Capillary electrophoresis and hydrophilic interaction chromatography coupled to mass spectrometry for anionic metabolic profiling

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ISBN 978-90-393-6154-2

Capillary electrophoresis and hydrophilic interaction chromatography coupled to mass spectrometry for anionic metabolic profiling

Capillaire elektroforese en hydrofiele interactie chromatografie
gekoppeld aan massaspectrometrie voor het profileren
van anionische metabolieten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
vrijdag 4 juli 2014
des middags te 2.30 uur

door

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geboren op 23 april 1985
te Utrecht

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Introduction

1.1 Metabolic profiling

Metabolites are the intermediates and products of metabolism. The chemical reactions of metabolism are organized into metabolic pathways, which may be studied by the analysis of individual metabolites. Over the last decades, there has been a strongly growing awareness that the complexity of biological networks and pathways is better addressed by quantitatively analyzing multiple components simultaneously. From this more holistic perspective, the concept of systems biology has emerged with the goal to study the complex interactions between genes, proteins and metabolites in a biological system. These components can be assessed by, respectively, genomics, proteomics and metabolomics approaches.

Metabolomics is an essential part of the so-called 'omics' cascade and involves the analysis of all low-molecular-weight compounds present in a biological sample. The identities, concentrations and fluxes of metabolites result from gene and protein expression, and environmental factors (e.g. diet and bacteria), which together determine the phenotype of an organism. The general objective of metabolomics studies is to unravel biochemical pathways by detecting differences or changes in metabolite levels between groups of samples. This approach has been coined by various terms and definitions which are used interchangeably and include metabolic profiling, fingerprinting and footprinting.

Metabolomics has been carried out following targeted and untargeted strategies. In a targeted approach, a subset of preselected metabolites is analyzed in order to monitor potential changes in concentrations and probe biochemical processes. In an untargeted approach, the goal is to profile as many metabolites as possible without *a priori* knowledge of their identity in order to reveal new potential biomarkers. In this thesis, the term 'metabolic profiling' is used for the process of untargeted analysis of metabolites in biological systems. Global metabolic profiling is challenging, since metabolites can have diverse physicochemical properties and may be present in a broad concentration range [1-4].

A metabolic profiling study typically consists of several consecutive steps, which are depicted in Figure 1.1. The process starts with a biological question and the design of experiments. Subsequently, samples are collected, treated and stored in a way that assures representativeness. Prior to analysis, samples are prepared in order to meet requirements for appropriate analysis by the selected analytical methodology. Different sample preparation procedures are used for the diverse biological matrices met in metabolomics. For instance, urine samples sometimes may only have to be diluted and centrifuged. Analysis of blood or plasma samples often requires removal of proteins, e.g. by precipitation. Extraction of metabolites is frequently necessary

for effective metabolic profiling of plants, bacteria and cells. After analysis of the pretreated samples with one or more analytical techniques, preprocessing of the obtained data is carried out. An alignment procedure may be needed to allow a correct comparison of metabolite profiles. Thereafter, peak picking algorithms are often used in order to select significant signals that are related to metabolite features. Normalization of measured abundances for detected metabolites can be applied to correct for system and inter-sample differences in analyte responses. Normalization is especially indicated when urine samples are analyzed, since there may be a substantial variation in total urine volume among subjects. After data preprocessing, multivariate data analysis is commonly performed to reveal potential compounds that discriminate the studied groups of samples. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are among the most used multivariate techniques to find significant differences in an unsupervised and supervised way, respectively. Thereafter, assignment of detected discriminatory molecular features to real metabolite structures is intended. This can be quite challenging and additional identification strategies might be needed. Finally, when distinctive metabolites have been identified, interpretations towards their possible role in biochemical pathways are attempted, potentially leading to biomarker discovery [5].

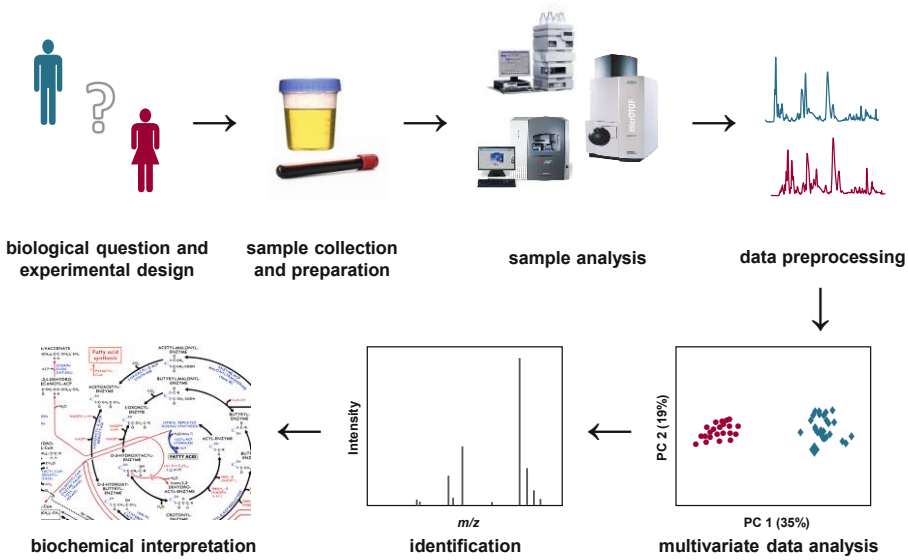

Figure 1.1 Typical workflow of metabolic profiling studies.

Several analytical methodologies have been developed and used for the analysis of metabolites in biological samples. Nuclear magnetic resonance (NMR) spectroscopy is a major tool in metabolomics, capable of detecting metabolites in unmodified biological samples. Gas chromatography (GC) and liquid chromatography (LC), both in combination with mass spectrometry (MS), are also common techniques in contemporary metabolomics. NMR spectroscopy is a non-destructive and highly selective analytical technique providing detailed information on molecular structures. NMR spectroscopy has a relatively poor concentration sensitivity and, therefore, low-abundant metabolites might not be detected. GC–MS provides highly efficient separation of various types of metabolites with good sensitivity. The major drawback of GC is the labour-intensive derivatization procedure required for the analysis of non-volatile and polar metabolites. LC–MS is very suitable for the analysis of unlabelled metabolites in aqueous samples. Still, the coverage of ionic and polar metabolites by reversed-phase (RP)LC – i.e. the most common mode of LC – is limited, since these compounds elute with or near the column dead time. In order to cover the highly polar metabolome, more recently, capillary electrophoresis (CE) and hydrophilic interaction chromatography (HILIC) have been introduced as separation techniques in MS-based metabolic profiling studies [4]. This thesis studies the optimization, feasibility and applicability of CE–MS and HILIC–MS for the analysis of polar anionic metabolites. Before outlining the scope of the research, some basic aspects of CE and HILIC, and of their combination with MS are treated.

1.2 CE

CE is a separation technique based on differential electromigration of compounds in an applied electric field. Different modes of operation can be employed, namely, capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (cIEF), capillary isotachophoresis (ITP) and micellar electrokinetic chromatography (MEKC). The instrumental set-up for all these separation modes is virtually the same and basically consists of a narrow-bore capillary (internal diameter of typically 25-75 μm), buffer vials, electrodes, a high voltage power supply, and a detector. The fundamental difference between the various CE modes arises from the composition of the buffer or background electrolyte (BGE) in which the separation takes place. The studies described in this thesis have been confined to CZE – which often is denoted as just CE – in which separation takes place in a simple electrolyte solution. As outlined below, the charge-to-size based separation mechanism of CZE is highly useful for the separation of ionogenic metabolites.

Electrophoresis has been defined as the movement of charged particles in an electric field. The velocity (v) of a charged compound depends on the applied field strength (E) and the electrophoretic mobility (μ_e) of the ionic compound, which is determined by the viscosity of the solution (η), and the size (radius, r) and charge (q) of the ion.

$$v = \mu_e E \qquad \text{[Eq. 1.1]}$$

$$\mu_e = \frac{q}{6\pi\eta r} \qquad \text{[Eq. 1.2]}$$

It follows that small and highly charged compounds have high electrophoretic mobilities, whereas large and barely charged compounds have a low electrophoretic mobility. Electrophoretic separation is based on differences in charge-to-size ratios among analyzed molecules.

Electroendosmosis causes a bulk flow of the BGE through the capillary as a result of a potential difference between the inner surface of the capillary wall and the BGE. CE is most often performed in fused-silica capillaries of which silanol groups on the inner surface of the wall can be deprotonated. This results in a negative charge which attracts cations from the BGE forming a diffuse double layer and creating a potential difference (zeta potential). When a high voltage is applied across the capillary, the cations of the electrical double layer are attracted towards the cathode drawing the rest of the BGE with them, and thereby generating an electroosmotic flow (EOF). The magnitude of the EOF depends on the degree of deprotonation of the silanol groups, and thus, on the pH of the applied BGE.

The observed or apparent mobility (μ_{app}) of analytes is the sum of the electrophoretic mobility (μ_e) and electroosmotic mobility (μ_{eof}). Negatively charged compounds are attracted by the anode and will have an electrophoretic mobility opposite to the electroosmotic mobility. However, often the electroosmotic mobility is larger than the electrophoretic mobility, and therefore, all compounds will migrate towards the detector at the site of the cathode. Positively charged compounds will reach the detector first, followed by neutral compounds (migrating with the EOF) and negatively charged compounds, as indicated in the electropherogram depicted in Figure 1.2 [6].

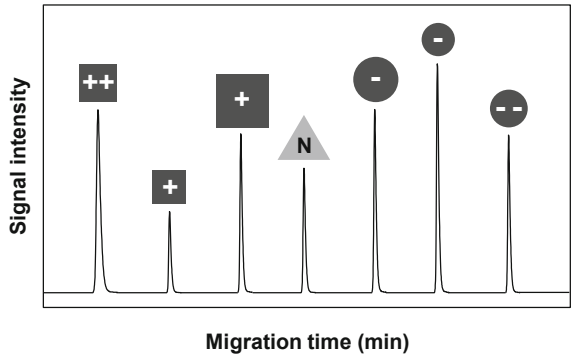

Figure 1.2 Electropherogram showing the migration order of cations, anions and neutral compounds.

The charge of the inner capillary wall can be altered by coating its surface with charged or neutral agents. For instance, coating of the inner capillary wall with a positively charged polymer reverses the EOF and thereby the migration order of the analytes. Furthermore, the use of coated capillaries might prevent the adsorption of analytes and matrix components when analyzing biological samples, and may improve the reproducibility of the CE system [7]. As in CE peak broadening predominantly relies on longitudinal diffusion of the analytes, the high voltages applied (10-30 kV) lead to high peak efficiencies (plate numbers of 100,000-1,000,000) and high resolution of charged compounds can be obtained in relatively short analysis times. Furthermore, consumption of chemicals is low and only small amounts of sample are required.

1.3 HILIC

HILIC has emerged as an increasingly popular separation technique for the analysis of polar compounds. In HILIC, highly hydrophilic stationary phases are applied. Columns with bare or chemically-modified silica stationary phases are most often used. Modified silica chemistries include amino-, amide-, diol-, cyanopropyl-, poly(succinimide)- and cyclodextrin-bonded phases. Also organic polymer columns and monolithic columns have been applied in HILIC. These diverse column materials provide a wide range of selectivity. The mobile phase employed in HILIC–MS contains an organic solvent (most frequently acetonitrile) and an aqueous acetate or formate buffer. The separation mechanism of HILIC is multimodal and not fully understood. The principal mechanism is believed to

involve partitioning of analytes between the mobile phase and a stagnant water layer on the surface of the stationary phase (Figure 1.3). Polar compounds have higher affinities for the stagnant water layer, and are thereby more retained. Increasing the water content of the mobile phase decreases the affinity for the stagnant water layer, resulting in faster elution of the compounds. The formation of the stagnant water layer is dependent on the water content of the mobile phase. At least 2.5% of water is necessary to establish the aqueous layer. Besides partitioning, also other retention principles are involved in HILIC, including electrostatic interactions, hydrogen bonding and weak hydrophobic interactions. These retention principles partly depend on the type of stationary phase. For instance, using bare silica and amide-bonded columns, compounds are mainly separated by partitioning. Stationary phases containing acidic and/or basic groups causes retention of charged metabolites by electrostatic interactions under appropriate pH conditions [8-11]. As HILIC provides interesting selectivities for polar compounds, it has been introduced in metabolomics as a complementary separation technique for RPLC.

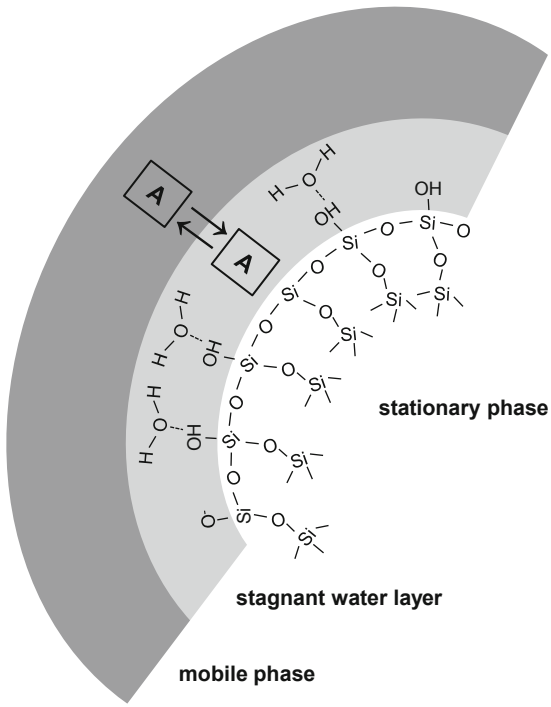

Figure 1.3 Schematic representation of the partitioning of an analyte [A] between the mobile phase and a stagnant water layer on the surface of the stationary phase.

1.4 CE–MS and HILIC–MS

CE and HILIC can be hyphenated to MS for the mass-selective detection of analytes. Most frequently, electrospray ionization (ESI) is used to ionize the compounds and to evaporate the BGE or mobile phase employed in CE and HILIC, respectively. ESI can be operated in positive or negative ionization mode. Various interfaces have been developed of which the co-axial sheath-liquid interface is commonly used in CE–MS (Figure 1.4A) [12, 13]. The fused-silica capillary is contained in a stainless steel tube and forms the spray tip. A flow of sheath liquid is applied through the tube to make contact with the BGE emerging from the capillary, and thus close the electrical circuit necessary for the separation process in CE. The sheath liquid is typically a mixture of an organic solvent and water with the addition of a salt (mostly the BGE) to establish conductivity. A nebulizing gas aids the evaporation of the solvent and formation of an electrospray. The interface used to couple HILIC to MS is more simple, because there is no sheath liquid required. The HILIC effluent enters the stainless steel capillary and a co-axial flow of nebulizing gas assists the ESI (Figure 1.4B).

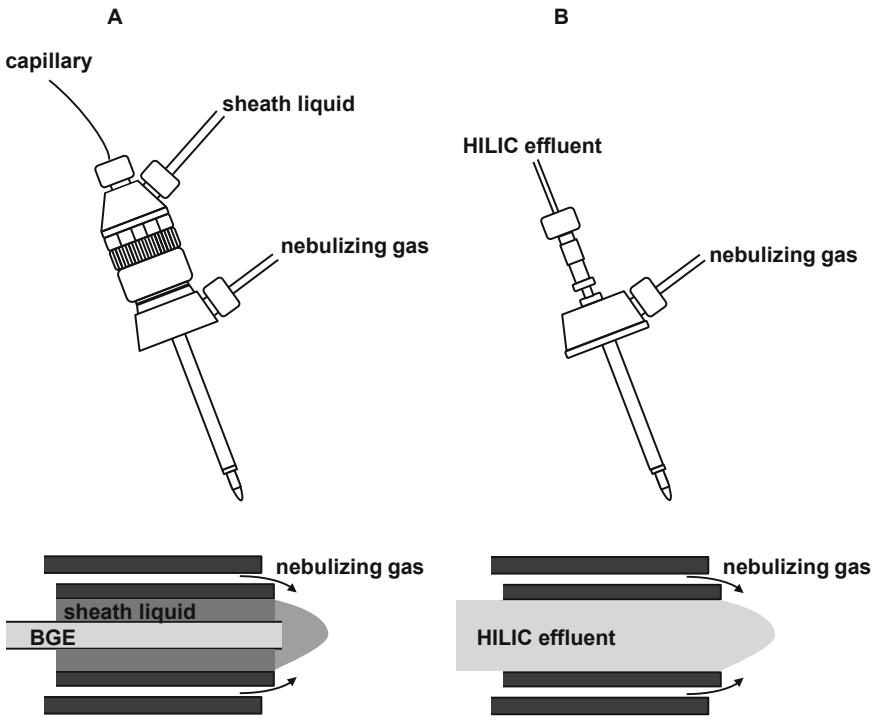

Figure 1.4 Co-axial sheath-liquid sprayer used in CE–MS (A) and a co-axial LC sprayer used in HILIC–MS (B).

The BGE and HILIC effluent exit the capillary and under the influence of the applied electrospray voltage form a Taylor cone from which a spray of fine droplets is formed. Solvent evaporation from the droplets is enhanced by a flow of dry gas. When the repulsive force of the excess of charge equals the cohesive force of the surface tension (Rayleigh limit), the droplets break into multiple small droplets (coulombic explosion). This process repeats until the solvent is completely evaporated and analyte ions end up in the gas phase and can enter the mass spectrometer [14, 15].

