagine, ornithine, aspartate, N-acetyl-spermidine, cytidine, and aspartyl-lysine. In MRKO, the independent role of GR in response to cortisol has been observed. However, it was observed that the involvement of the MR receptor in WT fish larvae cooperates in the modulation of these metabolites, showing slight increases in the percentage changes in the case of sarcosine, alanine, cytosine, N-acetylspermidine, and cytidine, as well as slight decreases in the levels of serine, asparagine, ornithine, and aspartyl-lysine (**Table 2**). These findings support the essential functions described for GR in energy homeostasis, stress response, and inflammation. Thus, it has also been observed that the cooperation between GR and MR is important for homeostasis.

Cortisol-generated stress has been associated with increased reactive oxygen species (ROS) and nitrogen species (RNS) leading to oxidative and nitrosative stress [36]. Ornithine is a substrate for putrescine production and complements polyamine metabolism, which may explain the decrease in ornithine levels during both GR activation and GR:MR cooperation in response to stress. Polyamines such as putrescine and spermidine are essential for decreasing reactive oxygen spice (ROS) damage in animals [37] and for protecting from stress, allowing the flow of K1 current, controlling brain glutamate receptors implicated in learning and memory [38]. On other hand, we found higher increased levels of N1-acetylspermidine when GR is activated, however, we did not find alterations in putrescine and spermidine levels. Spermidine is a substrate of spermidine/spermine N^1-acetyltransferas (SSAT), which is responsible for promoting acetylation at N1 in the presence of acetyl-CoA resulting in N1-acetylspermidine which in turn is converted to putrescine. These results suggested that GR promotes the acetylation of spermidine by altering the regulatory metabolism of polyamines [39]. In addition, ornithine is a substrate for proline production and contributes to the formation of glutamate. Glutamate is the main excitatory neurotransmitter in the central nervous system, and its regulation is important for maintaining the balance of the glutamate cycle. It has been reported that glutamatergic dysfunction causes neuropsychiatric conditions such as stress, anxiety and depression [40].

As previously mentioned, cortisol exposure causes increased oxidative stress resulting from the generation of ROS contributing to neuronal cell death. Glutathione (GSH) acts as an antioxidant defense and is responsible for the direct scavenging of ROS. During ROS scavenging, glutathione interacts with radicals such as superoxide radical anion, nitric oxide, or hydroxyl radical. On the other hand, GSH is an electron donor for the reduction of peroxides in the GPx reaction with the final oxidation product being glutathione disulfide (GSSH). However, GSH is

regenerated from GSSH through the reaction catalyzed by glutathione reductase [44]. In our study, decreased levels of GSSH were found in response against stimulation in WT fish larvae, whereas in MRKO the percentage change was not significant. This suggests the importance of the role of MR related to the activation of the antioxidant protection system.

Another metabolite that was modulated by the effects of stress was an N-methylated derivative of glycine. Sarcosine is produced from glycine via Glycine N-methyltransferase which utilizes a methyl group from S-adenosylmethionine (SAM) [45]. Sarcosine has been described to be an inhibitor of glycine transporter-1, increasing the levels of glycine, an inhibitor of rapid neurotransmission. Sarcosine is also responsible for enhancing the function of the N-methyl-D-aspartate receptor [45]. Previous studies have shown that sarcosine has a neuroprotective effect as it is effective in reducing oxidative stress [46]. These findings could justify the increase in sarcosine levels in response to stress during GR and MR activation and their cooperation with each other.

Moreover, decreases in asparagine and aspartate levels have been observed in response to cortisol stimulation in both GR activation and GR:MR involvement. Aspartate is converted to asparagine through asparagine synthetase using glutamine in response to cellular stress [41]. Glutamate and aspartate are both excitatory neurotransmitters, with aspartate as the most abundant in the central nervous system. Additionally, aspartate is converted to alanine via aspartate 4-decarboxylase [42, 43].

On the other hand, acetylcholine levels were found to be increased under stress conditions in MRKO-F compared to MRKO-V, whereas in WT-F compared to WT-V acetylcholine was not significant. This could suggest the involvement of GR in mediating acetylcholine levels. Acetylcholine is the main neurotransmitter reported to be released under stress conditions. These findings are consistent with previous work in which elevated levels of acetylcholine were observed in depressed individualS.

Interestingly, serine levels were found to be significantly decreased in WT-F and MRKO compared to their respective controls. Serine is a precursor of phospholipids, ATPs, and nucleic acids; serine is also used to produce SAM, which is responsible for donating ubiquitinated methyl groups to produce NADPH, which maintains cellular redox homeostasis [48].

These results suggest that the regulation of the balance between redox reactions within a cell depends on GR and MR and their cooperation together.

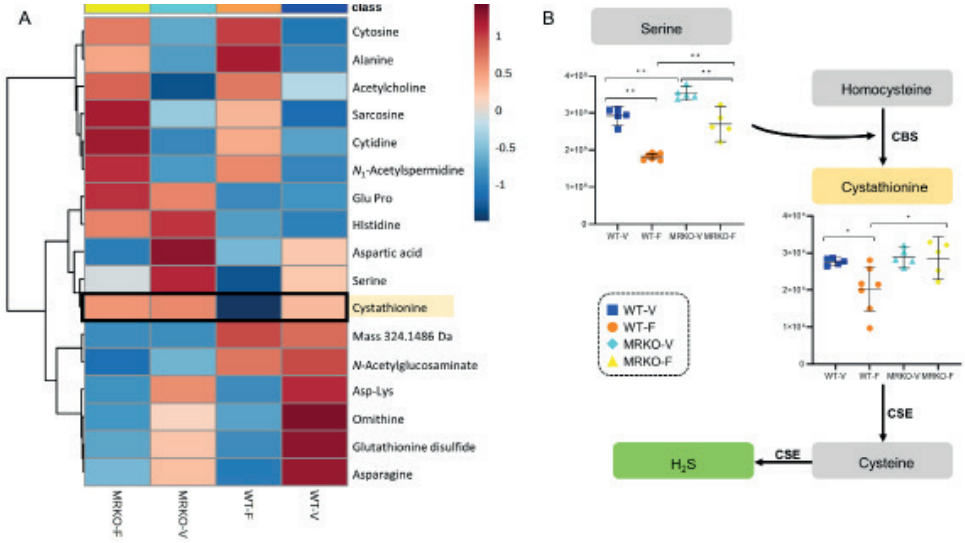


Figure 4. A) Heatmap of the 16 differentially modulated metabolites in metabolomic analysis of cortisol-stimulated WT or MRKO versus their respective controls. In each column the average concentrations of the samples, and each row represents a metabolite. The color coding within the heatmap represents the relative fold change of each metabolite between groups. Metrics used for the analysis were the Euclidean distance measure and Ward's clustering algorithm using MetaboAnalyst 5.0 software. B) Biogenesis of H2S by CSE or CBS.

In contrast to all metabolites, cystathionine regulation was observed to be MR-dependent only. Cystathionine is produced by cystathionine beta synthase (CBS) an enzyme that catalyses the first step of the transsulfuration pathway, leading to the production of cystathionine from serine and homocysteine. It has been described that under stress cystathionine levels are decreased consistent with what was observed in cortisol treated WT larvae in this study. Here we show that this decrease is likely due to MR as the effect of cortisol on cystathionine was abolished when MR was absent. Cystathionine can then be converted to cysteine and by extension H2S by the enzyme cystathionine gamma lyase (CSE) to produce H2S from cysteine and homocysteine (**Figure 4**) [49]. H2S is associated with many physiological processes, such as inflammation, neuromodulation and apoptosis and abnormal levels are associated with Alzheimer's diseases, liver cirrhosis and diabetes [50]. The data here suggests that MR may be a key regulator of enzymes involved in cystathionine production and subsequently impact key physiological processes.

Finally, in this study we have shown the role of MR in the response to cortisol at the metabolic level, which is consistent with a metabolic role previously described for MR in zebrafish larvae [47].

Conclusions and perspectives

In this work, a CE-MS-based analytical workflow has been developed for the profiling of polar ionogenic metabolites in extracts from zebrafish larvae with acceptable performance metrics. The workflow has been used successfully used for the profiling of metabolites in extracts from wild-type and zebrafish with a ubiquitous MR-knockout, providing insights into the role of MR and GR in mediating the effects of cortisol.

Through the present study, besides the ability of GR to modulate metabolism independently of MR, we observed the cooperation of both receptors in maintaining the balance of the metabolome, and that part of this profile is regulated by MR. Indeed, differential regulation of cystathionine by MR has been identified, and although this metabolite is known to be modulated during stress. Taken together, this technique has the potential to identify novel pathways and mechanisms of action in zebrafish larvae.

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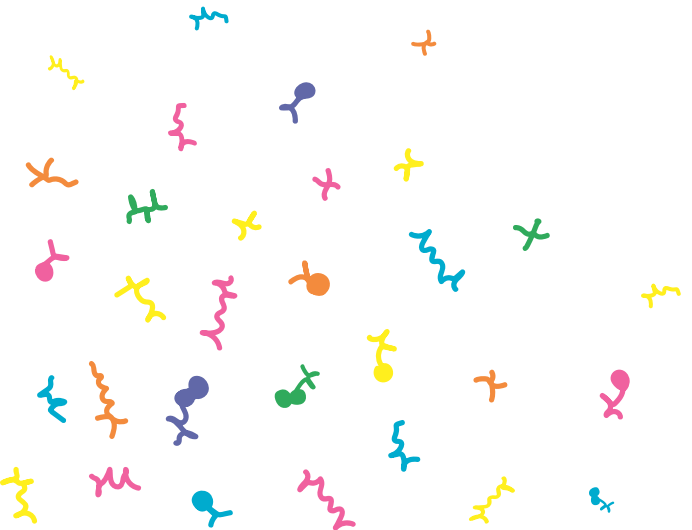
Supplementary information

The supplementary files are available upon request.



Chapter 7

Concluding remarks and perspectives

The ultimate aim of metabolomics research is to obtain an answer to a well-defined biomedical or clinical question. At present, the analytical techniques commonly employed for metabolomics studies fall short for addressing volume-restricted biological questions. Therefore, in this thesis, the key aim was the development of reliable and sensitive microscale analytical methods and workflows in order to enable the study of questions intrinsically dealing with low sample amounts. To realize this ambition, capillary electrophoresis-mass spectrometry (CE-MS) was selected as analytical technique given its potential to separate a wide range of (polar) ionogenic compounds in tiny sample amounts with a high separation efficiency and selectivity, in order to show that CE can be made as suitable as LC-MS for metabolomics. At the start of this thesis, various CE-MS methods had been developed for profiling (endogenous) metabolites in small-volume biological samples in the years prior to the start of the thesis. However, the studies reported mainly focused on showing the proof-of-principle of CE-MS for this purpose. The work described in this thesis can be considered a seamless follow-up of the prior art with a special emphasis on showing the actual applicability of CE-MS-based workflows for volume-restricted metabolomics studies. More specifically, the utility of new or refined CE-MS workflows is shown for both targeted, quantitative analysis and for non-targeted profiling of endogenous metabolites in low amounts of biological sample in order to answer relevant biological questions. With the established methods, we focused on the profiling of (highly) polar ionogenic metabolites in different material-limited matrices that are of interest for neuroscience research, such as brain fluids, low amounts of HepG2 cells and zebrafish larvae models. A major goal was to demonstrate that the technical reproducibility, detection sensitivity and metabolic coverage of CE-MS are sufficient to allow use in biomedical applications, and current misconceptions about these in CE-MS are not valid anymore [38]. All of these aspects will be discussed in the following sections, including some remaining challenges and future perspectives.

Conclusions

Reproducibility of CE-MS for metabolomics

One of the most important aspects in metabolomics studies is the reproducibility of an analytical method, which remains a critical factor in cross-laboratory comparative studies using heterogeneous instruments. In this regard, the migration time (or retention time for chromatographic techniques) is an important parameter as it ensures a reliable comparison of metabolic profiles, including examining samples for subtle changes in patterns. Even though a well-known drawback of CE-MS is the variability in migration time between runs, the conversion of migration times to effective electrophoretic mobilities has already shown to be highly beneficial towards reliable metabolite identification in a repeatable manner [137]. At

the beginning of this thesis, the actual reproducibility of CE-MS using effective electrophoretic mobility as the key parameter for compound identification was further evaluated in an interlaboratory study. As demonstrated in **chapter 3**, the Metabo-ring study included 16 CE-MS systems, divided over 13 independent laboratories from 11 countries, with high instrumental and methodological heterogeneity between the participating labs. The only prerequisite for the trial study was the preparation of the acidic BGE according to a protocol.