In the research reported in this thesis, mainly a time-of-flight (TOF) mass spectrometer is used. In a TOF mass analyzer ions are accelerated and move via a reflectron to the detector with a velocity dependent on their mass. Lighter ions travel faster than heavier ions. TOF mass spectrometers have a high resolving power and mass accuracy. Furthermore, TOF-MS has a fast acquisition rate and a large range of masses can be measured [5, 16]. Therefore, TOF-MS is well suited for the profiling of compounds in biological samples. Analytes with highly similar masses can still be resolved and the obtained accurate masses can strongly aid in the provisional identification of unknown compounds, e.g. by using chemical-formula software and databases.

1.5 Scope and outline

Many metabolites in biological samples are highly polar, and therefore, CE and HILIC seem very suitable for metabolic profiling. At the time of the start of this research project, the potential of CE–MS in metabolomics had been recognized. CE–MS strategies commonly dealt with the profiling of cationic metabolites in various types of biological samples. For basic compounds, an acidic BGE (e.g. formic or acetic acid) was used in combination with a bare or coated fused-silica capillary. Positively charged capillary coatings prevent analyte adsorption, reverse the EOF and provide a large separation window for cationic compounds. Negatively charged coatings were also used to achieve fast separations of cationic metabolites within a restricted migration window [17].

As stated above, most of the reported CE–MS methods for metabolomics focused on the analysis of basic and zwitterionic metabolites using positive ESI. It should be noted that a large part of metabolites in biological fluids is acidic and can only be ionized efficiently using negative ESI. These compounds will not be covered by CE–MS using positive ionization mode. In order to allow separation and detection of anionic metabolites, CE–MS should be carried out using a BGE with a pH of 7.5 or higher applying negative ESI. The relatively small number of studies dealing with

negative ionization CE–MS of metabolites, mostly published by the group of Soga, typically employed ammonium acetate BGEs (pH 7.5–10.0). Both bare fused-silica (BFS) capillaries and capillaries coated with a positively charged polymer were used. Regrettably, compared to positive ESI, metabolite responses in negative ionization mode were often relatively low, leading to rather poor sensitivity in metabolomics applications. Moreover, certain anionic compounds showed distorted peak shapes and low migration-time reproducibilities, and analyses were often carried out by applying pressure on the inlet buffer vial. However, pressure-induced CE analysis results in a loss of separation power. In addition, reversal of voltage, as needed when using positively charged coatings, may lead to corrosion of the steel needle causing current drops and clogging of the capillary [18].
Still, the optimization and application of negative ionization mode CE–MS in metabolic profiling studies is important, expanding the coverage of metabolites and potentially providing essential information on biochemical processes. Therefore, the primary objective of the studies described in this thesis was to establish the potential of CE–MS for anionic metabolic profiling. In order to achieve this goal, the initial part of the work was devoted to the improvement of the performance of negative mode CE–MS for metabolite analysis. Possibilities to enhance analyte detectability were studied, and separation and interfacing conditions were optimized. In order to evaluate the applicability and usefulness of the newly developed CE–MS method, relevant human and rat urine samples were analyzed. Furthermore, the CE–MS data acquired for anionic metabolites were extensively compared with results obtained with HILIC–MS in negative mode. At the start of this research project, HILIC–MS of metabolites had also been applied primarily in positive ionization mode. Therefore, HILIC–MS was first studied and optimized to achieve reproducible profiling of anionic metabolites. Particular attention was paid to the composition of the mobile phase to maximize ionization efficiencies and MS detection intensities using negative ESI. The gradient of the mobile phase and sample solvent were evaluated to obtain a large separation window with good peak shapes and low limits of detection (LODs) of metabolites, which is of utmost importance in metabolomics studies.

An overview of studies involving CE and one or more other analytical techniques for comparative and comprehensive metabolic profiling is given in ***Chapter 2***. The aim is to elaborate and possibly clarify the potential and added value of CE in relation to other more common methodologies for metabolomics studies. General aspects, including sample preparation, analytical set-up, data analysis and metabolite

coverage are discussed. Moreover, the various combinations of methodologies are systematically outlined, and conditions and characteristics are summarized in tables.

The development of a new CE–MS method for the profiling of anionic metabolites is presented in ***Chapter 3***. Possibilities to improve ionization efficiencies of anionic metabolites are investigated by testing various BGEs and sheath liquids via infusion experiments. The applicability of the BGE and sheath liquid providing the highest analyte responses is studied by CE–MS of a representative test metabolite mixture. The method is further optimized taking metabolite separation, migration-time and peak-area reproducibility, and LODs of test metabolites into consideration. Also the performance of the CE–MS method for urine analysis is investigated. Comparisons are made with two regular CE–MS methods to appreciate the new method's merits.

Chapter 4 covers the comparison of the optimized CE–MS method with HILIC–MS for anionic metabolic profiling considering reproducibility, sensitivity, metabolite coverage and separation selectivity. The HILIC–MS method is optimized by carrying out infusion experiments to select a mobile phase providing optimal signal intensities of anionic test metabolites. The influence of concentration and pH of the mobile phase on metabolite signals is investigated. Moreover, the effect of the slope of the gradient, injection solvent and injection volume on metabolite separation are evaluated. The performance of the optimized HILIC–MS method is assessed in terms of sensitivity, linearity and repeatabilities of peak areas and retention times of test metabolites. The HILIC–MS and CE–MS methods are also evaluated for the analysis of urine comparing the number of detected molecular features and assessing peak area and retention time repeatabilities of selected urinary compounds. Numbers of common and unique molecular features are considered, and differences in separation selectivity of CE–MS and HILIC–MS are assessed by comparing migration and retention times of common features. In addition, the CE and HILIC behavior of putatively identified common features are evaluated involving their physicochemical properties.

The performance and applicability of the CE–MS and HILIC–MS methods for anionic metabolic profiling are further explored in ***Chapters 5 and 6***, respectively. Urine samples of rats receiving the antibiotics penicillin G and streptomycin sulfate for 0, 4 or 8 days are analyzed to study microbial-host co-metabolism. A mixture of all these urine samples is used as quality control (QC) sample to monitor the repeatability of the applied methods. Univariate and multivariate data analysis is performed to study the differences between antibiotic-treated and control rats. Discriminatory compounds are revealed and putatively identified. The biological relevance of these compounds is discussed. Furthermore, comparisons are made

with results obtained with ^{1}H NMR spectroscopy [19] and other studies using different analytical methodologies, in order to demonstrate the potential added value of CE–MS (*Chapter 5*) and HILIC–MS (*Chapter 6*). The methodologies are compared based on the number, identity and relative abundance of compounds found to be different between antibiotic-treated and control rats.

Chapter 7 describes the feasibility of CE–MS and HILIC–MS for the analysis of anionic metabolites involved in the inborn errors of metabolism citrullinemia, medium chain acyl-coenzyme A dehydrogenase (MCAD) deficiency and 3-methylcrotonyl-coenzyme A carboxylase (MCC) deficiency. Urine samples from patients and their matched controls are analyzed after minimal sample pretreatment. PCA is performed to study differences in metabolite abundances between urine samples from patients and controls. The compounds found to be discriminatory between patients and controls are compared with known biomarkers of the studied disorders. In addition, as a proof-of-principle discriminatory compounds of MCAD deficiency are identified with HILIC–MS/MS.

In the **final chapter**, some general conclusions are drawn with respect to the performance of CE–MS and HILIC–MS for anionic metabolic profiling. Differences between these analytical methodologies are discussed and future perspectives are presented.

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II

The role of capillary electrophoresis in metabolic profiling studies employing multiple analytical techniques: an overview

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G.W. Somsen
G.J. de Jong

Submitted

Abstract

Capillary electrophoresis–mass spectrometry (CE–MS) is increasingly used for the targeted and untargeted analysis of metabolites in biological samples. CE–MS has shown to be particularly useful for the profiling of highly polar metabolites without the need for derivatization and/or extensive sample preparation. In order to appreciate the potential and added value of CE amongst more conventional analytical techniques for metabolomics, this paper gives an overview of reported studies in which CE or CE–MS has been used next to flow-injection MS, gas chromatography (GC)–MS, liquid chromatography (LC)–MS, and/or nuclear magnetic resonance (NMR) spectroscopy for the analysis of metabolites. The first part of the review discusses particular aspects of the analytical methodologies used for comparison, such as sample preparation, separation conditions, MS detection and data analysis. An extensive overview of the comparative metabolic profiling studies involving multiple analytical techniques, including CE, is given in the second part of this review. The particular conditions and characteristics of each study are described and summarized in tables. Furthermore, results of the various studies are discussed and compared, and selected examples are treated. Based on the outcomes of the comparative studies, it is concluded that CE is highly complementary to the other analytical technologies and can add essential information on the metabolic profile of various biological samples.

2.1 Introduction

Metabolomics encompasses the identification and quantification of low-molecular weight endogenous compounds in biological samples, such as cells, tissues and body fluids. Metabolomics provides information on biochemical processes and phenotypes of organisms, and can be applied using a targeted and an untargeted strategy. In a targeted approach, specific (classes of) metabolites are analyzed with the aim to determine differences in their concentration between samples. In an untargeted approach, global profiling of as many metabolites as possible is attempted in order to reveal new potential biomarkers [1-4]. Global metabolic profiling is challenging since metabolites have very diverse physicochemical properties and can occur in a broad concentration range in biological systems. In the last years, many analytical techniques have been developed to profile metabolites in biological samples [5]. Ideally, the analytical techniques are rapid, sensitive, reproducible, requiring no or simple sample preparation, and capable of analyzing nearly all metabolites. Not surprisingly, today not one analytical technique meets all these requirements.

Nuclear magnetic resonance (NMR) spectroscopy is most commonly used in metabolic profiling studies, especially ^{1}H NMR spectroscopy. NMR spectroscopy is a rapid non-destructive analytical technology which provides information on the identity and concentration of metabolites, without the need for prior separation. A drawback is its limited sensitivity. Mass spectrometry (MS) detection is highly sensitive and provides information on metabolite masses. When mass spectrometers with a high mass accuracy and resolution are used, putative identification based on accurate mass might be possible. MS is often coupled to separation techniques in order to distinguish isobaric compounds and to reduce sample matrix effects (e.g. ion suppression) [6]. Gas chromatography (GC) and liquid chromatography (LC) are two chromatographic separation techniques which are often combined with MS. In GC, metabolites are analyzed in the gas phase and separated with high resolution. For GC analysis, compounds need to be volatile which can be achieved using pre-column chemical derivatization procedures. Derivatization procedures are time-consuming and labour intensive and may introduce artifacts (e.g. incomplete derivatization) [7]. The basic separation principle in LC is the difference in partition of compounds between a mobile liquid phase and a stationary phase. Different separation modes exist, of which reversed-phase (RP) LC is most often applied in metabolomics studies. RPLC is not well suited for the analysis of highly polar and charged compounds which elute in the column dead time. Hydrophilic interaction chromatography (HILIC) has been introduced for the profiling of these polar

compounds providing complementary metabolite information to RPLC. In HILIC, besides partitioning between the mobile phase and a stagnant water layer on the surface of the stationary phase, compounds are also retained by electrostatic interactions with the polar stationary phase employed [8, 9].

The various analytical techniques are most suited for different (classes of) metabolites. Therefore, platforms applying multiple analytical techniques have been employed to obtain a comprehensive coverage of the metabolome and to compare the outcomes and characteristics of the techniques. Recently, the separation technique capillary electrophoresis (CE) has been added to the analytical toolbox for metabolomics and studies which involve CE next to other analytical techniques have been carried out. CE–MS has been introduced as a powerful technique for the profiling of highly polar and charged metabolites. Compounds are separated based on their charge-to-size ratio and only very small amounts of sample and separation electrolytes are required [10]. CE–MS has the potential to provide complementary information to other more common methodologies, thereby increasing the metabolite coverage. In order to evaluate the contribution and added value of CE in metabolomics, this paper gives an overview of metabolic profiling studies, involving CE and one or more other analytical techniques, which have been reported until December 2013 [11-59]. In some studies, a comparison between CE and the other analytical technologies has been made, whereas in the other reported studies CE and the other techniques have only been applied to analyze different metabolite classes and to obtain a comprehensive coverage of the metabolome. The review starts with a discussion on general aspects of the techniques applied in the reported comparative metabolic profiling studies, including analytical set-up, sample preparation, MS detection and data analysis. In the second part, a detailed overview of studies comparing multiple analytical techniques including CE is given. Characteristics of each study are summarized in tables. Moreover, results of the reported studies are discussed and exemplified. The non-comparative studies are briefly treated to consider which classes of metabolites can be efficiently analyzed with CE and the other applied methodologies.

2.2 General aspects of analytical methodologies used in comparative metabolic profiling studies

A typical metabolic profiling study involves multiple steps as depicted in Figure 2.1. After the experimental design, sample collection and preparation, the samples are analyzed with one or more analytical methodologies. Acquired data are analyzed after several preprocessing steps, such as alignment, normalization and scaling.

Multivariate data analysis is applied to determine differences in metabolite concentrations between different groups of samples. In the final steps, identification of metabolites of interest is pursued and their role in biochemical pathways is interpreted. General aspects of the analytical techniques used in comparative metabolic profiling studies are discussed below.

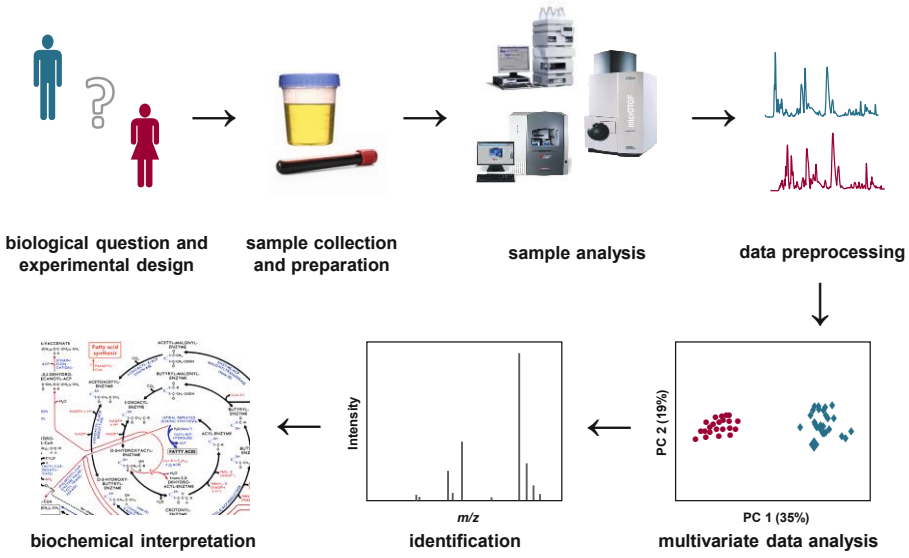

Figure 2.1 Typical workflow of metabolic profiling studies.

2.2.1 Methods

Predominantly, three CE methods have been used in comparative profiling studies to separate cationic, anionic and neutral metabolites. Cationic compounds have been analyzed using a background electrolyte (BGE) containing formic acid with a pH of approximately 2 [12, 15, 18, 20, 23-25, 28, 43-45]. Anionic metabolites are most often separated in a BGE containing ammonium acetate, but ammonium carbonate and formate have been used as well. The pH of the employed BGEs with ammonium salts varied from 7.5 to 10.0 [11-14, 22, 26, 43, 45]. Neutral metabolites have been analyzed with (micellar) electrokinetic chromatography ((M)EKC) using sodium dodecyl sulfate (SDS) and sulfated β-cyclodextrin [19, 21, 27, 53, 54]. Separations have usually been carried out in bare fused-silica (BFS) capillaries [12, 15, 18-23, 25-28, 43-45, 53-55], although coated capillaries have been applied in some studies [11, 13, 14, 19, 24, 43, 45, 53, 54]. The use of

capillaries coated with positively charged polymers reverses the electroosmotic flow (EOF), and hence, the migration order of the metabolites. Furthermore, coated capillaries are applied to diminish adsorption of matrix components to the inner wall of the capillary, resulting in a more reproducible EOF and analyte migration times required for reliable comparison of obtained metabolite profiles [60, 61].

Sample pretreatment may be necessary for good analytical CE performance. In global metabolic profiling studies, minimal sample preparation is desirable to prevent metabolite losses. The sample pretreatment depends on the nature of the samples. Types of samples that have been analyzed in comparative metabolic profiling studies involving CE are urine, plasma and extracts of bacteria, cells, plants and tissues. When analyzing urine with CE, samples are often diluted in a ratio of 1:1, v/v with BGE, water or a combination of both, sometimes followed by centrifugation [13, 19, 24, 53-55]. Sample pretreatment of plasma for CE analyses involves protein precipitation by the addition of organic solvents followed by centrifugation. Subsequently, the supernatant is normally dried and resuspended in water prior to analysis [13, 23]. Metabolic profiling of bacteria, cells, plants and tissues requires more complicated sample preparation. After cell culture, cells are washed with phosphate buffered saline. Cells are then disrupted and after various centrifugation and ultrafiltration steps, samples are ready for analysis [20, 28]. Plants, bacteria and tissues are harvested after collection. Phospholipids from cell membranes and proteins are removed, and metabolites are extracted using water, methanol and/or chloroform. Subsequently, extracts are dried and dissolved in appropriate injection solvents [11, 12, 14, 15, 21, 22, 25-27, 44, 45].