In **chapter 3**, we show that the conversion of migration times into effective electrophoretic mobilities resulted in a variability of 3.1% compared to a variability of 10.9% when using the relative migration time scale, while employing the same BGE composition. The preparation of the BGE was found to be crucial in order to acquire reproducible results for the effective mobility, as especially the electrophoretic mobilities of slow-migrating compounds are heavily influenced by slight changes in the acidity of the BGE. This stresses the importance of standardized protocols for BGE preparation when performing (comparative) metabolomics studies, especially for less experienced users of CE-MS.

The Metabo-ring study addresses the main weakness of the commonly used relative migration time (RMT) approach, as the RMT is still influenced by fluctuations of the electroosmotic flow (EOF). We found that the RMT approach can only be used in a reliable fashion for compounds with a similar migration velocity as the internal standard, but increases in bias for compounds that migrate significantly faster or slower (up to 40% bias). This indicates that in order to reliably compare interlaboratory RMT values, multiple internal standards with different migration velocities need to be included in the analysis. In contrast, migration time correction using electrophoretic mobility showed similar bias (<5%) over a broad migration time range using merely two internal standards, due to a neutral marker that corrects for the EOF mobility. This shows the strength of using electrophoretic mobility as a parameter for direct interlaboratory data comparison, but also for metabolite identification using effective mobility libraries. Moreover, the ability to directly compare effective mobilities between different labs regardless of instrumental and methodological heterogeneity shows the advantage of CE-MS for global metabolomics compared to another commonly used analytical technique for the analysis of polar metabolites i.e. hydrophilic interaction liquid chromatography (HILIC)-MS. When using HILIC-MS, there is often a lack of standardization in operating parameters such as the column material, the injection solvent and the (preparation of) mobile phase that causes variable separation conditions, which makes interlaboratory comparison of retention times tedious.

Overall, the CE-MS Metabo-ring study revealed the potential of using the effective electrophoretic mobility as a universal and reliable criterion of peak identification in metabolomics. Even though this study predominantly focused on the profiling of cationic metabolites, it is anticipated that the same strategy could also be applied to anionic metabolic profiling. However, the robust and sensitive analysis of anionic metabolites has proven to be quite challenging, as is discussed in **chapter 5**.

Sensitivity enhancement

The prerequisite of low sample volumes for analysis makes CE-MS an attractive tool for the analysis of volume-limited samples. Nevertheless, due to the limited loading capacity of CE and concentration-sensitive detection of ESI-MS, CE-MS is often perceived as non-suitable for trace metabolomics [38]. However, the loading capacity of CE has been addressed effectively by the use of in-capillary sample preconcentration techniques. In **chapter 4**, we present an in-capillary preconcentration procedure with sample stacking based on dynamic pH junction to profile endogenous metabolites in rat brain microdialysis samples. The preconcentration was optimized systematically by response surface design (RSM), and allowed for a sample injection corresponding of about 291 nanoliter (compared to the typical 18 nanoliter).

The method was used to directly analyze microdialysates in a highly repeatable way employing minimum sample pretreatment (1:1 dilution in BGE). This limited sample preparation is especially beneficial when dealing with very low sample volumes, as an increased number of sample preparation steps may increase the bias due to handling errors and metabolite loss. Detection limits were in the low nanomolar range for amino acid neurotransmitters, already showing the methods potential for trace-sensitive brain metabolomics studies. Moreover, CE-MS could definitely yield comparable detection limits as compared to conventional LC-MS methods, with the main difference being that for CE only a volume of about 30-300 nL is injected, while 5-10 µL in the sample vial is sufficient for injection, whereas typically 300-10000 nL is injected in a conventional LC-MS method. This makes using CE-MS beneficial when the sample amount is very limited.

In this thesis, we employed the CE-MS method to analyze 48 endogenous compounds in rat brain microdialysis samples, of which 25 metabolites were (provisionally) identified and quantified. These metabolites include amino acids and related compounds, which are associated with glutamatergic, GABAergic and glycinergic systems, which play a key role in the pathophysiology of many neurological and neuropsychiatric diseases, such as epilepsy [338] and major depressive disorder [339]. The ability of our method to provide absolute quantification of these metabolites from different time points of *in-vivo* brain extracellular fluid samples

demonstrates the potential of CE-MS to gain better understanding of the underlying neurochemistry of neurological diseases, including how treatments might regulate these causes. Additionally, besides rat brain microdialysis samples, this method could also easily be implemented to directly profile a range of other low-volume high-conductivity sample matrices including tears, sweat or brain microdialysates from smaller animal models such as mice.

In this study, a volume of 10 µL was present in the sample vial to ensure reliable sample injection. However, when only nanoliters are injected into the CE system from the sample vial, the volume mismatch may limit the analytical performance of CE-MS for material-limited metabolomics studies. The development novel interfacing designs, the modification of sample vials [340, 341] or the use of mineral oils [342], allow for even lower sample volumes in the sample vial (1-2 µL). To apply direct sample injection with sample stacking on such an adapted system would be highly beneficial for brain microdialysis studies, where an improved temporal resolution is an important factor in order to profile neurotransmitters and neuromodulators over time.

Improved metabolic coverage

For cationic metabolic profiling, well-established CE-MS protocols have been developed and employed to analyze large sample cohorts. On the other hand, the development of a robust CE-MS approach for anionic metabolic profiling is still an ongoing development. Over the last decade, efforts have been made to develop analytical techniques for the selective and sensitive analysis of acidic ionogenic metabolites. In **chapter 5A**, an overview is provided of the recent methodological developments in this specific research area. Based on the examination of reported literature, we concluded that there is still room for the development of a highly sensitive and reliable CE-MS method for anionic metabolic profiling. Therefore we investigated the use of chemical derivatization to order to profile anionic metabolites in low amounts of HepG2 cells. In **chapter 5B**, we introduced a pre-column derivatization CE-MS method for the analysis of anionic metabolites using exactly the same conditions as employed for the analysis of basic metabolites. In this method, we use a novel chemical derivatization reagent; trimethylmethaneaminophenacetyl bromide (TmAmPBr), which is an adaptation of the commercially available dimethylaminophenacyl bromide (DmPABr) reagent that has already been successfully employed to label primary amines, secondary amines, thiols and carboxyls in (RP)LC-MS workflows [95, 290]. The tertiary amine group of DmPABr is replaced by a quaternary amine group in TmAmPBr to ensure a permanent positive charge on the derivatives, which is beneficial in terms of ionization efficiency and minimizes ion suppression in the ESI source. The original

reaction conditions from Willacey *et al.* [95] were adapted and altered to ensure reliable derivatization in presence of the quaternary amine, and to improve detection sensitivity in CE-MS. The derivatization strategy was further optimized using RSM.

The optimized method provided detection limits in the mid- to low nM range for a range of carboxylic acid containing metabolites, showing a 10-100 times sensitivity improvement compared to the state-of-the-art CE-MS methods that use negative ionization mode. Therefore, this work demonstrates that CE-MS with the use of pre-capillary TmMPABr derivatization of small organic acids can compete with the latest GC-MS and RPLC-MS techniques when it comes to sensitivity. To showcase its applicability for low-volume biological samples, the method was employed to profile organic acids in low amounts of HepG2 cells (ranging from 5000 to 2.5 × 10^5 cells). This shows the suitability of the method to study a range of diseases associated to abnormalities in tricarboxylic acid cycle (TCA) metabolites [343].

Based on the similar reaction properties compared to the DmPABr reagent, it is anticipated that the novel derivatization procedure can be further expanded to a wider selection of metabolites, potentially even allowing the analysis of both acidic and basic metabolites in a single CE-MS run using the same separation conditions. However, this requires further evaluation of labelling patterns and potential competition between functional groups.

Overall, this proof-of-principle study shows the potential of CE-MS with pre-capillary derivatization to study TCA cycle and energy metabolism in material limited samples. However, before the method can be used for actual metabolomics research, the aspects discussed above require some further attention.

The applicability of CE-MS biomarker discovery

The utility of the CE-MS workflows developed in **chapters 4 and 5B** is shown for targeted metabolomics, thereby providing absolute quantification of a chosen subset of metabolites related to neurological and neurodegenerative diseases, i.e. amino acids in **chapter 4**, and organic acids in **chapter 5B**. This approach takes advantage of the comprehensive understanding of a vast subset of metabolites and the known biochemical pathways to which they contribute. On the other hand, non-targeted metabolomics involves global profiling of the metabolome, and allows us to compare metabolic profiles in diseased groups and healthy control groups in order to gain a better understanding of the mechanism of disease onset and progression. Therefore, in this thesis, we also investigated the applicability of CE-MS to perform non-targeted metabolomics.

In **chapter 6** we demonstrate a non-targeted metabolomics workflow for the profiling of endogenous metabolites in extracts from individual zebrafish larvae and pools of small numbers of larvae. We utilized the CE-MS conditions first presented in **chapter 3** (10% acetic acid as BGE), and devoted special attention to an efficient and effective sample homogenization and extraction strategy. Zebrafish larvae pools with lowering numbers of larvae per pool (40, 20, 16, 12, 8, 4, 2 and 1 larva) were analyzed, yielding 84 metabolites detected for 40 zebrafish, and still 29 detected metabolites in a single zebrafish larvae. This already shows the potential of CE-MS to perform metabolomics studies in one single zebrafish larva. Additionally, when a stacking procedure as presented in **chapter 4** is used, even more metabolites can be detected in a single larva. For metabolite identification purposes, we used electrophoretic mobilities and compared these to a library containing over 600 metabolite entries [137]. As an extra layer of annotation confidence, two different fragmentor voltages (100V and 200V) were applied to induce in-source fragmentation using a TOF-MS instrument [311].

The applicability of the method to differentiate metabolites between groups was demonstrated by using a zebrafish larvae stress model. For this purpose, wildtype and ubiquitous mineralocorticoid (MR)-knockout zebrafish larvae were used, which were exposed to an exogenous cortisol treatment. Up to now, it has been barely investigated what the specific role is of MR during stress on metabolite levels, and which mechanisms might be important in this association. Using multivariate data analysis, different metabolic profiles could be obtained for all four groups, clearly indicating that MR has a role in stress response. This preliminary work shows the potential of our CE-MS workflow to be used in neuroendocrinology studies, which encompasses the study of the interactions between hormones and the brain. An interesting next step for this research would be to include zebrafish larvae behavior, and to investigate how hormones such as cortisol affect the metabolome and related behavioral changes.

It should be noted that due to its great diversity and complexity, it is impossible to analyze the complete metabolome within a biological system using a single analytical technique, and therefore often multiple analytical techniques are employed to acquire maximal biochemical information. In that regard, it would be interesting to perform multi-platform metabolomics on the zebrafish stress model, thereby including both polar and non-polar metabolites. This would be possible when using for example a two-phase extraction strategy (i.e. Bligh and Dyer extraction), and analyze the aqueous layer using CE-MS, and the organic layer using RPLC-MS or NACE-MS. This will provide a more comprehensive view on the metabolome of zebrafish larvae when exposed to stress.

Future perspectives

Further improving identification capability

As for now, electrophoretic mobility is still rarely used as a direct parameter of metabolite identification. However, a recently developed software tool that converts the migration-time scale into an effective electrophoretic mobility scale, designated as ROMANCE [152], might further encourage research groups to start using effective mobilities as additional parameter for metabolite identification in their analyses, either via targeted or non-targeted metabolomics. The development of such free, easy to use software has already led to the construction of compound libraries that include electrophoretic mobility values under specific BGE compositions [137]. When electrophoretic mobilities are added into universal databases such as the Human Metabolome Database (HMDB) [55] or METLIN [56], another compound-dependent and reliable value will be provided, thus complementing accurate mass and fragmentation patterns. Additionally, electrophoretic mobilities are easier to use in order to predict new molecules than retention time in the case of HILIC-MS.

Another interesting property to improve metabolite identification in CE-MS is collision cross-section (CCS), which was introduced with the development of ion mobility (IM-)MS. IM-MS adds an extra dimension of separation based on conformational differences, making it particularly interesting for the analysis of compounds that are typically not (well) separated by CE [286, 344]. Even though this is not widely used for metabolomics studies yet, we anticipate that IM-MS will be more extensively applied for metabolomics in the near future.

The importance of standardized procedures

The lack of standard operating procedures and data workflows that are fit for purpose may have hindered the widespread use of CE-MS in metabolomics. Relevant technological and practical aspects for the metabolomics workflows presented in **chapters 4 and 5B** are highlighted in detailed protocol papers [345] and [346]. Besides written protocol papers, an essential recent development the availability of peer-reviewed video articles [162, 347-349]. The availability of standard operating procedures ensuring accurate and reproducible results would be highly favorable in order to encourage researchers to actively consider CE-MS for metabolomics studies.

Furthermore, the recent CE-MS Metabo-ring trial showcased in **chapter 3** clearly revealed that this methodology can be used in a relatively simple way, even by groups that do not have (any) experience with metabolomics research.