In GC, metabolites are separated using capillary columns with various polymer stationary phases of 0.1-0.25 μm thickness. In the reported comparative studies, helium has been used as carrier gas with a flow rate of 0.6-1.3 mL/min and a temperature program has been employed starting at 50-80°C and finishing at 300-330°C. Analytes have to be volatile, and therefore, a derivatization procedure is often needed [11-15, 43-45]. In the comparative studies applying GC–MS methods, a two-stage derivatization process has most commonly been employed. In the first step, ketone groups are converted to oximes by methoxyamine hydrochloride dissolved in pyridine. Subsequently, a silylation step is performed in which active hydrogens on OH, SH and NH groups are replaced with trimethylsilyl [11-15, 43-45]. For the latter, the derivatization agent N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) is most often used, sometimes in combination with trimethylchlorosilane (TMCS) [12, 15]. Compared to sample preparation for CE

analysis, this process is labour intensive and time-consuming with total analysis times up to 31 hours [12].

For LC analyses, RPLC is most commonly employed using packed columns with 1.7-1.8 μm particles (ultra performance (UP)LC). Only in a few reported studies, columns with larger particles were applied [18, 43-45]. Mostly, RPLC separations are performed under gradient elution using a mobile phase of 0.1% formic acid in water and acetonitrile [19, 20, 24, 25, 28]. For HILIC–MS analyses, various stationary phases have been used in comparative metabolic profiling studies, including bare silica [20, 25] and amino-bonded silica [43]. Metabolites are separated under gradient elution using mobile phases of water and acetonitrile, containing ammonium salts [20, 25, 43]. The sample preparation for the LC methods is similar to sample pretreatment for CE analysis. For analysis of metabolites in plants, bacteria, cells and tissues, an extraction procedure is carried out [20-22, 25-28, 44], whereas plasma and urine samples are diluted and centrifuged. Urine is diluted with water [18, 19, 24], whereas plasma is mixed with acetonitrile to remove proteins [23].

The ^{1}H NMR methods employed in the comparative studies are very straightforward. Only urine samples were analyzed which are diluted with a phosphate buffer of pH 7.4 to circumvent pH-induced signal shifts. The internal standard 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP) could be used for all detected metabolites, because ^{1}H NMR signals are directly proportional to molar proton concentrations. Treated samples are just transferred into NMR tubes and one-dimensional spectra are recorded on 600 or 800 MHz instruments employing water signal suppression [53, 54, 56].

The different applied sample preparation methods complicate the comparison of the analytical techniques used for metabolic profiling. A specific procedure may show bias towards metabolites. Different sample preparation methods, therefore, may result in the detection of different metabolites. For an ideal comparison, the sample pretreatment should be exactly the same for the various compared analytical techniques. However, this is not always possible, since specific sample preparation procedures are necessary for reliable performance of the various techniques.

The various analytical methodologies show a large variety in applied sample injection volumes. Injection volumes in CE are only a few nanoliters, which makes CE eminently suited for the analysis of volume-limited samples like cerebrospinal fluid. However, the attainable CE sensitivity is generally compromised. Injection volumes in GC and LC analyses are larger. In GC–MS, 1-2.5 μL of the samples is

injected at a temperature of 230-280°C in a splitless mode [12, 14, 43] or at a split ratio of 1:5-10 [44], 1:15 [13-15], 1:20 [11] or 1:40 [45]. Typical injection volumes in LC analyses are 1-10 μL.

2.2.2 MS detection

In a few studies which compare CE with other analytical techniques, metabolites are detected with ultraviolet (UV) spectroscopy [19, 21, 22, 27, 53, 54]. UV detection requires complete chromatographic or electrophoretic separation to reliably determine absolute changes in metabolite concentrations. However, this is complicated by the large number of metabolites present in biological samples. Furthermore, unambiguous identification of compounds is difficult in CE–UV, even when standards are available. Therefore, MS detection of metabolites is favored. When using MS, complete separation is often not required, because mass information is obtained. Electrospray ionization (ESI) which is a soft ionization method producing spectra with no or little fragment-ions, is mostly used in CE–MS and LC–MS to allow the transfer of metabolites into the mass spectrometer. In contrast, electron ionization (EI) is used in GC–MS. EI is a hard ionization technique providing detailed mass spectra showing fragmentation to give molecular structural information. Various type of mass spectrometers are used in the different comparative metabolic profiling studies. Most often, time-of-flight (TOF) or quadrupole (Q)-TOF detection is used to obtain accurate masses necessary for putative identification of metabolites of interest. In many studies comparing GC or LC with CE, different mass spectrometers are hyphenated to the applied separation techniques [11, 13-15, 18, 20, 24-26, 28, 44]. Gika *et al.* have demonstrated that the analysis of a sample on an LC-system coupled to different mass spectrometers will result in the detection of different metabolites [62]. Hence, a comparison of the results obtained with two different separation techniques coupled to mass spectrometers should be done meticulously. The use of different mass spectrometers can also be advantageous. For example, the use of Fourier transform-ion cyclotron resonance (FT-ICR)-MS will result in the detection of more accurate masses compared to masses obtained with TOF mass spectrometers [57]. Mass spectrometers can also be used to perform tandem MS measurements. In these ways, more structural information can be obtained which aids the identification of potential biomarkers.

In comparative studies, so far CE has been coupled to MS via a co-axial sheath-liquid interface. In this set-up a sheath liquid is used at a relatively high flow-rate (3-5 μL/min) to provide the electric contact at the ESI emitter tip. Unfortunately,

this also results in dilution of the sample, and thereby, a loss of sensitivity [63]. Recently, various interfaces have been developed in order to increase the attainable sensitivity of CE–MS, especially interfaces with a flow-through microvial [64] or a sheathless design [65-68]. The performance of sheathless CE–MS for metabolic profiling has been evaluated with various biological samples [65, 69-71]. More compounds are detected when using a sheathless interface, as illustrated by the base-peak electropherograms in Figure 2.2 [65, 69, 70]. Moreover, a better sensitivity is achieved for many compounds with sheathless CE–MS (up to 30-fold improvement) [65, 69, 70], although for some compounds lower limits of detection (LODs) were obtained using a sheath-liquid interface [65, 69].

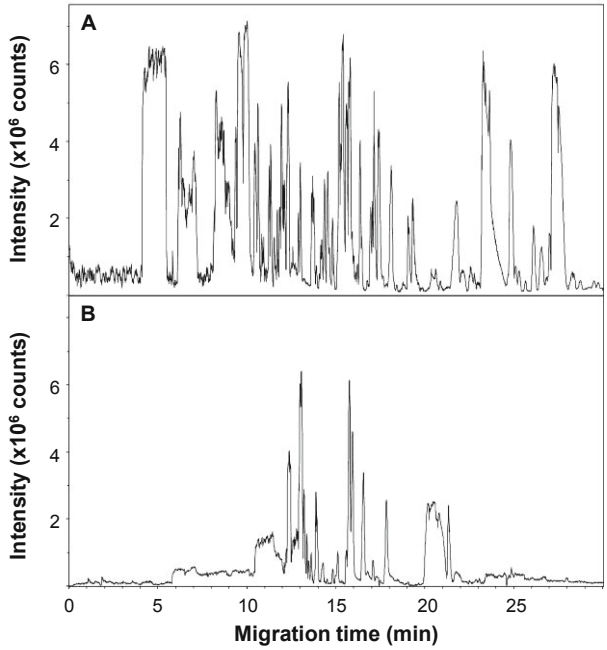

Figure 2.2 Base-peak electropherogram (*m/z* 50-450) of human urine obtained with (A) sheathless CE–MS using a porous tip sprayer and (B) CE–MS using a coaxial sheath-liquid interface. A BGE of 10% acetic acid (pH 2.2) was used and samples were injected for 30 s with a pressure of 2.0 psi (A) or 0.5 psi (B) (1% of the capillary volumes) [65].

The potential of these new CE–MS methodologies applying sheathless interfacing for improvement of metabolite coverage is high, but robustness still needs to be demonstrated in long-term metabolic profiling studies. Furthermore, all sheathless

CE–MS studies have been conducted in positive ionization mode. However, a large part of metabolites is acidic and can only be ionized efficiently in negative ionization mode. Also in many comparative profiling studies using a sheath-liquid interface, CE–MS is carried out in positive ionization mode [15, 18, 20, 23-25, 28, 44]. To increase the coverage of metabolites, the applicability of (sheathless) CE–MS with negative ESI needs to be further investigated.

2.2.3 Data analysis

Metabolic profiling generates large numbers of variables and complex data sets. Chemometric analysis is essential for a correct interpretation of the acquired data. Before performing multivariate data analysis, a few data preprocessing steps have to be carried out which are similar for all analytical techniques. These steps include baseline correction, alignment, peak detection and normalization of obtained metabolite profiles. In CE, shift of migration times of metabolites in successive analyses might be an issue. There is often a higher variability in migration times in CE as compared to retention times in GC and LC, and chemical shifts in ^{1}H NMR spectroscopy. However, it has been demonstrated that these problems can be overcome effectively by applying proper alignment algorithms [55, 72]. Migration-time shifts can be corrected for allowing reliable comparison of metabolite profiles. Metabolite profiles have been compared using principal component analysis (PCA) [11-13, 18, 19, 24, 27, 44, 54-56] and (orthogonal) partial least squares discriminant analysis (PLS-DA) [12, 13, 18, 19, 24, 44, 53, 54, 56]. Samples derived from different groups may be discriminated and potential biomarkers might be revealed. In order to determine whether observed differences originate from biological variance and not from analytical variance, a quality control (QC) sample, which is a mixture of aliquots of (a part of) all samples, has been used. A QC sample is repeatedly analyzed to assess the stability of the system and determine the quality of the obtained data [18, 20, 25, 44, 55].

Identification of potential biomarkers and elucidating their role in biochemical pathways is the last step in the data analysis procedure. For GC–MS, there are extensive databases with retention indices and mass spectral data, making compound identification very well feasible. This is certainly not the case for CE and LC where standardization of separation and MS conditions is not trivial. In CE and LC, compounds can be putatively identified by comparing recorded accurate masses with masses listed in metabolomics databases [73-75]. However, a single mass may result in multiple possibilities for identity, since many metabolites have similar masses. In CE–MS, some possibilities might be eliminated based on observed

migration times. Electrophoretic mobilities, and thus migration times, of metabolites can be predicted based on their charge-to-size ratios under the applied CE–MS conditions [76, 77]. Interpretation of NMR spectra can be complicated. Since no separation is carried out, there is an enormous overlap of analyte signals. Lack of preseparation also makes NMR susceptible to incorrect data interpretation due to potential signals derived from chemicals or solvents used in sample preparation.

2.3 Overview of comparative studies

Over the last decade, in total 48 comparative and comprehensive metabolic profiling studies involving CE have been reported [11-59]. In 22 studies, the CE performance and results have been compared with results obtained with GC, LC, ^{1}H NMR spectroscopy and/or flow-injection MS [11-15, 18-28, 43-45, 53-56]. In this review, particular attention is paid to these comparative studies. In the other 26 studies, no actual comparison was made between CE and other analytical technologies. In the latter studies, the specific analytical techniques have been applied to analyze different metabolite classes and to obtain a more comprehensive coverage of the metabolome. These 26 studies are covered with respect to classes of covered metabolites, but not treated exhaustively.

2.3.1 CE and GC

So far, seven studies have been described applying both CE–MS and GC–MS to profile metabolites present in bacterial, plant and yeast extracts, and in plasma and urine samples [11-17]. In five of these studies, a comparison between CE–MS and GC–MS was made and the characteristics of these studies are shown in Table 2.1 [11-15].

The use of both analytical techniques resulted in the detection of more metabolites demonstrating the complementarity of CE–MS and GC–MS for metabolic profiling. For instance, Jumtee *et al.* employed a targeted CE–MS method to analyze nucleotides, coenzyme A compounds and sugar phosphates, because these compounds could not be detected with the applied GC–MS method. In contrast, sugars, sugar alcohols, fatty acids and sterol derivatives were only analyzed with GC–MS. Some amino acids and organic acids were determined with both methodologies and the obtained relative metabolite levels were highly similar. The advantage of GC–MS was the higher reproducibility in most cases, whereas the separation of isomers (e.g. isocitrate and citrate) could only be achieved with CE–MS [11]. Also in a study by Timischl and colleagues, the analytical variance was smaller with GC–MS, and observed LODs and metabolite levels were similar.

There was only a difference in the measured level of adenosine monophosphate. Higher amounts were found with GC–MS, because of the degradation of adenosine triphosphate and adenosine diphosphate under the applied GC–MS conditions. Phosphorylated compounds are thermally instable, and for that reason, sugar phosphates were also in this study only determined with CE–MS [14].

An inter-laboratory comparison of CE–MS and GC–MS for metabolic profiling of amino acids in plant cell cultures was made by Williams *et al.* Again, there was a high correlation between metabolite concentrations determined with CE–MS and GC–MS. However, using GC–MS not every amino acid could be analyzed. Another advantage of the applied CE–MS method was the three times higher throughput, because it requires, in contrast to GC–MS, no extensive derivatization procedure [15].

Besides the metabolic profiling studies of bacterial, plant and yeast extracts, also urine and plasma samples of rats were analyzed after methamphetamine-induced acute intoxication [13]. As also seen in other studies, GC–MS showed a low sensitivity for sugar phosphates and separation of isocitrate and citrate could not be achieved. Therefore, CE–MS was applied for the analysis of these anionic metabolites in particular. Only eight metabolites in urine and nine metabolites in plasma were detected with CE–MS. The authors do not explain this limited number of detected and identified metabolites. Notably, the injection volume is relatively small (6 nL). In contrast, untargeted profiling using GC–MS revealed 496 peaks in urine and 353 peaks in plasma, of which 82 and 62 could be identified, respectively [13].

Kim *et al.* studied the metabolism of soybeans during metabolism using CE–MS and GC–MS. Metabolites were extracted with two different procedures. Extraction was carried out with methanol and water-methanol-chloroform (1:3:1 v/v/v) for CE–MS and GC–MS, respectively. In addition, extracted metabolites were derivatized for GC–MS analysis. In positive mode CE–MS 49 metabolites were annotated and in negative ionization mode 38 metabolites were identified. Also in GC–MS 87 compounds were identified, despite the fact that in CE–MS more molecular features were detected. Some metabolites could be detected with both analytical methodologies. There was an overlap between GC–MS and CE–MS of 24 and 14 compounds for the positive and negative ionization mode, respectively. This implies that 49 metabolites could be uniquely detected with GC–MS and 36 compounds were only found with CE–MS. Using GC–MS, more fatty acids and carbohydrates were detected, whereas with CE–MS more nucleosides were observed. So, applying both techniques increased the coverage of the metabolome tremendously [12].

Others also used CE–MS and GC–MS to profile the metabolome comprehensively, but without comparing the two techniques [16, 17]. Toya *et al.* profiled metabolites in a non-targeted approach using CE–MS and in a targeted approach using GC–MS. With CE–MS, mainly sugar phosphates were detected, whereas GC–MS was used to analyze proteinogenic amino acids [16]. In contrast, Urano *et al.* used GC–MS for non-targeted metabolic profiling and CE–MS was used to identify and quantify 14 amino acids [17]. The authors do not give reasons for their choices of the applied analytical methods.

2.3.2 CE and LC

The most often combined use of two analytical techniques for comprehensive and comparative metabolic profiling involving CE is a combination with LC [18-42]. The performance of CE and LC for metabolic profiling was evaluated and compared in ten studies by analyzing various types of samples (Table 2.2) [18-28], and detection was conducted with MS [18-20, 23-26, 28] or UV spectroscopy [19, 21, 22, 27].

Garcia-Perez *et al.* used CE and UPLC for the analysis of urine to study schistosomiasis in mice [19]. After a data-analysis procedure applying PCA and (orthogonal) PLS-DA on obtained metabolic profiles, *Schistosoma mansoni* infected and control mice could be discriminated with both CE and LC [19]. The discrimination by CE–UV and UPLC–MS (Figure 2.3) was based on the same metabolites, namely hippuric acid and phenylacetylglycine [19]. Ramautar *et al.* used CE–MS and UPLC–MS to study gender differences in urinary metabolite profiles of human [24]. Using multivariate data analysis, men and women could be distinguished. In contrast to Garcia-Perez *et al.*, Ramautar and colleagues found different classifying metabolites with CE and LC [24]. Ten times more discriminatory compounds were observed with UPLC–MS than with CE–MS (300 versus 27). The identified metabolites found with CE showed no retention when analyzed with UPLC. Furthermore, with CE–MS m/z values between 100 and 150 were observed, whereas with UPLC–MS, 95% of the compounds had m/z values above 150 [24].

Also in other studies, more features were found with LC compared to CE [18, 23, 27, 28]. Alberice *et al.* observed on average 75 and 684 urinary compounds using CE–MS and LC–MS, respectively. Of these compounds, 14 and 13 identified metabolites were found to be statistically related to the stage and recurrence of bladder cancer. 6-Keto-decanoyl carnitine was the only discriminatory metabolite which was revealed with both CE–MS and LC–MS [18]. Valdés *et al.* found on

average differences in 69 and 113 compounds in cancer cell extracts after treatment with dietary polyphenols with CE–MS and LC–MS, respectively [28]. For the characterization of rhubarb species, 50 and 90 peaks were detected with CE and LC using UV spectroscopy [27]. Minami *et al.* analyzed mouse plasma samples to study circadian oscillations in metabolite concentrations within 24 hours providing information about the so-called internal body time. More compounds were detected with UPLC–MS. However, CE–MS was only carried out in positive ionization mode, whereas with UPLC–MS the samples were also analyzed in negative ionization mode. Of the detected compounds, more metabolites were identified based on CE–MS data. Different discriminatory compounds were found with UPLC–MS and CE–MS. For instance, lysophosphatidylcholines were mainly observed with UPLC–MS, whereas CE–MS indicated more amino acids and metabolites of the urea cycle to be significantly different [23].