Multivariate optimization for analytical workflows

As mentioned earlier, the workflows in **chapters 4 and 5B** were optimized in a systematic way by employing RSM. RSM is a Design of Experiments (DoE) that entails a multivariate optimization tool that encompasses mathematical and statistical techniques in order to study the relationship between multiple input variables (factors) and key output variables (responses). In comparison to the classical one-variable-at-a-time (OVAT) approach, RSM allows for the simultaneous evaluation of multiple factors during method optimization. A major advantage of RSM over OVAT is the reduced number of experiments, saving both time and experimental resources. Additionally, as is showcased in this thesis, RSM allows to investigate the interaction between factors to get a deeper understanding of a process and identify which factors have the largest influence on the response. In contrast, the interaction effects would most likely been overlooked when using the traditional OVAT approach, as it is highly influenced by the order in which the factors are optimized. The two presented studies in this thesis show that RSM is a powerful and efficient tool for method optimization in metabolomics.

However, RSM is not yet widely used in research labs, mainly because it is perceived as a complex methodology that requires deep understanding of statistics and mathematics. Furthermore, elements such as selecting a proper design are critical in obtaining the optimal characterization of a process. Therefore, it is important that general guidelines are provided on how to design DoE and how to interpret the acquired data. A recent study tested over multiple DoE strategies incorporating over 500.000 experimental simulations [350], reported their findings and provided general recommendations. The development of these guidelines, together with easy-to-use statistical software packages and tutorials may encourage the widespread use of RSM in the near future.

Quantitative performance of CE-MS

Besides its qualitative performance, the ability of an analytical technique to quantify metabolite concentrations in a reliable manner is of upmost importance for metabolomic studies. For non-targeted metabolomics studies, typically a relative quantification strategy is sufficient to capture changes in the metabolome after perturbations to the biological system in question. On the other hand, for targeted metabolomics studies, absolute quantification of metabolite concentrations is often preferred. In MS-based targeted metabolomics workflows, attention should be paid towards (standardized) sample preparation strategies to minimize ion suppression and improve the quantitative accuracy. Furthermore, the addition of (isotope labelled) internal standards for peak area correction is essential in targeted quantitative metabolomics. However, especially isotope labelled standards can be

very costly, and in practice often only a few internal standards are used to correct for a range of metabolites, as is for example demonstrated in **chapter 4,** where 5 internal standards were used to quantify 25 metabolites. A great potential of using a derivatization approach as described in **chapter 5**B is the use of isotope-coded-derivatization. This relatively easy and cost-efficient strategy allows the addition of a stable-isotope-labelled internal standard for every metabolite, as for example shown by Willacey *et al*. [95].

Recently, Harada et al. demonstrated the quantitative performance of CE-MS through the analysis of over 8000 human plasma samples [270]. However, before CE-MS could be adapted for reliable absolute quantification in a large-scale (routine) clinical setting, we believe it is necessary to set up an additional interlaboratory study to assess the quantitative performance of CE-MS.

Electrokinetic-based preconcentration

As has been demonstrated in **chapter 4**, significant sensitivity enhancement can be achieved relatively simple and fast by (temporarily) manipulating the electrophoretic mobility of metabolites. Besides dynamic pH junction, another promising in-capillary preconcentration strategy for the analysis of low concentrations of metabolites in low-volume samples is electrokinetic supercharging (EKS). EKS is based on electrokinetic injection and combines the preconcentration techniques of field-amplified sample injection and transient isotachophoresis. Wells *et al*. applied EKS for neurotransmitter analysis in volume-limited tissue samples from rat brain tissue and whole Drosophila [112], thereby reaching detection limits as low as 10 picomolar, showing a 1000 times improvement compared to pH junction. These detection limits show the potential of this EKS for profiling of low-abundant catecholamine neurotransmitters such as dopamine and serotonin, which are already important targets for therapeutic drugs. However, even though EKS could yield very low detection limits, its application in actual biological samples is still limited. This is probably because the relatively low repeatability and low recovery of EKS due to the injection bias of electrokinetic injection as a result of varying salt concentrations. Therefore, EKS is not directly applicable to high conductive sample matrices. Moreover, the effectiveness of EKS is significantly higher for metabolites with a high electrophoretic mobility such as lysine or GABA, and shows hardly any sensitivity improvement for low-mobility metabolites such as aspartic acid or inosine. Nevertheless, it is still an interesting procedure to further study and optimize, by for instance adding a desalting step as recently reported by Liao *et al*. [351] prior to electrokinetic sample injection to improve injection repeatability.

Another interesting electromigration based preconcentration strategy is electroextraction (EE). Instead of an in-capillary preconcentration strategy, EE is performed prior to sample injection and takes place in an immiscible liquid-liquid system consisting of an organic phase and an aqueous phase. Extraction takes place from the donor phase to the acceptor phase through the application of an electric field, either with (electromembrane extraction, EME) or without a filter phase (EE). Over the last years, EE and EME have been coupled on-line to various analytical techniques, showing its potential for automated high-throughput analysis of low-volume biological samples [352]. Recently, Oedit *et al*. developed a fully automated two-phase electroextraction system coupled on-line to CE-MS for the analysis of urine samples [353], which resulted in low nanomolar detection limits. The on-line coupling of EE to CE-MS allows to utilize a sample volume of merely 300 nanoliter, greatly lowering the amount of sample needed for analysis, thereby showing its potential for implementation in chip-based systems. It should be noted, that as with the electrokinetic procedure described above, also for EE the salt concentration in the sample plays an important role in its efficiency.

To further improve the sensitivity of the method presented in **chapter 5**B and reach detection limits that allow single cell analysis, it would be interesting to explore electrophoretic-based preconcentration strategies as discussed above for this purpose. The high selectivity of electrokinetic injection would be highly beneficial in combination with the permanent positive charge on the derivatives, even further lowering ionization suppression caused by (uncharged) matrix.

Alternative interfacing designs

In this thesis, a conventional, commercially available co-axial sheath-liquid interface was used for coupling CE to MS. The sheath-liquid interface has proven itself as a robust system for metabolomics, and has shown its applicability the last few years for large-scale metabolomics studies in urine- [354] and plasma samples [270, 355, 356]. However, employing a sheath-liquid interface leads to sample dilution at the ESI sprayer tip, causing a reduction in sensitivity. As a result, over the past few years some significant developments have been made in CE-MS interfacing techniques [108, 262].