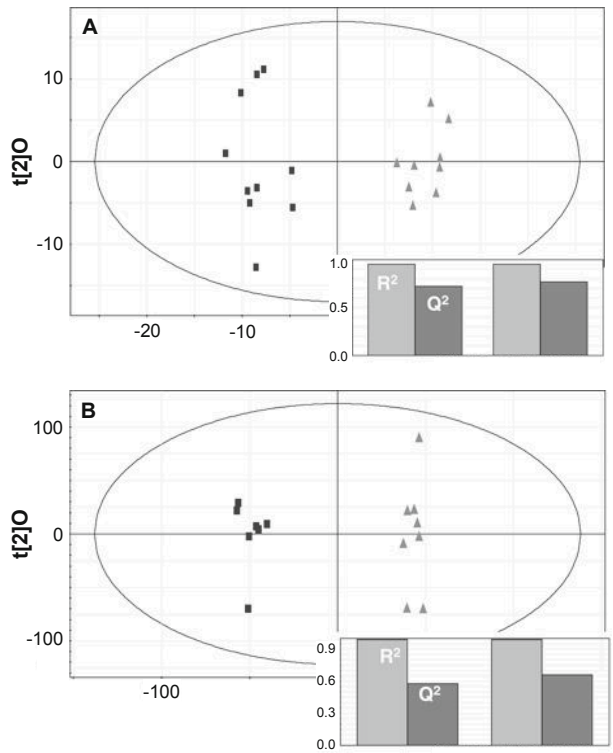

Figure 2.3 Chemometric analysis (orthogonal PLS-DA) of urinary metabolic profiles of *Schistosoma mansoni* infected (triangles) and control mice (squares) obtained with CE–UV (A) and UPLC–MS (B) [19].

Recently, CE was compared to both RPLC and HILIC for metabolic profiling of parasite tissues and colon cancer cells [20, 25]. The results of Saric *et al.* [25] were similar to the results obtained by Minami *et al.* [23]. More peaks were observed with both LC methodologies, but also in this study CE–MS was only operated in positive ionization mode [25]. Furthermore, relatively less metabolites were identified with LC (RPLC <2%, HILIC 6%) compared to CE (32%). In total, 142 metabolites were identified of which nine were detected across all methodologies (mainly amino acids), showing the complementarity of the applied analytical techniques [25]. However, it should be noticed that LC–MS measurements were performed on a Q-TOF mass spectrometer, whereas a TOF mass spectrometer was used for CE–MS analyses. As indicated before, different compounds can be detected when analyzing the same sample on various mass spectrometers [62].

Ibáñez *et al.* compared RPLC–MS, HILIC–MS and CE–MS for metabolic profiling of colon cancer cells using different mass spectrometers, providing 2176, 1077 and 2890 features, respectively. So, the number of molecular features with HILIC–MS was much lower. However, samples were diluted more for HILIC–MS analysis, because analyzing less diluted samples resulted in detector saturation. Overall, 32, 12 and 22 compounds were identified with respectively RPLC, HILIC and CE being significantly different in colon cancer cells treated with dietary polyphenols. Of these compounds, six were observed with both CE and RPLC (adenosine, creatinine, leucine, oxidized glutathione, phenylalanine, tyrosine) and two with HILIC and RPLC (11-aminoundecanoic acid, 3-ketolactose). There was no overlap in altered compounds detected with CE and HILIC [20].

Jia and co-workers used an entirely different approach by designing an offline LC–CE system to improve the separation of compounds in bacterial extracts that could not be completely resolved by only one of the systems [21, 22]. First, compounds were separated on a octadecylsilylated monolithic column. Compounds with no or limited retention (i.e. charged compounds) were subsequently analyzed with dynamic pH junction CE. The separation of retained metabolites (i.e. non-charged compounds) was enhanced by applying sweeping MEKC. The improved separation was required to detect and identify more compounds using detection with UV spectroscopy [21].

In the majority of the studies involving CE and LC, both techniques were applied in order to increase the number of detected metabolites without comparing the performance of the methodologies [29-42]. In one study, LC–MS was used to analyze plasma samples of type 1 diabetic children, whereas CE–MS was used to analyze urine samples from the same patients [29]. In many studies, CE is used as

the principal analytical technique and LC was used in addition to detect and quantify sugars [30-33], lipids [31, 34, 35], γ-glutamyl peptides [36], thiol compounds [37], purine nucleotides [38] or prostaglandins [39]. Furthermore, LC was used to determine metabolite recovery [40] or the degree of enzyme phosphorylation [41].

The choice to analyze specific metabolites with LC instead of CE was due to sensitivity, the polarity of compounds and whether compounds can be charged under the applied CE conditions. For instance, Osanai *et al.* used LC–MS/MS to quantify purine nucleotides, because the concentrations were too low to analyze these metabolites with CE–MS [38]. Lipids are very apolar and have a poor solubility in aqueous BGEs that are often used in CE analyses. Those compounds are easier assessed with RPLC [31, 34, 35]. Dissociation constants of many sugars are relatively high (pK_a >12), and therefore, the sugars are not charged under the experimental CE conditions which are most commonly applied. It is possible to analyze sugars with CE as charged compounds by employing BGEs with a high pH or as neutral compounds by employing MEKC. In the described comprehensive metabolic profiling studies, concentrations of various sugars were preferably determined with HILIC–MS [30-33]. Sugimoto *et al.* also analyzed prostaglandins with LC because of a lack of charge under the chosen CE conditions [39]. However, prostaglandin E_2 and $F_{2\alpha}$ have carboxylic acid groups which are deprotonated at pH 8.5 used in this study. Furthermore, the choice for the applied LC system is questionable since the prostaglandins of interest were not resolved.

2.3.3 CE, GC and LC

The combined use of CE with both GC and LC is also employed in order to analyze and cover as many metabolites present in biological samples [43-52]. In three studies a comparison is made between the various employed analytical techniques (Table 2.3) [43-45].

Saheki *et al.* performed CE–MS and GC–MS to analyze and identify metabolites extracted from mouse livers. LC–MS and enzymatic methods were used for the quantification of specific metabolites and for the confirmation of the results obtained with GC–MS and CE–MS. With CE–MS a broader range of metabolites could be analyzed compared to GC–MS. For example, more metabolites which play key roles in the urea cycle and the tricarboxylic acid (TCA) cycle were detected with CE–MS. Furthermore, the applied CE–MS method appeared to be more sensitive than the GC–MS method. A few differences were found between results obtained with CE–MS, LC–MS and enzymatic methods. Some compounds appeared to be

significantly changed as determined with one method, whereas this effect was not observed with the other method. The applied methods might not be sensitive enough to detect certain metabolites or to observe small differences in concentration [45].

Naz and co-workers used CE–MS, GC–MS and LC–MS for untargeted fingerprinting of lung tissue [44]. Substantial more compounds could be detected and identified with LC–MS (1115 metabolites), compared to CE–MS (85 metabolites) and GC–MS (69 metabolites). However, LC–MS was also operated in negative ionization mode, whereas CE–MS and GC–MS were only operated using positive ESI. The greatest overlap in detected compounds was observed with GC–MS and LC–MS (21 metabolites), followed by the combined use of CE–MS and LC–MS (20 metabolites). Common metabolites detected with GC–MS and LC–MS were mainly fatty acids, sterols and sugars, whereas amino acids were found with both CE–MS and LC–MS. Overall, seven metabolites were observed with all three analytical technologies, namely glutamic acids, hypoxanthine, valine, nicotinamide, proline and pyroglutamic acid.

An extensive comparison of CE–MS, GC–MS and LC–MS was performed by Büscher *et al.* [43]. For each analytical platform, two different methods were developed to analyze a mixture of 91 metabolites, covering important metabolic pathways, with and without added yeast extracts (Table 2.3). Two commonly used CE–MS methods were employed to profile anionic and cationic metabolites using a BGE containing ammonium acetate and formic acid, respectively. Before GC–MS analysis, metabolites were derivatized with both MSTFA and N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (TBDMS-FA). The two LC–MS methods were ion-pairing LC and HILIC. Comparisons of all the analytical methodologies were made based on the following criteria: metabolite coverage, matrix effects (by assessing signal intensities of test metabolites without and with the addition of ^{13}C-labeled yeast extracts), separation of isomers and general considerations, including overall performance, time and costs. The coverage of the metabolites with GC–MS was substantially lower than with CE–MS and LC–MS (Figure 2.4A and 2.4B). Of all analyzed metabolites, 33 compounds could be measured on all platforms and three compounds could not be analyzed with either methodology. Except the CE–MS method for profiling cationic metabolites, all methods were largely affected when ^{13}C-labeled yeast extracts were added to the test mixture (Figure 2.4C). Matrix effects in CE–MS for cationic compounds were less apparent, because the separation of metabolites was sufficient to avoid ion suppression caused by co-elution of interfering metabolites.

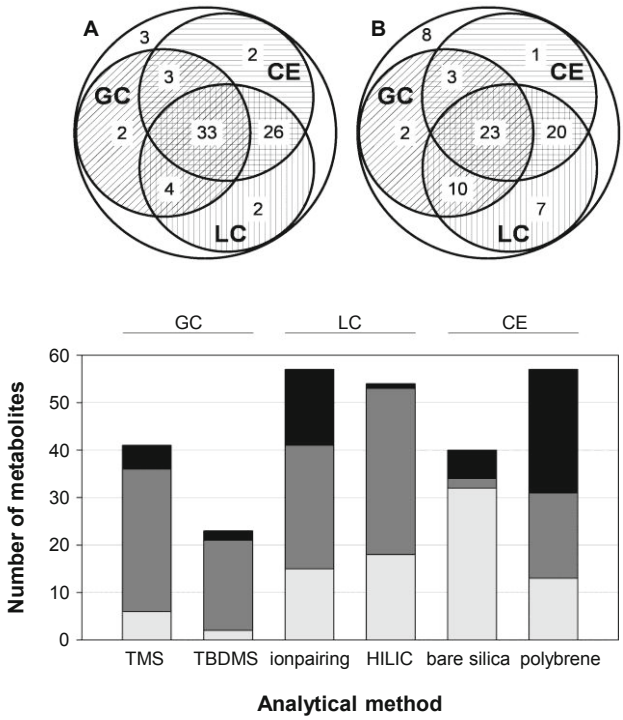

Figure 2.4 Coverage of 91 reference standards without (A) and with (B) added ^{13}C-labeled yeast extracts and the number of metabolites affected by matrix effects (C). Light gray, not affected; dark grey, affected by matrix effects, but measurable in the presence of ^{13}C-labeled yeast extracts; black, not measurable in the presence of ^{13}C-labeled yeast extracts. Structural isomers were counted as one metabolite [43].

The separation of isomers was possible with the applied GC–MS and LC–MS methods. For instance, GC–MS after derivatization with TBDMS-FA was capable to distinguish citrate and isocitrate. The separation of these metabolites was not obtained with the two applied CE–MS methods [43]. This is contradictory to the results of Shima *et al.* [13] and Jumtee *et al.* [11], who employed CE–MS to resolve these two compounds (Section 2.3.1). In all three studies, a BGE containing 50 mM ammonium acetate (adjusted to pH 8.5 and 9.0) was used. The main difference was the applied coating on the inner wall of the capillary. Shima *et al.* [13] and Jumtee *et al.* [11] used capillaries of which the internal surface was modified with anionic groups, whereas Büscher *et al.* [43] used a coating of positively charged polymers.

Therefore anionic metabolites, like citrate and isocitrate, were measured after and before the EOF, respectively. The separation window is larger in the former case, thereby allowing the separation of the isomers. The predominant difference between the CE–MS, GC–MS and LC–MS platforms was the robustness of the methods. Retention times in GC–MS and LC–MS were very stable from sample to sample, whereas shifts in migration times were more common with CE–MS. For this reason and because of the high coverage of analyzed metabolites, Büscher *et al.* recommend the use of LC–MS, which is best complemented with GC–MS. However, the evaluation of the complementary of the analytical techniques for metabolic profiling is limited as a small number of test metabolites was analyzed and no biological samples were examined. The yeast extracts were not used for metabolic profiling, but only for determining the extent of matrix effects [43].

In seven studies, the analytical techniques were not compared extensively, but were only applied for the comprehensive profiling of the metabolome [46-52]. For instance, in two studies LC–MS was used for global metabolic profiling of urine samples to study adverse effects of various drugs, including hepatotoxicity. The separation power and sensitivity of the LC–MS method for amino acids and steroids were not good enough, and therefore, these metabolites were quantitatively analyzed using CE–MS and GC–MS [46, 47], respectively. Furthermore, urinary concentrations of oxysterols and bile acids were determined applying GC–MS as targeted approach [47]. The additional targeted CE–MS and GC–MS analysis of the urine samples resulted in the detection of more compounds discriminating between control rats and drug-treated rats [46, 47]. In two other studies, GC was connected to a sulfur chemiluminescence detector for the analysis of sulfur-containing gasses. Also CE–MS and LC–MS were only used in a targeted approach to measure various compound classes of metabolites [48, 49]. Recently, a combination of LC–MS and GC–MS was used to profile specific metabolite classes [50, 51]. Using GC–MS mainly volatile compounds and fatty acids were analyzed, whereas LC–MS was applied for the analysis of non-volatile metabolites. In these studies, CE–MS was used for the identification and quantification of primary metabolites [50, 51]. Kusano *et al.* combined the results obtained with CE–MS, GC–MS and LC–MS in one data set to study genetically modified tomatoes. Only the metabolite coverage with the three techniques was compared. The largest number of metabolites was detected with LC–MS, whereas with CE–MS the lowest number of metabolites was revealed. Overall, the largest coverage of metabolites was obtained with the combination of the three analytical methodologies [52].

2.3.4 CE and NMR spectroscopy

The combined use of CE and NMR spectroscopy is rarely applied in metabolic profiling studies (see Table 2.4) [53-56]. Garcia-Perez *et al.* studied the differences in the urinary metabolome of control mice versus *Schistosoma mansoni* infected mice [53] and slc26a6 null mice [54]. The influence of an infection caused by *Schistosoma mansoni* on urinary metabolic profiles have also been analyzed with a combination of CE–UV and UPLC–MS (see above) [19]. A few compounds were detected with both CE and ^{1}H NMR spectroscopy, including creatine, creatinine, hippuric acid, citric acid, phenylacetylglycine and hydroxyphenylpropionylsulfate. The two techniques also uniquely identified a few compounds. For instance, taurine and methylamines were detected with ^{1}H NMR spectroscopy, whereas oxalic and nitric acid were detected with CE. Remarkably, indoxyl sulfate was in one study only detected with CE, whereas in the other study this compound was only observed with ^{1}H NMR spectroscopy.

The identification in CE was complicated since only UV detection was used, thereby limiting the comparison between CE and NMR. Using PLS-DA based on metabolic profiles obtained with both techniques, control mice could be distinguished from *Schistosoma mansoni* infected mice and slc26a6 null mice. If the CE and NMR data obtained from the metabolic profiles were combined into one PLS-DA model, the predictivity of the model was increased, facilitating the discrimination of the urine samples [53, 54].

Recently, Kok *et al.* analyzed urine samples from antibiotic-treated rats with CE–MS to study microbial-host co-metabolism, which had previously been analyzed with ^{1}H NMR spectroscopy [55, 56]. In CE–MS, uncoated capillaries and a BGE containing triethylamine were used for the separation of metabolites and MS detection was performed in negative ionization mode. Urine samples were diluted with the BGE in a proportion of 1:1, v/v prior analysis [55]. For NMR analyses, sample pretreatment was similar to the one carried out by Garcia-Perez *et al.*, since urine was mixed with a phosphate buffer (pH 7.4), containing TSP [56]. Metabolic profiles from rats treated for 0, 4 or 8 days with antibiotics could be distinguished with both CE–MS and ^{1}H NMR spectroscopy using multivariate data analysis. Fifteen urinary compounds were found with CE–MS to be down-regulated upon antibiotic treatment, whereas two compounds were up-regulated [55]. Using ^{1}H NMR spectroscopy, the urinary concentrations of twenty-five metabolites were altered after the administration of antibiotics, of which seven had a lower abundance. This is illustrated in a typical coefficient plot in Figure 2.5 [56]. Thirty-eight compounds were uniquely found with one of the two analytical techniques,

exemplifying the complementarity of CE–MS and ^{1}H NMR spectroscopy in metabolic profiling studies. Only hippuric acid and indoxyl sulfate were observed with both CE and ^{1}H NMR spectroscopy [55, 56]. In the studies conducted by Garcia-Perez *et al.* these compounds could also be detected with both CE and ^{1}H NMR spectroscopy [53, 54].

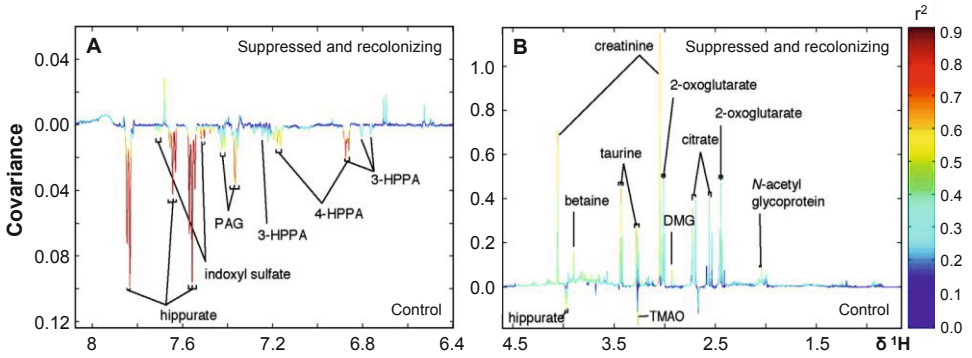

Figure 2.5 Coefficient plot showing differences in urinary ^{1}H NMR profiles obtained from control and antibiotic-treated rats (suppressed and recolonizing). Metabolites which were down-regulated (A) and up-regulated (B) upon treatment are indicated [56].

2.3.5 Other combinations of analytical techniques

A few metabolic profiling studies have been conducted in which CE–MS is used in combination with flow-injection MS [57-59]. In two studies, concentration changes in energy-related metabolites in the brain were visualized by imaging MS using a matrix-assisted laser desorption/ionization (MALDI)-TOF mass spectrometer [58, 59]. Quantitative information obtained with imaging MS was limited since only relative changes could be observed. Therefore, CE–MS was used to determine and validate the absolute quantities of metabolites of interest. However, as sample pretreatment before CE–MS analysis, tissues were homogenized in order to extract metabolites, resulting in the loss of information on the spatial distribution of these metabolites in various regions in the brain. So, the information available on the changes in energy metabolism in the brain was increased by taking advantage of both analytical strategies [58, 59].

Leon and co-workers used a combination of CE–MS and FT-ICR-MS for the metabolome analysis of transgenic maize [57]. Highly accurate masses can be obtained using FT-ICR-MS, which enables the assignment of the elemental

composition of compounds. On average 170 and 420 compounds were identified in positive and negative ionization mode, respectively. However, isomers could not be distinguished because no separation was applied before detection. Therefore, CE–MS was used to gain information based on electrophoretic mobilities of metabolites. For example, a compound with *m/z* 150.055 was found with FT-ICR-MS. This mass corresponds to the molecular formula $C_8H_8NO_2$ and can originate from three compounds, namely 4-hydroxymandelonitrile, 2-formylaminobenzaldehyde and 5,6-dihydroxyindole. CE–MS was performed using a BGE of 5% formic acid in water (pH 1.9) and this resulted in the detection of one compound with an *m/z* value of 150.06. Only 5,6-dihydroxyindole is positively charged under these experimental conditions and will migrate faster than the EOF. So, the additional use of CE–MS provides a more complete picture in metabolic profiling studies, especially in discriminating isomers which cannot be resolved with flow-injection MS. Coupling CE to FT-ICR-MS is a possibility to use the advantages of both techniques simultaneously. However, this will probably result in a loss of sensitivity, thereby decreasing the number of potential biomarkers [57].

2.4 Concluding remarks

As demonstrated in the various comparative metabolic profiling studies, CE–MS has a clear added value in relation to GC, LC, NMR spectroscopy and flow-injection MS. Applying multiple techniques increases the overall coverage of metabolites. CE was shown to be highly complementary to the other analytical methodologies by detecting polar and ionogenic metabolites which were not revealed by other analytical techniques. In various metabolic profiling studies employing multiple techniques, phosphorylated compounds, amino acids, metabolites from the TCA and urea cycle, nucleotides and nucleosides were efficiently analyzed by CE. The analysis of these classes of metabolites could not be completely fulfilled with the applied GC–MS and/or LC–MS methods. In contrast, GC–MS and LC–MS appeared to be more suitable for the profiling of more apolar compounds, such as sugars, fatty acids, sterols, steroids and bile acids. No general conclusions can be drawn on differences in metabolite coverage between CE and NMR spectroscopy, due to the limited number of comparative studies.

CE was in favor for the separation of structurally highly related metabolites and isomeric compounds. Better separation of metabolites may result in less matrix effects since the ionization of compounds is less suppressed.

A low migration-time reproducibility and limited sensitivity are often reported as drawbacks for CE compared to the other applied analytical techniques in metabolic

profiling studies. Migration-time shifts can partly be prevented by the use of coated capillaries, resulting in more stable CE methods. Moreover, it has been shown that proper alignment procedures can substantially diminish shifts in migration time allowing reliable comparison of metabolite profiles. The limited sensitivity is mainly caused by the relatively small injection volumes in CE compared to the other methodologies. Efforts have been made to improve the sensitivity of CE–MS by applying chromatographic and/or electrophoretic concentration techniques. In this way, larger sample volumes (if available) can be injected. The sensitivity might also be increased by the development and application of new CE–MS interfaces. The use of sheathless CE–MS has already demonstrated that significantly higher signal intensities can be obtained and more molecular features can be found, but its robustness has to be proven in long-term metabolomics studies. Improvements in CE–MS performance may further increase its added value and give it a more prominent role in metabolic profiling studies.

Table 2.1 Overview of studies comparing CE–MS and GC–MS

Capillary electrophoresis		**Gas chromatography**		**Sample**	**Results of comparison**	**Ref.**
Capillary	*BGE*	*Column*	*Derivatization*			
FunCap-CE type S (50 µm x 80 cm)	50 mM NH_4Ac (pH 9.0)	CP-SIL 8 CB low bleed (0.25 µm, 0.25 mm x 30 m)	methoxyamine and MSTFA	Plant extracts	CE: 71 compounds GC: 64 compounds 24 compounds in common	[11]
Fused-silica (50 µm x 100 cm)	1 M FA 20 mM NH_4Form (pH 10.0)	RTX-5 SIL (0.25 µm, 0.25 mm x 30 m)	methoxyamine, MSTFA and TMCS	Bean extracts	CE (+): 49 compounds CE (-): 38 compounds GC: 87 compounds 24 (+) and 14 (-) compounds in common	[12]
FunCap-CE type S (50 µm x 80 cm)	50 mM NH_4Ac (pH 9.0)	CP-SIL 8 (0.25 µm, 0.25 mm x 30 m)	methoxyamine and MSTFA	Rat plasma and urine	CE: 9 (plasma) and 8 (urine) compounds GC: 62 (plasma) and 82 (urine) compounds Levels determined with both CE and GC correspond	[13]
PolyE-323 coated (50 µm x 100 cm)	50 mM NH_4Ac (pH 8.7) in 5% MeOH	ZB-AAA (0.1 µm, 0.25 mm x 15 m) RXI-5 MS (0.25 µm, 0.25 mm x 30 m)	chloropropyl formate methoxyamine and MSTFA	Bacterial extracts	GC: smaller analytical variance Comparable LODs	[14]
Fused-silica (50 µm x 70 cm)	1 M FA	DB-5 MS (0.25 µm, 0.25 mm x 60 m)	methoxyamine, MSTFA and TMCS	Plant extracts	CE: 21 amino acids GC: 15 amino acids Excellent correlation of levels	[15]

(+): positive ionization mode; (-): negative ionization mode; BGE: background electrolyte; CE: capillary electrophoresis; FA: formic acid; GC: gas chromatography; H_2O: water; LOD: limit of detection; MeOH: methanol; MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide; NH_4Ac: ammonium acetate; NH_4Form: ammonium formate; Ref.: reference; TMCS: trimethylchlorosilane.

Table 2.2 Overview of studies comparing CE and LC

Capillary electrophoresis			**Liquid chromatography**			**Sample**	**Results of comparison**	**Ref.**
Capillary	*BGE*	*Detection*	*Column*	*Mobile phases*	*Detection*			
Fused-silica (50 μm x 100 cm)	0.8 M FA in 10% MeOH	MS (TOF)	Ascentis Express C_{18} (2.7 μm, 2.1 x 50 mm)	(A) 0.5% FA in H_2O (B) ACN	MS (Q-TOF)	Human urine	CE: 14 discr. comp. LC: 13 discr. comp. 1 common discr. comp.	[18]
Fused-silica (50 μm x 40 cm) Polyacrylamide-coated (50 μm x 57 cm)	25 mM borate, 75 mM SDS and 1.435% (m/v) sulfated β-CD (pH 9.5) 200 mM phosphate (pH 6.1) in 10% MeOH	UV	Acquity C_{18} (ns μm, 2.1 x 100 mm)	(A) 0.1% FA in H_2O (B) 0.1% FA in H_2O-ACN (5:95, v/v)	MS (Q-TOF)	Mouse urine	CE: Q^2, 0.77; R^2, 0.95 LC: Q^2, 0.66; R^2, 0.99 2 common discr. comp.	[19]
Fused-silica (50 μm x 80 cm)	1 M FA	MS (TOF)	Zorbax C_{18} RRHT (1.8 μm, 2.1 x 50 mm) Zorbax HILIC Plus HT (1.8 μm, 2.1 x 50 mm)	(A) 0.1% FA in H_2O (B) 0.1% FA in ACN (A) 10 mM NH_4Form (pH 5) (B) ACN	MS (Q-TOF)	HT29 colon cancer cell extracts	CE: 212 discr. comp. RPLC: 210 discr. comp. HILIC: 214 discr. comp. CE+RPLC: 6 common CE+HILIC: 0 common RPLC+HILIC: 2 common	[20]
Fused-silica (75 μm x 50 cm) Fused-silica (50 μm x 50 cm)	160 mM borate (pH 9.4) 50 mM SDS and 50 mM phosphoric acid in 20% ACN	UV	Monolithic silica-ODS (0.2 x 250 mm)	(A) MeOH (B) 30 mM phosphate buffer (pH 3.0)	UV	Bacterial extracts	More comp. separated with both CE and LC	[21]
Fused-silica (75 μm x 50 cm)	100 mM ammonium carbonate (pH 9.6)	UV	Monolithic silica-ODS (0.2 x 500 mm)	(A) MeOH-30 mM NH_4Ac (pH 4.0) (95:5, v/v) (B) MeOH-30 mM NH_4Ac (pH 4.0) (2:98, v/v)	UV	Bacterial extracts	More comp. separated with both CE and LC	[22]

Table 2.2 Continued

Fused-silica (50 µm x 100 cm)	1 M FA	MS (TOF)	Zorbax SB-C_{18} RRHT (1.8 µm, 2.1 x 50 mm)	(A) 0.1% acetic acid in H_2O (B) MeOH	MS (Q-TOF)	Mouse plasma	CE (+): 153 discr. comp. LC (+): 142 discr. comp. LC (-): 176 discr. comp.	[23]
PB-DS-PB coated (50 µm x 100 cm)	1 M FA (pH 2.0)	MS (TOF)	Acquity C_{18} BEH (1.7 µm, 2.1 x 100 mm)	(A) 0.1% FA in H_2O (B) 0.1% FA in ACN	MS (Q-TOF)	Human urine	CE: 27 discr. comp. LC: 300 discr. comp. 4 common discr. comp.	[24]
Fused-silica (50 µm x 65 cm)	0.8 M FA (pH 1.8) in H_2O-MeOH (8:2, v/v)	MS (TOF)	Acquity C_{18} BEH (1.8 µm, 2.1 x 100 mm) Acquity HILIC BEH (1.7 µm, 2.1 x 100 mm)	(A) 0.1% FA in H_2O (B) 0.1% FA in ACN (A) 0.1% FA in ACN-200 mM NH_4Ac (95:5, v/v) (B) 0.1% FA in ACN-20 mM NH_4Ac (1:1, v/v)	MS (Q-TOF)	Parasite tissue extracts	CE(+): 114 features RPLC(+): 5183 features RPLC(-): 5420 features HILIC(+): 2281 features HILIC(-): 1726 features 9 comp. detected with all platforms	[25]
Fused-silica (50 µm x 100 cm)	50 mM NH_4Ac (pH 7.5)	MS (Q)	Asahipak ODP-50 2D (2 x 150 mm)	5 mM NH_4Ac	MS (triple Q)	Bacterial extracts	CE: better resolution LC: low recovery rate	[26]
Fused-silica (50 µm x 62.5 cm)	15 mM tetraborate, 15 mM phosphate and 30 mM SDC in 30% ACN	UV	Acquity C_{18} BEH (1.7 µm, 2.1 x 100 mm)	(A) 0.05% phosphoric acid in H_2O (B) ACN	UV	Plant extracts	CE: 50 peaks LC: 90 peaks CE: less sensitive and reproducible	[27]
Fused-silica (50 µm x 80 cm)	1 M FA	MS (TOF)	Zorbax C_{18} RRHT (1.8 µm, 2.1 x 50 mm)	(A) 0.1% FA in H_2O (B) 0.1% FA in ACN	MS (Q-TOF)	K562 cell extracts	CE: 69 discr. comp. LC: 113 discr. comp.	[28]

(+): positive ionization mode; (-): negative ionization mode; ACN: acetonitrile; BGE: background electrolyte; CD: cyclodextrin; CE: capillary electrophoresis; comp.: compounds; discr.: discriminatory; DS: dextran sulfate; FA: formic acid; H_2O: water; HILIC: hydrophilic interaction chromatography; MeOH: methanol; MS: mass spectrometry; NH_4Ac: ammonium acetate; NH_4Form: ammonium formate; ns: not specified; PB: polybrene; Q: quadrupole; Ref.: reference; RPLC: reversed-phase liquid chromatography; SDC: sodium deoxycholate; SDS: sodium dodecyl sulfate; TOF: time-of-flight; UV: ultraviolet spectroscopy.

Table 2.3 Overview of studies comparing CE–MS, GC–MS and LC–MS

Capillary electrophoresis		**Gas chromatography**		**Liquid chromatography**		**Sample**	**Ref.**
Capillary	*BGE*	*Column*	*Derivatization*	*Column*	*Mobile phases*		
Fused-silica (50 μm x 100 cm)	1 M FA	HP-5 MS (0.25 μm, 0.25 mm x 30 m)	MSTFA TBDMS-FA	Synergi Hydro RP (4 μm, 2.1 x 150 mm)	(A) 10 mM tributylamine and 15 mM acetic acid (B) MeOH	Yeast extracts	[43]
SMILE (+) (ns)	50 mM NH_4Ac (pH 8.5)			Luna NH2 (5 μm, 2 x 250 mm)	(A) 20 mM NH_4Ac (pH 9.45) in 5% ACN (B) ACN		
Fused-sillica (50 μm x 96 cm)	0.8 M FA in 10% MeOH	DB-5 MS (0.25 μm, 0.25 mm x 30 m)	methoxyamine, BSTFA and TMCS	Poroshell 120 EC-C_8 (2.7 μm, 2.1 x 150 mm)	(A) 5 mM NH_4Form in H_2O (B) 5 mM NH_4Form in MeOH	Mouse and rat lung extracts	[44]
Fused-silica (50 μm x 100 cm)	1 M FA	5% phenylmethylsilicone (0.25 μm, 0.25 mm x 30 m)	TMCS and BSTFA	EZ:faast AAA-MS (4 μm, 3.0 x 250 mm)	(A) 10 mM NH_4Form in H_2O (B) 10 mM NH_4Form in MeOH	Mouse liver extracts	[45]
SMILE (+) (ns)	50 mM NH_4Ac (pH 8.5)						

ACN: acetonitrile; BGE: background electrolyte; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; FA: formic acid; H_2O: water; MeOH: methanol; MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide; NH_4Ac: ammonium acetate; NH_4Form: ammonium formate; ns: not specified; Ref.: reference; TBDMS-FA: N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide; TMCS: trimethylchlorosilane.

Table 2.4 Overview of studies comparing CE and ^{1}H NMR spectroscopy

Capillary electrophoresis			**^{1}H NMR spectroscopy**		**Sample**	**Results of comparison**	**Ref.**
Capillary	*BGE*	*Detection*	*Frequency*	*Buffer*			
Fused-silica (75 µm x 50 cm[a])	25 mM sodium borate, 75 mM SDS and 6.25 mM sulfated β-CD (pH 9.5)	UV	600.13 MHz	phosphate buffer (pH 7.4), containing 0.01% TSP	Mouse urine	CE (SDS): Q^2, 0.76 CE (phosphoric acid): Q^2, 0.59 NMR: Q^2, 0.82	[53]
Polyacrylamide-coated (50 µm x 57 cm)	0.2 M phosphoric acid (pH 6.2) in 10% MeOH					All data combined: Q^2, 0.88	
Fused-silica (50 µm x 40 cm[a])	25 mM sodium borate, 75 mM SDS and 6.25 mM sulfated β-CD (pH 9.5)	UV	800 MHz	phosphate buffer (pH 7.4), containing 1 mM TSP	Mouse urine	CE: Q^2, 0.34 NMR: Q^2, 0.39	[54]
						All data combined: Q^2, 0.45	
Polyacrylamide-coated (50 µm x 50 cm[a])	0.2 M phosphoric acid (pH 6.1) in 10% MeOH					6 common compounds	
Fused-silica (50 µm x 100 cm)	25 mM TEA (pH 11.7)	MS (TOF)	600.13 MHz	phosphate buffer (pH 7.4), containing 1 mM TSP	Rat urine	CE: 17 discr. comp. ^{1}H NMR: 25 discr. comp. 2 common discr. comp.	[55] [56]

BGE: background electrolyte; CD: cyclodextrin; CE: capillary electrophoresis; comp.: compounds; discr.: discriminatory; MeOH: methanol; MS: mass spectrometry; NMR: nuclear magnetic resonance; Ref.: reference; SDS: sodium dodecyl sulfate; TEA: triethylamine; TOF: time-of-flight; TSP: 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid; UV: ultraviolet spectroscopy.