An important event was the introduction of the sheathless porous tips based interface by Moini in 2007 [252], which has already proven itself the last few years for metabolomics analysis of material-limited samples [340, 357]. As an example, Zhang *et al*. [273] developed a sheathless CE-MS method for the profiling of nucleotides in an extract of only 500 HepG2 cells, corresponding to less than a single cell injected onto the capillary. Nevertheless, the porous tip has not yet been adopted as a routine CE-MS method, which is mainly due to the

complexity of system handling and the lack of capillary-to-capillary repeatability. Another interfacing trend is lowering the flow of the shealth-liquid interface. The use of an alternative CE-MS interfacing design was recently shown for single cell analysis. Lombard-Banek *et al*. reported a CE-MS method that allowed *in-vivo* single-cell proteomics and metabolomics in the same single cell in chordate embryos using X. *laevis* [269]. With a custom-build CE-μESI platform, quantitative proteo-metabolomic differences were observed between cells at the cleavage stage. This shows the potential of alternative CE-MS interfacing designs for single cell analysis, thereby including the ability to study cell heterogeneity in future metabolomics studies. However, special attention needs to be paid towards the efficient sampling and sample preparation of these minute sample amounts, as this is the most challenging and demanding process in single cell metabolomics [358].

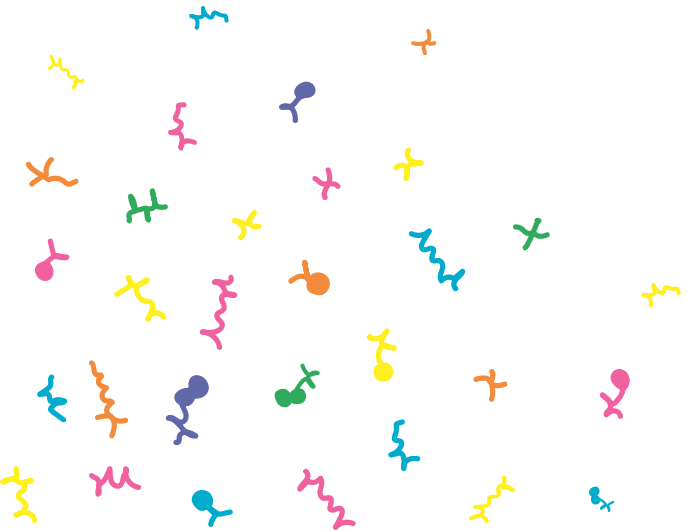
Translation to the clinic

For clinical metabolomics, the high-throughput analysis of dozens, hundreds or even thousands of biological samples is important. In particular, there is a requirement for fast and robust metabolomics workflows for material-limited samples. An important development in CE-MS analysis strategies is the multi-segment injection (MSI) approach [227], presented in 2013 by the research group of Britz-Mckibbin. MSI allows for serial injections of multiple samples within a single capillary, thereby greatly improving the sample throughput. Furthermore, when including a quality control (QC) sample in the same run, stringent quality control and batch correction can be performed. Recently, MSI-CE-MS potential for large-scale metabolomics was shown in a study including over a thousand serum samples [308], where metabolic fingerprints in serum samples from pregnant woman all over Canada. The samples were analysed for 7 months using standardized methodology and data treatment, and the results showed acceptable intermediate precision for a range of metabolites. Overall, this study demonstrated the value of MSI-CE-MS for performing large-scale high throughput metabolomics studies in a repeatable manner, thereby including successful correction for long-term signal drift and inter-batch variations.

A potential field where CE-MS could be especially useful is neonatal healthcare. Early and definitive diagnosis is critical for the clinical management of newborns and infants with inborn errors of metabolism or infections. Current newborn screening programs still face some challenges, such as high false positives and delayed diagnosis and an inability to screen for certain metabolic disorders. Another aspect requiring further research is using the volume-restricted material from newborns more effectively for screening purposes. During the end-phase of this thesis, we performed a pilot study where we demonstrated the utility of

sheathless CE-MS employing MSI for profiling of endogenous metabolites in volume-restricted serum samples from neonates. The study aims to assess the utility of CE-MS by determining creatinine levels in plasma collected from neonates at a clinical lab and comparing these findings with the assay used routinely in the clinical chemistry lab for this purpose.

In summary, in this thesis, with our developed methods we have shown the versatility of CE-MS and its applicability for a number of different sample matrices i.e. brain fluid, cells and tissues to perform targeted and non-targeted metabolomics studies related to neurological applications. In an ideal scenario, the data and knowledge from both the clinic and basic research is combined in order to gain a comprehensive understanding of disease pathophysiology, thereby facilitating personalized treatment. For the upcoming years, we anticipate a focus on further advancing CE-MS into a robust, quantitative and sensitive approach for metabolomics and bioanalysis studies in general, followed by an increase use of CE-MS in the areas of metabolomics, pharmaceutical analysis, clinical chemistry and biotechnology.



Addendum

References

List of abbreviations

Nederlandse samenvatting

Summary

Curriculum Vitae

List of publications

Dankwoord

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List of abbreviations

µPPPS	Micro Push-pull perfusion sampling
AKG	α-Ketoglutaric acid
BBB	Blood-brain barrier
BBD	Box-Behnken Design
CCD	Central Composite Design
CE	Capillary electrophoresis
CI	Chemical ionization
CITS	Citric acids
CMS	Capillary microsampling
CNS	Central nervous system
cOFM	Cerebral open flow microperfusion
CSF	Cerebrospinal fluid
DA	Dopamine
DBS	Dried blood spots
DI-MS	Direct infusion-MS
DLLME	Dispersive liquid-liquid microextraction
DmPABr	Dimethylaminophenacyl bromide
ECF	Extracellular fluid
EI	Electron ionization
EKS	Electrokinetic supercharging
EME	Electromembrane extraction
EPI	Epinephrine
FA	fatty acid
FASI	Field-amplified sample injection
FASS	Field-amplified sample stacking
FbI	Find by ion
FUM	Fumaric acid
GA	Glutaric acid
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GR	Glucocorticoid receptor
HDI	Hydrodynamic injection
ICD	Isotope-coded derivatization
IM	ion mobility
IPR	ion-pair reagent
kNN	k-nearest neighbours
LAC	Lactic acid
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOF	Lack-of-fit
LPME	Liquid-phase microextraction
MAL	Malic acid
MCE	Microchip CE

ME	Matrix effect
MEF	Matrif effect factor
MEPS	Microextraction in a packed syringe
MFE	Molecular features extraction
MIPs	Molecularly-imprinted polymers
MNA	Malonic acid
MOI	Microfluidic open interface
MR	Mineralocorticoid receptor
MRKO	MR-knockout
MVDA	Multivariate data analysis
MS	Mass spectrometry
MSI	multi-segment injection
MT	Migration time
NE	Norepinephrine
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
OXA	Oxaloacetic acid
PIESI	Paired ion electrospray ionization
PPPS	Push-pull perfusion sampling
PT-SPE	Pipette-tip solid-phase extraction
PYR	Pyruvic acid
QC	Quality control
RMT	Relative migration time
RSD	Relative standard deviation
RSM	Response surface methodology
SIL	Stable-isotope labelled
SL	Sheath-liquid
SPE	Solid-phase extraction
SPME	Solid phase microextraction
SUC	Succinic acid
TCA	Tricarboxylic acid
TEOA	Triethanolamine
TFME	Thin-film microextraction
tITP	Transient-isotachophoresis
TmAmPBr	trimethylmethaneaminophenacetyl bromide
TOF	Time of flight
TUS	Total useful signal
VAMS	Volumetric absorptive microsampling

Nederlandse samenvatting

Metabolomics is een krachtige tool om inzicht te verkrijgen in de complexiteit van de menselijke biologie en de pathofysiologie van ziekten. Door het metaboloom te analyseren, wat verwijst naar de verzameling van stofwisselingsproducten (met een massa <1500 Da) die aanwezig zijn binnen een biologisch systeem, kan direct de fysiologische status van een organisme worden verkregen. Metabolomics is naar voren gekomen als een veelbelovende techniek voor neurowetenschappelijk onderzoek, omdat het helpt om licht te werpen op de moleculaire mechanismen die ten grondslag liggen aan neurodegeneratieve en neuropsychiatrische ziekten. Een van de belangrijkste uitdagingen binnen (hersen)metabolomics is de betrouwbare en gevoelige analyse van volume-gelimiteerde biologische monsters. Daarom is het van cruciaal belang om betrouwbare en gevoelige analytische workflows te ontwikkelen die de studie van biomedische vragen mogelijk maken in kleine monster hoeveelheden. In dit proefschrift worden innovatieve analytische workflows op microschaal ontwikkeld, gebaseerd op capillaire elektroforese (CE) gekoppeld aan massaspectrometrie (MS), om metabole profielen te genereren in monsters met een beperkt volume. Tot op heden hebben MS-gebaseerde analytische workflows al potentie getoond voor neurologische metabolomics onderzoeken die te maken hebben met kleine monster volumes (**hoofdstuk 2**). CE-MS is echter nog steeds ondervertegenwoordigd in vergelijking met andere chromatografische technieken zoals vloeistofchromatografie (LC)-MS en gaschromatografie (GC)-MS, wat voornamelijk te wijten is aan misvattingen dat de techniek minder robuust, minder gevoelig en minder gebruiksvriendelijk is. Om deze reden is het uiteindelijke doel van dit proefschrift om aan te tonen dat de technische reproduceerbaarheid, detectiegevoeligheid en metabole dekking van CE-MS voldoende zijn om toepassing mogelijk te maken voor verschillende volume-gelimiteerde matrices die van belang zijn voor neurowetenschappelijk onderzoek.

Allereerst wordt een interlaboratoriumonderzoek uitgevoerd dat het welbekende probleem van migratietijd variabiliteit in CE-MS aanpakt door migratietijden om te rekenen naar de effectieve mobiliteit schaal (**hoofdstuk 3**), en daardoor de reproduceerbaarheid van CE-MS aanzienlijk vergroot. Bovendien toont de studie de geschiktheid aan van het hanteren van de effectieve mobiliteit parameter als een universeel en betrouwbaar criterium voor piek identificatie in metabolomics. In **hoofdstuk 4** wordt een methode gepresenteerd die gebruik maakt van on-line preconcentratie van monsters op basis van ‘dynamic pH-junction’ om de detectielimieten in CE-MS te verbeteren. De methode wordt op een systematische manier geoptimaliseerd met behulp van een Design of Experiments (DoE)-strategie, en is toepasbaar voor de directe profilering van endogene metabolieten in volumegelimiteerde (10 µL) microdialyse monsters van rattenhersenen.

Detectielimieten in het lage nanomolaire bereik worden bereikt voor aminozuur neurotransmitters, wat de potentie van deze methode voor neurologische studies aantoont.

Het volgende hoofdstuk van dit proefschrift richt zich specifiek op op CE-MS gebaseerde metabolomics onderzoeken voor de analyse van polaire anionische verbindingen (doorgaans metabolieten met alleen een carbonzuur als functionele groep), welke normaliter uitdagend te analyseren zijn met behulp van CE-MS (**hoofdstuk 5**). Eerst behandelt **hoofdstuk 5A** de recente methodologische ontwikkelingen op het gebied van anionische metabolieten profilering door middel van CE-MS, inclusief een overzicht van recente toepassingen in verschillende onderzoeksgebieden. Als vervolg hierop rapporteert **hoofdstuk 5B** een nieuwe chemische derivatisering procedure die een permanente positieve lading geeft aan zure metabolieten. Dit maakt hun selectieve analyse mogelijk onder dezelfde omstandigheden als die voor basische metabolieten, waardoor hun detectielimieten aanzienlijk worden verbeterd. De bruikbaarheid van de methode wordt gedemonstreerd voor de analyse van energiemetabolisme-gerelateerde metabolieten in kleine hoeveelheden HepG2-cellen.

In **hoofdstuk 6** ligt de focus op het gebruik van CE-MS voor untargeted metabolomics. Het hoofdstuk presenteert een CE-MS-workflow voor metabole profilering van extracten van individuele zebravis larven en samengevoegde monsters van lage aantallen larven. De workflow wordt gebruikt om de specifieke rol van de mineralocorticoid receptor tijdens stress op metaboliet niveaus te bestuderen, en te achterhalen welke mechanismen hierbij van belang kunnen zijn. Multivariate data-analyse wordt gebruikt om differentiële metabolieten te identificeren en onthult verschillende metabolische profielen voor alle groepen. Bovendien suggereren de gegevens dat de mineralocorticoid receptor een belangrijke regulator kan zijn van enzymen die betrokken zijn bij het cystathionine metabolisme en vervolgens belangrijke fysiologische processen beïnvloeden.

Ten slotte wordt in **hoofdstuk 7** een algemene conclusie en discussie gegeven over de ontwikkelde CE-MS workflows voor materiaal gelimiteerde biologische monsters. Het benadrukt het belang van het ontwikkelen van betrouwbare en gevoelige analytische methoden en workflows om de studie van biomedische vragen mogelijk te maken met behulp van een metabolomics benadering. Verdere mogelijke ontwikkelingen en mogelijke richtingen worden ook behandeld in dit hoofdstuk.