[a] Effective capillary length

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III

Sensitivity enhancement in capillary electrophoresis–mass spectrometry of anionic metabolites using a triethylamine-containing background electrolyte and sheath liquid

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Electrophoresis, 32 (2011) 3016-3024

Abstract

Analyte responses in capillary electrophoresis–electrospray ionization-mass spectrometry (CE–ESI-MS) using negative ionization are frequently relatively low, thereby limiting sensitivity in metabolomics applications. In order to enhance the ionization efficiency of anionic metabolites, background electrolytes (BGEs) and sheath liquids of various compositions were evaluated. Pressure-induced infusion and CE–MS experiments showed that addition of triethylamine to the BGE and sheath liquid enhanced analyte intensities. A BGE consisting of 25 mM triethylamine (pH 11.7) and a sheath liquid of water-methanol (1:1, v/v) containing 5 mM triethylamine was selected, providing separation and detection of ten representative test metabolites with good reproducibility (migration time RSDs<1%) and linearity (R^2>0.99). This BGE yielded lower limits of detection (0.7-9.1 μM) for most test compounds when compared to common CE–MS methods using a BGE and sheath liquid containing ammonium acetate (25 and 5 mM, respectively). CE–MS of human urine revealed an average amount of 231 molecular features in negative ionization mode when triethylamine was used in the BGE and sheath liquid, whereas 115 and 102 molecular features were found with an ammonium acetate-containing BGE and sheath liquid, employing a bare fused-silica (BFS) and Polybrene-dextran sulfate-Polybrene (PB-DS-PB) coated capillary, respectively. With the CE–MS method using triethylamine, about 170 molecular features were observed that were not detected with the ammonium acetate-based CE–MS methods. For more than 82% of the molecular features that were detected with the triethylamine as well as the ammonium acetate-containing BGEs (i.e. common features), the peak intensities were higher using triethylamine with gain factors up to 7. Overall, the results demonstrate that BGEs and sheath liquids containing triethylamine are quite favorable for the analysis of anionic metabolites in CE–MS.

3.1 Introduction

Metabolomics involves the comprehensive analysis of endogenous metabolites in a biological system. Global profiling of metabolites in a biological fluid is challenging since there is a wide variety of metabolite compound classes having diverse physicochemical properties. Moreover, metabolites occur in a broad concentration range in a biological system [1-3].

Various analytical techniques have been applied to profile metabolites in biological fluids, including gas chromatography (GC), nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography (LC) and capillary electrophoresis (CE) [4, 5]. None of the technologies is individually capable of providing a comprehensive coverage of metabolites. Using GC, a number of metabolite classes can be analyzed in a reproducible way with good resolution and sensitivity. However, GC does not allow direct analysis of highly polar compounds, and therefore, often requires extensive sample preparation and derivatization procedures to yield volatile and thermostable compounds [6, 7]. NMR is a rapid and non-destructive technique providing detailed structural information of metabolites without the need for separation. Unfortunately, low-abundant metabolites may be missed as the concentration sensitivity of NMR is relatively poor [8]. A wide range of types and concentrations of metabolites can be analyzed and quantified with LC coupled to mass spectrometry (MS) [9]. However, common reversed-phase (RP) LC is not well suited for the separation of highly polar and ionic compounds, which elute in the column dead time and thus are not well covered. Recently, hydrophilic interaction chromatography (HILIC) has been introduced for the analysis of polar metabolites in metabolomics. The results are promising, but retention-time and peak-area repeatabilities as well as plate numbers may be less favorable with HILIC [10, 11]. CE–MS employing electrospray ionization (ESI) using a sheath-liquid interface is a useful technique for the profiling of highly polar and charged metabolites present in biological fluids [12, 13]. The CE mechanism is based on charge-to-size ratio – and thus highly complementary to RPLC – and can provide very narrow peaks. With CE, compounds can be separated in a fast way with limited sample pretreatment, requiring only minute amounts of sample and small volumes of separation electrolytes. However, the sensitivity of CE–MS is limited, unless electrokinetic or chromatographic preconcentration techniques are used.

For a full coverage of ionogenic metabolites, CE–MS should be performed in both positive and negative ionization mode. Most of the reported CE–MS methods for metabolomics focus on the analysis of basic and zwitterionic metabolites using positive ESI and applying formic acid as background electrolyte (BGE) [14].

Notably, a large part of metabolites in biological fluids is acidic and can only be ionized efficiently using negative ESI, and thus will be missed in CE–MS in positive ionization mode. In order to allow their separation and detection, CE–MS of anionic metabolites should be carried out using a BGE with a pH above 7.5. Most commonly, ammonium acetate is used as BGE (20-50 mM, pH 7.5-10.0) and sheath liquid additive (5 mM), applying bare fused-silica (BFS) capillaries or capillaries coated with a layer of positively charged polymer [15-24]. Unfortunately, when compared with positive ESI, metabolite responses in negative ionization mode are often low, thereby limiting sensitivity in metabolomics applications. Reduced MS signals for anions have been attributed to analyte ionization suppression by acetate ions present in the BGE and sheath liquid [21, 25]. To circumvent this loss of sensitivity, transformation of anionic metabolites into cationic compounds by derivatization or complexation has been proposed [25, 26]. With these methodologies, sensitivity indeed improved for anionic compounds and more favorable detection limits were achieved applying CE–MS in positive ionization mode. However, derivatization procedures increase sample pretreatment complexity and losses of metabolites can occur due to incomplete derivatization. Furthermore, not every anionic compound can be derivatized efficiently.

In the present study, possibilities to improve ionization efficiencies of anionic metabolites in CE–ESI-MS were investigated by testing various BGEs and sheath liquids. Pressure-induced infusion experiments have been performed to assess responses of test metabolites in negative ESI. The applicability of the BGE and sheath liquid providing the highest ionization efficiencies was studied by CE–MS of a metabolite test mixture, and the compositions of the BGE and sheath liquid were further optimized. Aspects like metabolite separation, and migration-time and peak-area reproducibility of the test metabolites were considered. The applicability of the optimized system was ultimately evaluated by analyzing human urine samples. Migration-time repeatability of spiked test metabolites and the number of molecular features were determined. Comparisons were made with two regular CE–MS systems that have been used so far for profiling of anionic metabolites in negative ionization mode employing BGEs containing ammonium acetate.

3.2 Materials and methods

3.2.1 Chemicals

Acetonitrile, ammonium bicarbonate, ammonium formate, ammonium hydroxide (25% solution), isopropanol, methanol, glutaric acid, hippuric acid, pyroglutamic

acid, uric acid and uridine were obtained from Fluka (Steinheim, Germany). Polybrene (hexadimethrine bromide, PB), dextran sulfate (DS), morpholine, sodium hydroxide (NaOH), flavin adenine dinucleotide (FAD), glucose-6-phosphate (G6P), glutathione and L-proline were purchased from Sigma Aldrich (Steinheim, Germany). Triethylamine was from Fisher Scientific (Loughborough, UK) and piperidine from Alfa Aesar (Kalsruhe, Germany). Ammonium acetate and citric acid were supplied by Merck (Darmstadt, Germany). Water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA) prior to use.

3.2.2 Test mixture and human urine samples

Stock solutions (1 mM) of the metabolites hippuric acid, proline, glutathione, FAD, pyroglutamic acid, G6P, uridine, glutaric acid and citric acid were prepared in deionized water. Uric acid (1 mM) was dissolved in 10 mM ammonium hydroxide. Stock solutions of the metabolites were mixed and diluted with deionized water to obtain a test mixture in which each metabolite was present at the appropriate concentration (5-100 μM).

Human urine samples were collected from a healthy volunteer. Prior to analysis, urine samples were centrifuged at 48,000 rcf for 1 minute and mixed with BGE (1:1, v/v). To study possible matrix effects, human urine samples were spiked with FAD, G6P and glutathione by adding aliquots of the stock solutions to obtain concentrations ranging from 5 to 100 μM.

3.2.3 CE–MS

CE–MS experiments were carried out on a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton, USA) coupled to a quadrupole-time-of-flight (Q-TOF) mass spectrometer (micrOTOF-QII, Bruker Daltonics, Germany) using a sheath-liquid electrospray interface from Agilent Technologies (Waldbronn, Germany).

The optimized CE–MS system employed a BGE of 25 mM triethylamine and a sheath liquid of 5 mM triethylamine in water-methanol (1:1, v/v). Fused-silica capillaries (Polymicro Technologies, Phoenix, USA) had a total length of 100 cm and an internal diameter of 50 μm. New fused-silica capillaries were rinsed with 1 M NaOH for 20 min and water for 10 min at 20 psi. Between runs, capillaries were flushed with acetic acid (10% solution) for 3 min, water for 2 min and BGE for 1 min, applying a pressure of 50 psi. Hydrodynamic injections were performed using a pressure of 0.5 psi for 30 s. The separation voltage was 30 kV and the capillary temperature was set at 20°C. The sheath liquid was delivered by a 10 mL

gas-tight syringe of Hamilton (Reno, USA) using a syringe pump (KD Scientific, Holliston, USA). The following interface and MS conditions were used: sheath liquid flow, 5 μL/min; dry gas temperature, 180°C; dry gas flow, 4 L/min; nebulizer pressure, 10 psi; ESI voltage, 4 kV. Data were acquired in the mass range 50 to 550 m/z with a rate of 1 Hz.

The optimized CE–MS method using a BGE of triethylamine was compared with regular CE–MS methods employing ammonium acetate as BGE (25 mM; adjusted to pH 9.0 with ammonium hydroxide) and a sheath liquid of 5 mM ammonium acetate in water-methanol (1:1, v/v). In one method BFS capillaries were used and in the other capillaries coated with a triple layer of PB-DS-PB were applied. The coating was prepared by rinsing successively with a 10% m/v PB solution for 30 min at 5 psi, water for 5 min at 20 psi, DS (3% m/v) for 30 min at 5 psi, water for 5 min at 20 psi and PB (10% m/v) at 5 psi during 30 min. In between runs, the coating was regenerated by flushing the capillary with a PB solution (10% m/v) for 1 min at a pressure of 50 psi, followed by rinsing with water for 2 min and BGE for 1 min. Sample injection and capillary temperature were kept the same as for the CE–MS system using triethylamine as BGE. The separation voltage was 30 kV and -30 kV for the bare and coated capillary, respectively. The interface and MS conditions were the same as for to the CE–MS system with triethylamine as BGE, except for the ESI voltage (3.5 kV).

3.2.4 Data analysis

CE–MS data were processed using DataAnalysis software of Bruker Daltonics. For urine samples, molecular features revealed by CE–MS were determined with the 'Find Molecular Features' function of the software. A molecular feature is characterized by a unique combination of m/z value and migration time, and is considered to result from one sample component. Theoretically, one compound could produce more than one molecular feature, for example when fragmentation or adduct formation occurs during ionization. The following parameters were selected to determine molecular features: signal-to-noise (S/N) threshold, 3; correlation coefficient threshold, 0.7; minimum compound length, 10 spectra; smoothing width, 1. This implies that signals that persistently had an S/N ratio above 3 for at least 10 s were considered to be a genuine peak and originate from a molecular feature.

The optimized CE–MS method was compared with two common CE–MS methods for anionic metabolites (see above) by the analysis of the same urine sample. The total number of observed molecular features was determined for each

method. Method comparison was done pairwise (i.e. optimized versus BFS capillary with ammonium acetate, and optimized versus PB-DS-PB coated capillary with ammonium acetate) by determining the number of common molecular features (i.e. features observed with both CE–MS methods). For the determination of common molecular features, only m/z values were considered since migration times of metabolites will not be the same when using another BGE or a charged capillary coating. Molecular features were regarded the same if the difference of their respective m/z values was less than 10.0 mDa. A molecular feature with an m/z value that was detected with only one of the two CE–MS methods was considered to be unique for the respective method. If with one CE–MS method two molecular features with identical m/z values were observed, and with the other CE–MS method only one molecular feature with the same m/z value, than one common feature and one unique feature was counted. The urine data obtained with the CE–MS methods were also compared with respect to analyte response intensities. For this purpose, peak heights of common molecular features were determined from extracted-ion electropherograms, and peak-height ratios (optimized method versus other method) were calculated. In order to assure that signals originating from the same species were compared, only m/z values that correspond with a single molecular feature in both CE–MS methods were considered.

3.3 Results and discussion

3.3.1 Infusion experiments

The influence of the BGE and sheath liquid composition on signal intensities of anionic metabolites was first studied by infusion experiments. Solutions of the representative metabolites hippuric acid, G6P and uric acid (100 μM) in various BGEs were flushed through the capillary into the CE–MS sprayer by applying a constant pressure of 10 psi. Tested BGEs were solutions (25 mM) of ammonium acetate (pH 6.7), ammonium bicarbonate (pH 8.0), ammonium formate (pH 6.5), ammonium hydroxide (pH 10.6), morpholine (pH 10.5), piperidine (pH 12.0) and triethylamine (pH 11.7). These BGEs were chosen based on volatility and pH. A medium to high pH is preferred to deprotonate acidic metabolites and allow their CE analysis as anions. The pH of the BGEs was not adjusted, since additives influence analyte responses, thereby complicating the comparison of the metabolite ionization efficiencies in the various BGEs. A sheath liquid of water-methanol (1:1, v/v) was used during the BGE studies.

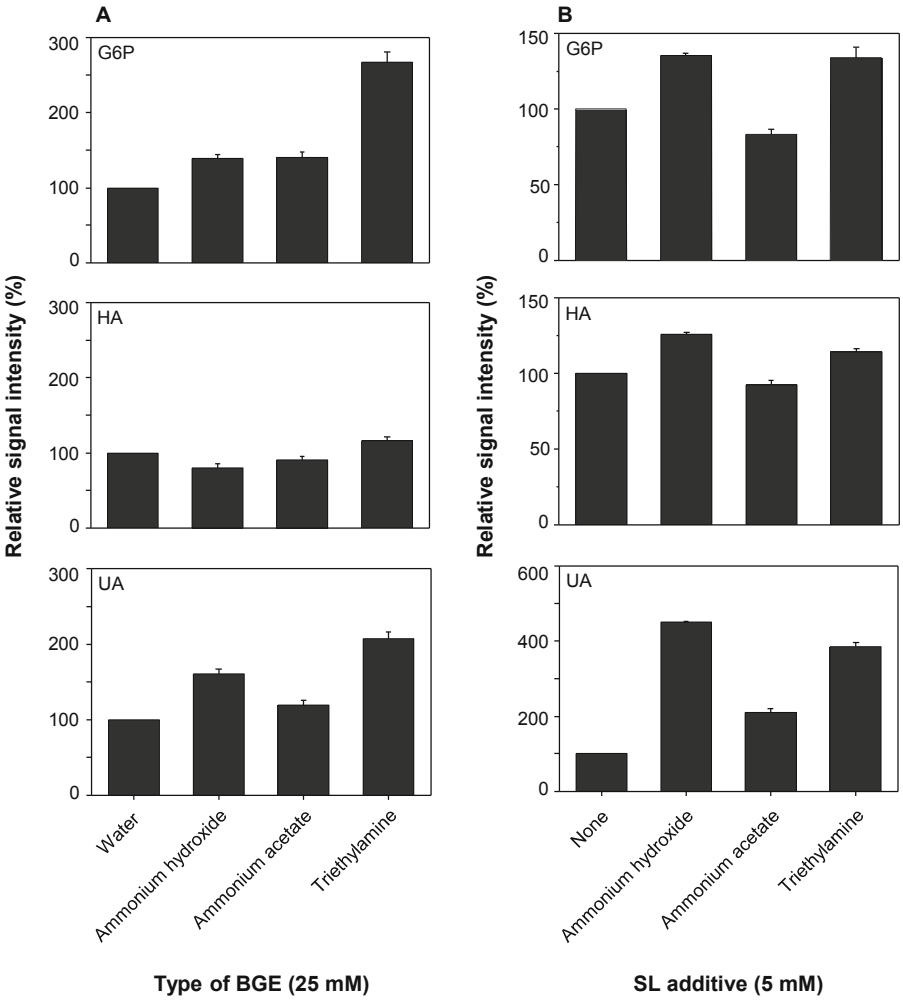

Figure 3.1 Effect of BGE and sheath liquid additive on signal intensities of anionic metabolites during infusion experiments using the CE–MS sheath-liquid interface. Metabolites, glucose-6-phosphate (G6P); hippuric acid (HA) and uric acid (UA); metabolite concentration, 100 μM. Conditions: (A) sheath liquid, water-methanol (1:1, v/v); (B) metabolites dissolved in water. Other conditions, see Experimental section.

Signal intensities obtained in water (no BGE) and using a sheath liquid without additive were set at 100%. Values are means ± SD (n=3).

Signal intensities obtained for the analytes in water were set at 100% (Figure 3.1A). Signals obtained for the test metabolites were higher than 100% when dissolved in the tested BGEs, except for the morpholine BGE, which provided lower analyte intensities than those obtained in pure water. The pH of the BGE induces the formation of preformed ions of the test metabolites, which will often aid their ESI. However, the signal intensities obtained for the test compounds using ammonium-containing BGEs were still quite modest. For instance, analyte responses in ammonium acetate were between 102 and 140% relative to responses in water. Signal intensities of the test metabolites dissolved in piperidine were in general higher (102-236%) than with ammonium acetate. Triethylamine as BGE showed optimum ionization efficiencies for the test metabolites with relative signal intensities ranging from 116 to 267%.

Sheath liquids of water-methanol (1:1, v/v) with the additives ammonium acetate, ammonium hydroxide and triethylamine (5 mM) were also tested. Sheath liquids containing ammonium hydroxide and triethylamine clearly provided higher ionization efficiencies for test compounds with respect to sheath liquids with ammonium acetate or without any additive (Figure 3.1B). For stable CE–MS performance, it is preferable to use the same components in the BGE and sheath liquid. Therefore, triethylamine was selected both for the BGE and as sheath liquid additive in order to enhance responses of anionic metabolites in negative ionization mode.

3.3.2 CE–MS optimization

The further optimization of the sheath liquid and the BGE was performed by CE–MS analysis of a test mixture of ten metabolites. Aspects like peak areas of the test metabolites, plate numbers and migration time repeatability were considered. The selected test metabolites were from various compound classes having different physicochemical properties (Table 3.1) and being representative for common metabolites in body fluids.

The type of organic solvents and the concentration methanol and triethylamine in the sheath liquid were investigated. Figure 3.2 shows the overall results for G6P which virtually reflect the general observed effects of the sheath liquid composition on analyte responses. Three solvents (acetonitrile, isopropanol and methanol) were tested as organic component of the sheath liquid in an 1:1 (v/v) ratio with water, including 5 mM triethylamine (Figure 3.2A). In combination with a BGE of 25 mM triethylamine (pH 11.7), the highest peak areas for the test metabolites were obtained with methanol in the sheath liquid, whereas with acetonitrile the lowest

signals were obtained (factor 1.2 to 2.4 lower with respect to methanol). Peak areas for the test compounds were optimal using sheath liquids containing 50% methanol or more (Figure 3.2B). However, the peaks for the various metabolites were broader when using a sheath liquid with 75% methanol. For instance, plate numbers for G6P were 147,000 and 116,000, using 50% and 75% methanol in the sheath liquid, respectively. The effect of the concentration triethylamine in a sheath liquid of water-methanol (1:1, v/v) was also studied. Increasing the concentration from 5 to 10 mM led to a decrease in peak areas of the test metabolites. The peak area of G6P, for example, decreased with 16% (Figure 3.2C).

Similar signal intensities were observed when a concentration of 2.5 or 5 mM triethylamine was used in the sheath liquid. However, the use of 2.5 mM triethylamine also negatively affected the stability of the CE–MS system (current drops) and migration-time repeatability of the test metabolites.

Four different triethylamine-containing BGEs were investigated: 10 mM (pH 11.6) and 25 mM triethylamine (pH 11.7), and 10 and 25 mM triethylamine (both adjusted to pH 11.0 with acetic acid). Plate numbers for the test metabolites in the BGEs were all above 50,000 with the highest plate numbers (up to 150,000) for the pH-adjusted BGEs. Unfortunately, the addition of acetic acid to the BGEs for pH adjustment caused a reduction of analyte responses. For example, peak areas obtained using 25 mM triethylamine adjusted to pH 11.0 were a factor 2 to 5 lower compared to 25 mM triethylamine (pH 11.7, not adjusted). Peak areas for the test metabolites were similar for the triethylamine BGEs (10 and 25 mM) without pH adjustment.

Table 3.1 Physicochemical properties of the test mixture metabolites

Compound	Classification	M_w[a]	pK_a[b]
L-proline	amino acid	115.0633	2.0; 10.6
Uridine	nucleoside	244.0695	9.5
Hippuric acid	organic acid	179.0582	3.6
DL-pyroglutamic acid	amino acid derivative	129.0426	3.3
FAD	nucleotide	785.1571	3.6; 10.0
G6P	sugar phosphate	260.0297	6.1; 12.5
Uric acid	purine	168.0283	5.4
Glutathione	tripeptide	307.0838	2.1; 3.6; 8.8; 9.7
Glutaric acid	dicarboxylic acid	132.0423	4.3; 5.4
Citric acid	tricarboxylic acid	192.0270	3.1; 4.8; 6.4

[a] Monoisotopic molecular weight

[b] Data obtained from the Handbook on Chemistry and Physics, 91st edition

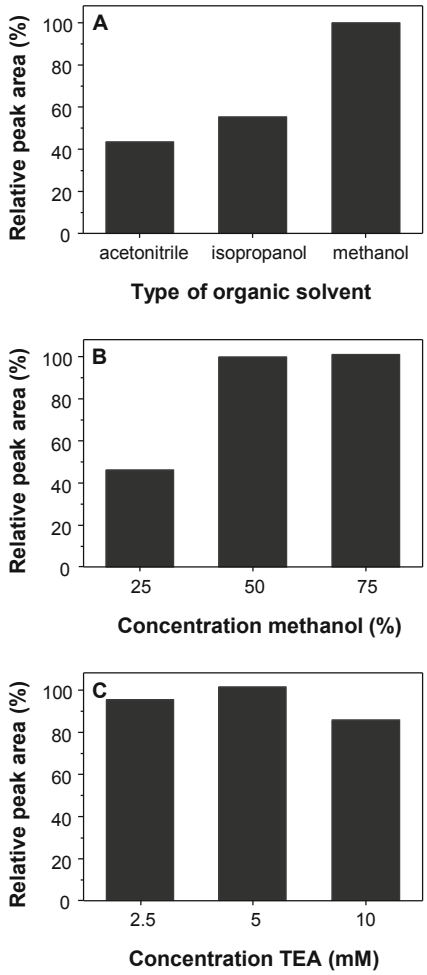

Figure 3.2 Influence of the type of organic solvent (A), the concentration methanol (B) and the triethylamine concentration (C) in the sheath liquid on the relative peak area of glucose-6-phosphate (100 μM) obtained during CE–MS. Conditions: Sheath liquid (A) 5 mM triethylamine in water-organic solvent (1:1, v/v); (B) 5 mM triethylamine in water-methanol in various proportions; (C) water-methanol (1:1, v/v) containing different concentrations of triethylamine. BGE, 25 mM triethylamine (pH 11.7); sheath liquid flow, 5 μL/min. Other conditions, see Experimental section.

However, when human urine samples were analyzed using a 10 mM triethylamine BGE (pH 11.6), relatively broad and asymmetric peaks were observed for spiked test metabolites. Increasing the BGE concentration to 25 mM triethylamine resulted in more narrow and symmetric peaks. Apparently, the ionic strength of the BGE should be sufficiently high for the analysis of human urine samples.
The studies described above led to an optimized CE–MS method employing a BGE of 25 mM triethylamine (pH 11.7) and a sheath liquid of water-methanol (1:1, v/v) containing 5 mM triethylamine. The performance of the selected CE–MS method and its applicability for urine analysis was further investigated (Section 3.3.3). To properly appreciate the method's merits, a comparison with two regular CE–MS methods for anionic metabolite analysis was made (Section 3.3.4).

3.3.3 Performance of the optimized CE–MS method

With the optimized method, all metabolites of the test mixture could be separated within 20 min (Figure 3.3A). The migration times of the test metabolites were highly reproducible with relative standard deviations (RSDs) below 1% for ten consecutive measurements. Peak area RSDs during these analyses were below 8% for all test metabolites. To assess the linearity of the CE–MS method, the test metabolite mixture was analyzed at concentrations ranging from 5-100 μM. Good linearity was observed for all test metabolites with coefficients of determination (R^2) above 0.995. Limits of detection (LODs, S/N ratio of 3) for the test metabolites ranged from 0.7 to 9.1 μM (Table 3.2). CE–MS analysis of human urine provided a complex profile of anionic metabolites (Figure 3.4A). Most compounds migrated between 7.5 and 23.5 min, providing a separation window of at least 16 min. In order to examine the migration-time repeatability and potential signal reduction effects in the presence of sample matrix, human urine samples were spiked with FAD, G6P and glutathione (5-100 μM). These compounds were selected because they had relatively low endogenous levels in the analyzed urine. Migration time RSDs for FAD, G6P and glutathione in urine were below 2% (n=10) and their MS responses were linear (R^2>0.99) in the tested range. The peak areas obtained for the test metabolites spiked in urine were slightly lower than peak areas for these compounds when dissolved at the same concentration in water. The slopes of the calibration curve in urine acquired for G6P, glutathione and FAD were, respectively, 2.2%, 8.1% and 12.6% lower than the slopes obtained for the aqueous solutions, indicating only modest suppression by matrix components. LODs for G6P, glutathione and FAD in diluted urine (1:1, v/v with BGE) were respectively 1.5, 2.2 and 0.8 μM.

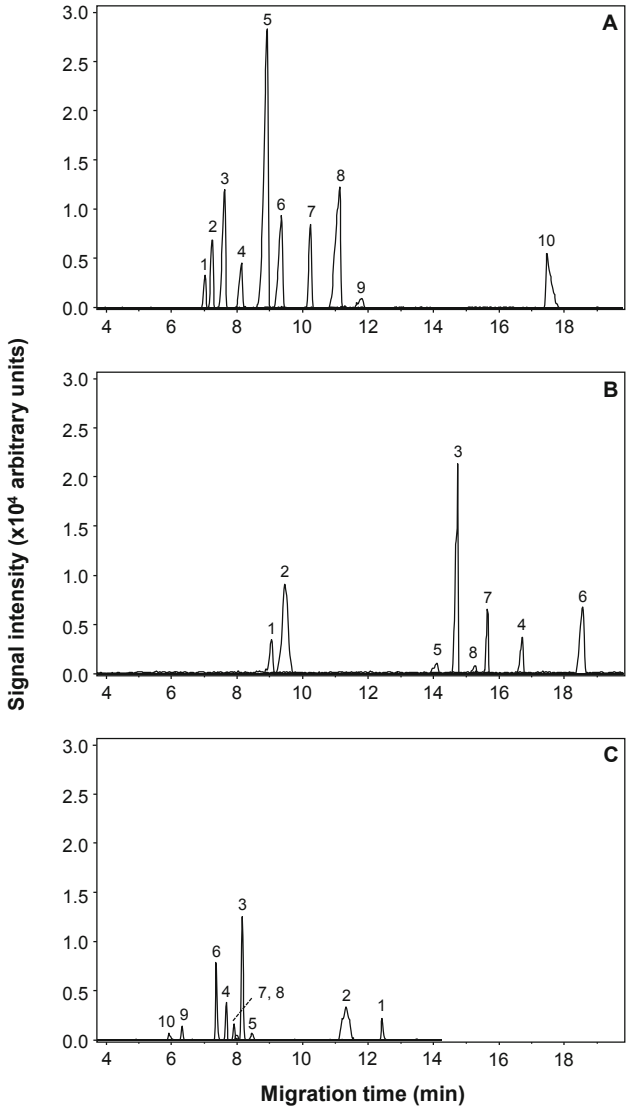

Figure 3.3 Extracted-ion electropherograms obtained during CE–MS of the test mixture of anionic metabolites. Metabolites: 1. proline (*m/z* 114.06); 2. uridine (*m/z* 243.06); 3. hippuric acid (*m/z* 178.05); 4. pyroglutamic acid (*m/z* 128.04); 5. flavin adenine dinucleotide (*m/z* 391.58); 6. glucose-6-phosphate (*m/z* 259.02); 7. uric acid (*m/z* 167.03); 8. glutathione (*m/z* 306.08); 9. glutaric acid (*m/z* 131.04); 10. citric acid (*m/z* 191.02); 100 μM each.
Conditions: (A) BGE, 25 mM triethylamine (pH 11.7); sheath liquid, 5 mM triethylamine in water-methanol (1:1, v/v); capillary, BFS; (B) BGE, 25 mM ammonium acetate (pH 9.0); sheath liquid, 5 mM ammonium acetate in water-methanol (1:1, v/v); capillary, BFS; (C) BGE, 25 mM ammonium acetate (pH 9.0); sheath liquid, 5 mM ammonium acetate in water-methanol (1:1, v/v); capillary, PB-DS-PB coating. Other conditions, see Experimental section.

Table 3.2 Detection limits (μM) for the test mixture metabolites dissolved in water using the optimized and common CE–MS methods

Compound	Triethylamine (BFS)[a]	Ammonium acetate (BFS)[b]	Ammonium acetate (PB-DS-PB coating)[c]
L-proline	4.0	2.6	8.7
Uridine	1.6	2.1	4.6
Hippuric acid	0.9	0.6	0.8
DL-pyroglutamic acid	3.2	7.9	8.1
FAD	0.7	5.5	10.6
G6P	1.5	3.0	3.2
Uric acid	3.5	3.6	6.5
Glutathione	2.1	6.1	12.0
Glutaric acid	9.1	n.d.	8.9
Citric acid	2.5	n.d.	11.5

Detection limits are based on an S/N ratio of 3. Conditions: (a) BGE, 25 mM triethylamine (pH 11.7); sheath liquid, 5 mM triethylamine in water-methanol (1:1, v/v); (b) BGE, 25 mM ammonium acetate (pH 9.0); sheath liquid, 5 mM ammonium acetate in water-methanol (1:1, v/v); (c) BGE, 25 mM ammonium acetate (pH 9.0); sheath liquid, 5 mM ammonium acetate in water-methanol (1:1, v/v). Other conditions, see Experimental section; n.d.: not detected.

The LODs found for the metabolites dissolved in diluted urine and in water (Table 3.2) were quite similar. Again, this indicates that the ionization suppression caused by the matrix was minimal with the CE–MS method using triethylamine. From the Human Metabolome Database it follows that common urine concentrations of most test metabolites are between 1 μM and 20 mM. Considering the obtained LODs in water and urine, and the minor ionization suppression observed using triethylamine as BGE, it is therefore likely that these metabolites can be detected in human urine samples. Indeed, for most test metabolites (i.e. their m/z values) peaks were detected in urine.

3.3.4 Comparison with regular CE–MS methods for anionic metabolites

The optimized CE–MS method was compared with two CE–MS methods which have been commonly used so far for profiling of anionic metabolites. These methods employ a BGE of 25 mM ammonium acetate (pH 9.0) and a sheath liquid of water-methanol (1:1, v/v) containing 5 mM ammonium acetate. One method uses BFS capillaries, whereas the other uses capillaries coated with a triple layer of PB-DS-PB [12, 21, 22, 24]. The test metabolites could be separated with all three CE–MS methods within 20 min (Figure 3.3). However, glutaric and citric acid

could not be detected using a BGE of ammonium acetate with a BFS capillary, probably because these metabolites are multiply charged and have a very small cathodic or even anodic overall electrophoretic mobility. The migration order of the metabolites was not completely the same for the methods employing BFS capillaries. This can be explained by the fact that the pH of the ammonium acetate BGE was adjusted to pH 9.0, whereas the pH of the triethylamine BGE was 11.7. This causes some metabolites, like FAD and glutathione, to be charged in a different degree in the respective BGEs and thus have different electrophoretic mobilities. Also potential interaction of protonated triethylamine with anionic metabolites may affect their electrophoretic migration. When applying a PB-DS-PB coated capillary, the migration order was roughly reversed when compared to the method employing a BFS capillary and ammonium acetate as BGE. This is due to the applied negative voltage and the 'reversed' electroosmotic flow (EOF) obtained with the PB-DS-PB coating. For all three CE–MS methods, migration time RSDs of the test compounds were below 1% for ten consecutive measurements. Overall, LODs for the test metabolites were clearly more favorable using triethylamine as BGE (Figure 3.3; Table 3.2). Only hippuric acid and L-proline could be detected at slightly lower concentrations when BFS capillaries were used with ammonium acetate as BGE.

In order to compare the applicability and to evaluate potential sample matrix effects, human urine was also analyzed with the three CE–MS methods (Figure 3.4). The signal intensity of the obtained overall metabolite profiles was clearly the highest when the triethylamine-containing BGE was used. Moreover, the largest separation window was obtained with this BGE. The CE–MS methods were compared with respect to the number of molecular features revealed during the analysis of human urine. With CE–MS using the BGE containing triethylamine, 231 molecular features were observed, whereas 115 and 102 features were detected with ammonium acetate as BGE using BFS and PB-DS-PB coated capillaries, respectively. The method employing triethylamine in the BGE and sheath liquid provides a significantly higher metabolic coverage. This is of great importance for finding relevant compounds that can distinguish various groups (e.g. healthy versus diseased).

The urine data obtained with the CE–MS method employing triethylamine were further compared pairwise with the data from the other two CE–MS methods, and the number of common and unique molecular features was established (see Section 3.2.4 for procedure).

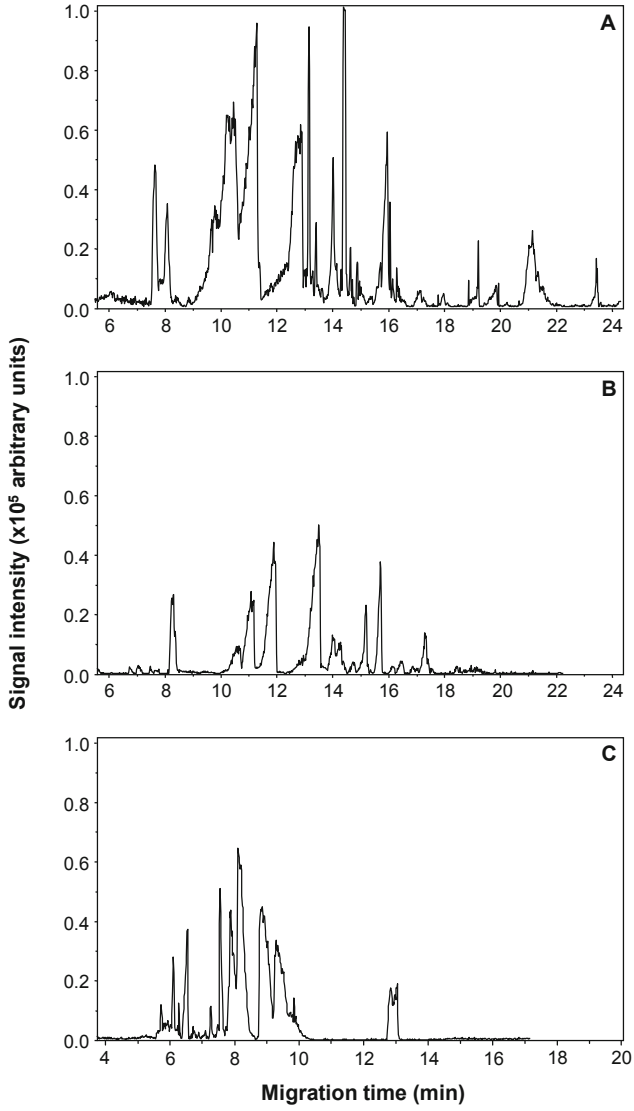

Figure 3.4 Base-peak electropherograms obtained during CE–MS of human urine.
Conditions: (A) BGE, 25 mM triethylamine (pH 11.7); sheath liquid, 5 mM triethylamine in water-methanol (1:1, v/v); capillary, BFS; (B) BGE, 25 mM ammonium acetate (pH 9.0); sheath liquid, 5 mM ammonium acetate in water-methanol (1:1, v/v); capillary, BFS; (C) BGE, 25 mM ammonium acetate (pH 9.0); sheath liquid, 5 mM ammonium acetate in water-methanol (1:1, v/v); capillary, PB-DS-PB coating. Other conditions, see Experimental section.