Summary

Metabolomics is a powerful tool that can provide a comprehensive insight into the complexity of human biology and the pathophysiology of diseases. By analyzing the metabolome, which refers to the complete set of small (endogenous) molecules (with a mass <1500 Da) present within a given biological system, a direct functional read-out of an organism's physiological status can be obtained. In particular, metabolomics has emerged as a promising approach for neuroscience research, as it helps to shed light on the molecular mechanisms underlying neurodegenerative and neuropsychiatric diseases. One of the main challenges in (brain) metabolomics studies is the reliable and sensitive analysis of volume-limited biological samples. Therefore, it is crucial to develop reliable and sensitive analytical workflows to enable the study of biomedical questions intrinsically dealing with low sample amounts. In this thesis, innovative microscale analytical workflows based on capillary electrophoresis (CE) coupled to mass spectrometry (MS) are developed in order to generate metabolic profiles in volume-restricted samples. Until now, MS-based analytical workflows have already shown potential for brain metabolomics studies that deal with low sample volumes (**chapter 2**). However, CE-MS is still underrepresented compared to other chromatographic techniques like liquid chromatography (LC)-MS and gas chromatography (GC)-MS, which is primarily due to misconceptions that the technique is less robust, less sensitive, and less user-friendly. Therefore, the ultimate aim of this thesis is to showcase that the technical reproducibility, detection sensitivity, and metabolic coverage of CE-MS are sufficient to allow use for different material-limited matrices of interest for neuroscience research.

First, an interlaboratory study is conducted that overcomes the well-known issue of migration time variability in CE-MS and thereby increases its reproducibility, by converting migration times to the effective mobility scale (**chapter 3**). Furthermore, the study showcases the suitability of the effective mobility parameter as a universal and reliable criterion for peak identification in metabolomics. In **chapter 4**, a method is presented that employs on-line preconcentration with sample stacking based on pH junction to improve detection limits in CE-MS. The method is systematically optimized using a design of experiments (DoE) strategy, and demonstrates to be applicable for the direct profiling of endogenous metabolites in volume-limited rat brain microdialysis samples of only 10 µL. Detection limits in the low nanomolar range are achieved for amino acid neurotransmitters, which demonstrates its potential for trace-sensitive neurological studies.

The next chapter of this thesis focuses specifically on CE-MS-based metabolomics studies for the analysis of polar anionic compounds, which are metabolites that

are typically difficult to analyze using CE-MS (**chapter 5**). First, **chapter 5A** covers recent methodological developments to improve anionic metabolite profiling by CE-MS, along with an overview of recent applications in different research areas. As a follow-up, **chapter 5B** reports on a novel chemical derivatization procedure that provides a permanent positive charge to acidic metabolites. This allows their analysis using the same conditions as used for basic metabolites, thereby significantly improving their detection limits. The method is demonstrated for the analysis of energy metabolism-related metabolites in low numbers of HepG2 cells.

In **chapter 6**, the focus is on utilizing CE-MS for non-targeted metabolomics. The chapter presents a CE-MS workflow for metabolic profiling of extracts from individual zebrafish larvae as well as pools of small numbers of larvae. The workflow is used in order to study the specific role of the mineralocorticoid receptor during stress on metabolite levels, and which mechanisms might be important in this association. Multivariate data analysis is employed to identify differential metabolites, and reveals distinct metabolic profiles for all groups. Furthermore, the data suggests that the mineralocorticoid receptor may be a key regulator of enzymes involved in cystathionine metabolism and subsequently impact key physiological processes.

Finally, in **chapter 7**, a general conclusion and discussion are provided on the developed CE-MS workflows for material-limited biological samples. It highlights the importance of developing reliable and sensitive analytical methods and workflows to enable the study of biomedical questions using a metabolomics approach. Further possible developments and potential directions are also discussed.

Curriculum Vitae

Marlien Admiraal – van Mever was born on July 11th 1994 in Alkmaar, The Netherlands. After obtaining the VWO diploma at the Jac. P. Thijsse College in Castricum in 2012, she started the Bachelor's program Pharmaceutical Sciences at the Vrije Universiteit Amsterdam. During the Bachelor's studies, she did an internship at the Vrije Universiteit Amsterdam at the department of Chemistry and Pharmaceutical Sciences, supervised by dr. Henk Lingeman. The research was focused on the chemical derivatization of sterols for their improved detection using liquid chromatography coupled to fluorescence detection. During this period, Marlien came into contact for the first time with analytical chemistry, and her interest was piqued for the research field. After obtaining the Bachelor's degree in July 2016, Marlien started the Master's program Chemistry, a joint degree between the University of Amsterdam/Vrije Universiteit Amsterdam. Within the Master's program Chemistry she specialized in Bioanalytical Sciences, following courses related to analytical separation techniques and mass spectrometry detection. During her Master's studies, she wrote a literature thesis describing the current use of synthetic antioxidants for the stabilization of lipid-rich foods, and how these can be effectively substituted by nontoxic, natural compounds with high antioxidative activity, supervised by prof. dr. ir. Jan-Gerd Janssen. Marlien performed her Master's internship again at the department of Chemistry and Pharmaceutical sciences under the supervision of dr. Rob Haselberg and prof. dr. Govert Somsen. The research was focused on the development of an analytical method to profile snake venoms, thereby using capillary electrophoresis as separation technique.

Following her graduation in 2018, Marlien started her PhD research at the Leiden University, Center for Metabolomics and Analytics (MAC), under the supervision of dr. Rawi Ramautar and prof. dr. Thomas Hankemeier. The research, which was financed by a VIDI grant from NWO awarded to dr. Ramautar, was focused on the development and application of microscale analytical workflows based on capillary electrophoresis mass spectrometry to profile endogenous metabolites in material-limited biological samples. Research results were presented on both national and international conferences through poster- and oral presentations, and resulted in nine papers as first author in international peer-reviewed scientific journals. During her PhD, Marlien dedicated a full academic year to teaching and supervision, and acquired a full University Teaching Qualification (BKO in Dutch). After completing her thesis, Marlien will continue her research as post-doctoral researcher at the MAC.

List of publications

(1) **van Mever, M**.; Hankemeier, T.; Ramautar, R. CE-MS for anionic metabolic profiling: An overview of methodological developments. Electrophoresis 2019, 40 (18-19), 2349. DOI: 10.1002/elps.201900115.

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* Authors contributed equally

$ Authors contributed equally

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