When comparing the triethylamine-based with the ammonium acetate-based CE–MS methods applying a BFS and PB-DS-PB coated capillary, the number of common molecular features was 67 and 58, respectively. The CE–MS method using triethylamine revealed 164 and 173 molecular features in urine that were not detected when ammonium acetate was used as BGE with the BFS and coated capillary, respectively. So, the amount of unique features was three to four times higher using a triethylamine-containing BGE, thereby considerably increasing the probability of finding relevant metabolites. The CE–MS methods employing ammonium acetate as BGE and using a BFS or coated capillary still yielded a number of 48 and 44 unique molecular features, respectively, with respect to the method using triethylamine. This might be explained by the difference in selectivity among the CE–MS methods due to the different BGEs and/or the use of a capillary coating. Altered selectivity may lead to a change in co-migration patterns of urine components, and thus, to other ionization efficiencies for some metabolites. It should be noted that not every feature represents a single compound, since also adduct ions and fragments of compounds are considered as a molecular feature. The occurrence of these ions might depend on the used BGE and so might be different among the three CE–MS methods.

In order to further evaluate the effect of the triethylamine-containing BGE and sheath liquid in a more quantitative manner, the ratios of peak heights of common molecular features were calculated for the optimized CE–MS method with respect to the two regular CE–MS methods (see Section 3.2.4). The results are summarized in a Box plot (Figure 3.5) which clearly indicates that on average the peak heights obtained for the common molecular features were considerably higher using triethylamine as BGE. Peak intensities for more than 82% of the common molecular features were higher with triethylamine in the BGE and sheath liquid, and the average ratio of the peak heights was 2.8 and 2.5, when comparing the triethylamine method with the ammonium acetate methods employing bare and coated capillaries, respectively. This significant increase in peak height is especially advantageous for the detection of low-abundant metabolites. The differences in observed peak heights can be attributed to less ionization suppression when ammonium acetate is replaced by triethylamine in the BGE and the sheath liquid. Another factor is the increased separation window when the triethylamine-containing BGE is used, leading to reduced co-migration of urine components, and thus, less ionization suppression.

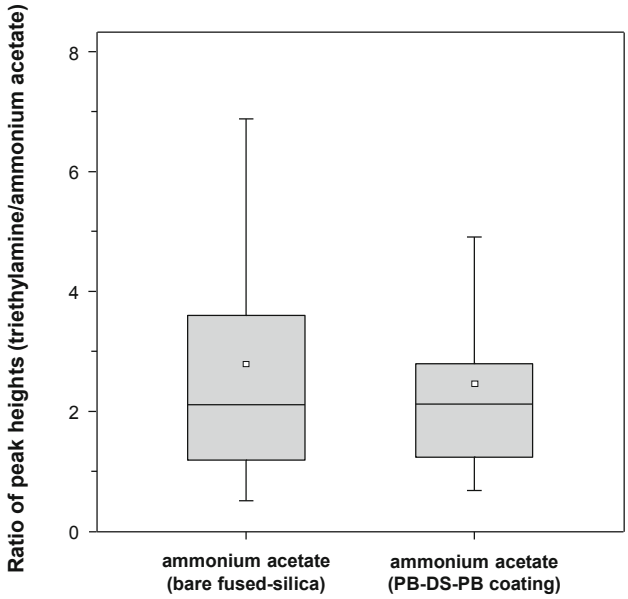

Figure 3.5 Box plot of the ratio of peak heights of common molecular features obtained during urine analysis with the CE–MS method employing triethylamine versus the methods employing ammonium acetate. The boxes represent the 25th percentile, median and 75th percentile. The whiskers represent the 5th and 95th percentile. Squares (□) indicate the mean ratio of the peak heights.

3.4 Concluding remarks

BGEs and sheath liquids of various compositions were evaluated to improve ionization efficiencies of anionic metabolites in CE–MS. The inclusion of triethylamine in the BGE and sheath liquid, and the exclusion of ammonium acetate, appeared to be an effective way to enhance metabolite responses in CE–MS in negative ionization mode. Using an optimized method employing a BGE of 25 mM triethylamine (pH 11.7) and a sheath liquid of water-methanol (1:1, v/v) containing 5 mM triethylamine, LODs of anionic metabolites could be improved significantly. Comparisons with common negative ionization CE–MS methods for metabolic profiling which use ammonium acetate in the BGE and sheath liquid showed that the metabolic coverage was considerably increased using triethylamine, achieving more than twice as much molecular features. Obviously, this is an essential aspect when metabolic profiling is pursued. The gain in sensitivity is most probably due to less ionization suppression by the BGE and sheath liquid when

using triethylamine instead of ammonium acetate. Moreover, the separation window is enlarged with the optimized method, which results in reduced co-migration of matrix components, and thus, less signal suppression.

It should be noted that no preconcentration of metabolites was carried out during this study. Consequently, detection limits could still be further improved by applying well-known principles as isotachophoresis, pH-mediated stacking and/or solid phase extraction [27, 28]. Another interesting way to further improve sensitivity for anionic metabolites might be the application of sheathless CE–MS interfacing. Recently, such an interface has shown to provide significant signal enhancement in peptide and protein analysis [29, 30].

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IV

Comparison of capillary electrophoresis–mass spectrometry and hydrophilic interaction chromatography–mass spectrometry for anionic metabolic profiling of urine

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Submitted

Abstract

In order to assess the utility of a recently developed capillary electrophoresis–mass spectrometry (CE–MS) method for the study of anionic metabolites in urine, a comparison was made with hydrophilic interaction chromatography (HILIC)–MS using negative electrospray ionization. After optimization of the HILIC conditions, a gradient employing 10 mM ammonium acetate (pH 6.8) in acetonitrile-water (5 min 90% acetonitrile followed by 90% to 50% acetonitrile in 10 min) was selected, providing baseline separation of five representative anionic test metabolites. Relative standard deviations (RSDs) for HILIC retention times and peak areas were below 0.2% and 7.7%, respectively, and detection limits were in the range of 0.04-2.21 μM. Metabolites in rat urine could also be analyzed in a reproducible way with retention time and peak area RSDs below 0.6% and 13.6%, respectively. The CE–MS and HILIC–MS methods were compared in terms of reproducibility, sensitivity, selectivity and coverage of the anionic urinary metabolome. In general, peak area RSDs were similar whereas HILIC–MS yielded better retention-time repeatability and up to 80 times lower detection limits (expressed in injected concentration) for test metabolites as compared to CE–MS. Rat urine analysis by HILIC–MS provided the detection of 1360 molecular features, compared to 347 molecular features revealed with CE–MS. Of these, a number of 144 molecular features were found with both HILIC–MS and CE–MS, which showed on average 10 times higher peak areas in HILIC–MS. The HILIC retention and CE migration times of the common features were clearly not correlated. The HILIC and CE behavior of the test metabolites and 16 putatively identified common features were evaluated involving their physicochemical properties, indicating a markedly different separation selectivity, and thus significant degree of orthogonality of HILIC and CE. It is concluded that with respect to HILIC–MS, CE–MS is capable of uniquely revealing a large number of anionic metabolites, which evidently adds to the overall metabolome coverage.

4.1 Introduction

Elucidation and understanding of biochemical pathways require accurate and specific determination of metabolite levels in biofluids and tissues. Metabolites may exhibit very diverse physicochemical properties and can be present in a wide range of concentrations [1]. Capillary electrophoresis (CE) coupled to mass spectrometry (MS) is particularly suitable for the direct profiling of highly polar and charged metabolites as predominantly present in urine. CE can provide efficient separation of metabolites based on their charge-to-size ratios without the need for analyte derivatization [2]. Recently, we have developed a CE–MS method for the profiling of anionic metabolites applying triethylamine in the background electrolyte (BGE) and sheath liquid [3]. This new method provided significantly higher signal intensities as compared to other CE–MS methods in negative ionization mode. Urine samples from antibiotic-treated rats and control rats could be distinguished, and potential biomarkers were revealed [4]. Having this new method available, the question arises to what extent CE–MS can provide complementary information on the anionic metabolome with respect to more common liquid chromatographic (LC) techniques for metabolic profiling.

LC–MS can provide information on the quantity of low-abundant metabolites without the need for analyte derivatization. Common reversed-phase (RP) LC, however, is less suited for the analysis of ionic compounds, unless ion-pairing agents are used to increase the retention [5]. Unfortunately, ion-pairing agents can cause substantial ionization suppression of compounds in MS and source contamination. Hydrophilic interaction chromatography (HILIC) allows profiling of polar compounds, providing complementary information to RPLC, as has been demonstrated by numerous metabolomics studies [6-19]. HILIC utilizes a highly organic mobile phase (often acetonitrile) containing water in combination with a polar stationary phase. Analyte separation is based on partitioning between the mobile phase and a layer of adsorbed water molecules on the stationary phase as well as electrostatic interactions with polar groups on the stationary phase [20-22]. Hence, in order to appreciate the utility of CE–MS for profiling of anionic metabolites, comparison with HILIC–MS seems indicated.

So far, only a few studies have been conducted in which both CE–MS and HILIC–MS were used for comparative and comprehensive profiling [17, 23-26]. Sugimoto *et al.* applied HILIC–MS and CE–MS to analyze edamame and Japanese sake [23, 24]. However, as HILIC–MS was used to profile sugars only, no conclusions could be drawn on the complementarity of HILIC–MS and CE–MS for untargeted metabolite profiling. Büscher and colleagues extensively compared CE,

LC and gas chromatography (GC) methodologies, all in combination with MS [25]. Metabolites were analyzed with GC–MS after two different derivatization procedures. Ion-pair RPLC and HILIC were used as LC separation modes and two CE–MS methods were employed for the analysis of cationic and anionic metabolites. A mixture of 91 test metabolites representing central carbon and energy metabolism was used for comparison. Of these test metabolites, 33 compounds could be detected by all three platforms. CE and LC showed the greatest overlap in metabolite coverage (26 compounds) and each analytical technique was capable to measure two compounds which could not be detected with the two other methodologies. The CE–MS, LC–MS and GC–MS methodologies were considered in general, that is, GC, HILIC, ion-pair RPLC and cationic and anionic CE methods were not individually compared. Moreover, the evaluation of the complementarity of the techniques for metabolic profiling was limited since it was based on a confined number of test metabolites in standard solutions [25]. Ibáñez and coworkers studied the effect of dietary polyphenols on the proliferation of colon cancer cells with CE–MS, HILIC–MS and RPLC–MS [26]. Saric *et al.* used the same techniques to analyze the metabolome of the *Fasciola hepatica* worm [17]. In both studies, a small part of the detected metabolite features was identified. There was no or a limited overlap of identified metabolites detected by CE–MS, HILIC–MS and RPLC–MS, indicating the potential complementarity of the analytical techniques [17, 26]. It should be noted, however, that Ibáñez *et al.* as well as Saric *et al.* employed positive electrospray ionization (ESI) for HILIC–MS and/or CE–MS, and thus could only compare the methodologies based on detected cationogenic compounds. Still, a significant number of urinary metabolites is acidic and can only be detected using negative ESI. Therefore, in order to achieve comprehensive profiling and to evaluate the complementarity of HILIC–MS and CE–MS, particular attention should also be paid to anionic metabolites.

In the present study, we compared a previously optimized CE–MS method with HILIC–MS for anionic metabolic profiling of urine samples. We first optimized a HILIC–MS method by carrying out infusion experiments to determine and evaluate the effect of different HILIC mobile phase compositions on the signal intensities of representative anionic test metabolites. An efficient gradient HILIC–MS method applying an acetonitrile-water mobile phase containing ammonium acetate was developed and the performance was assessed in terms of sensitivity, linearity, and repeatabilities of peak area and retention time. Furthermore, HILIC–MS was applied to urine analysis and the number of detected molecular features of urine components was determined. Outcomes were extensively compared with results

obtained with the earlier developed CE–MS method for anionic urinary profiling [3, 4]. Numbers of common and unique urinary molecular features were considered and MS responses of common features were compared. Moreover, differences in separation selectivity of CE–MS and HILIC–MS were assessed by comparing migration and retention times of test metabolites as well as putatively identified common features.

4.2 Materials and methods

4.2.1 Chemicals

Acetic acid, ammonium formate, ammonium hydroxide (25% solution), acetonitrile, formic acid, methanol, glutaric acid, hippuric acid, DL-pyroglutamic acid and uridine were obtained from Fluka (Steinheim, Germany). Sodium hydroxide (NaOH) and L-proline were purchased from Sigma Aldrich (Steinheim, Germany). Triethylamine was from Fisher Scientific (Loughborough, UK), piperidine was from Alfa Aesar (Karlsruhe, Germany) and ammonium acetate was supplied by Merck (Darmstadt, Germany). Water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA) prior to use.

4.2.2 Test mixture and rat urine sample

Stock solutions (50 mM) of the metabolites glutaric acid, hippuric acid, proline, pyroglutamic acid and uridine were prepared in deionized water. Stock solutions of the metabolites were mixed and diluted to obtain a test mixture in which each metabolite was present at the appropriate concentration (0.25-80 μM). Test metabolite mixtures were prepared in water-acetonitrile (1:4, v/v) and water for HILIC–MS and CE–MS analyses, respectively.

A mixture of aliquots of rat urine samples provided by AstraZeneca (Department of Drug Metabolism and Pharmacokinetics, Macclesfield, UK) [27] was prepared and stored at -80°C. Prior to analysis, the urine sample was thawed. For HILIC–MS analysis, urine was diluted with acetonitrile in a proportion of 1:4, v/v and centrifuged at 10,000 rcf for 10 min. When previously analyzed with CE–MS, the urine sample was only diluted with the BGE in a proportion of 1:1, v/v [3, 4].

4.2.3 HILIC–MS

All samples were analyzed on an LC system (Shimadzu, Kyoto, Japan) coupled online via an electrospray interface to a time-of-flight (TOF) mass spectrometer

(micrOTOF, Bruker Daltonics, Bremen, Germany) using a Waters XBridge™ Amide column (3.5 μm, 3.0 x 100 mm).
In the optimized HILIC–MS method, the test metabolite mixture and urine sample (5 μL) were injected and analyzed under gradient elution with mixing solvent A (10 mM ammonium acetate in water-acetonitrile (1:1, v/v)) and solvent B (10 mM ammonium acetate in water-acetonitrile (1:9, v/v)) in varying ratios at an overall flow rate of 0.5 mL/min. The gradient scheme was as follows: 0.0-5.0 min, 100% B; 5.0-15.0 min, from 100% B to 100% A; 15.0-20.0 min, 100% A; 20.1-30.0 min, 100% B. Column temperature was 45°C during separation.
The following LC–MS interface conditions were used: dry gas temperature, 180°C; dry gas flow, 4 L/min, nebulizer pressure, 50 psi; ESI voltage, 2 kV. Data were acquired in negative ionization mode in the mass range *m/z* 50-800 with a repetition rate of 1 Hz. Recorded mass spectra were internally calibrated using sodium acetate clusters which were detected within every run.

4.2.4 CE–MS

The CE–MS experiments were performed and described before [3, 4]. Briefly, CE–MS was carried out on a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton, USA) coupled to a micrOTOF mass spectrometer using a sheath-liquid electrospray interface from Agilent Technologies (Waldbronn, Germany). A BGE of 25 mM triethylamine (pH 11.7) and a sheath liquid of 5 mM triethylamine in water-methanol (1:1, v/v) were used. Fused-silica capillaries (Polymicro Technologies, Phoenix, USA) had a total length of 100 cm and an internal diameter of 50 μm. Hydrodynamic injections were performed using a pressure of 0.5 psi for 30 s. The separation voltage was 30 kV and the capillary temperature was set at 20°C. The sheath liquid was delivered with a flow rate of 5 μL/min by a 10 mL gas-tight syringe of Hamilton (Reno, USA) using a syringe pump (KD Scientific, Holliston, USA). The following CE–MS interface conditions were applied: dry gas temperature, 180°C; dry gas flow, 4 L/min; nebulizer pressure, 10 psi; ESI voltage, 4 kV. The negative ionization mode was used and data were acquired in the mass range 50 to 800 *m/z* with a rate of 1 Hz. Sodium formate clusters were used for internal calibration.

4.2.5 Data analysis

Data were processed using the DataAnalysis software of Bruker Daltonics. Using the 'Find Molecular Features' function of this software the number of molecular features obtained during urine analysis was determined. A molecular feature was

defined as the unique combination of an m/z value and retention time (HILIC–MS) or migration time (CE–MS), present in ten consecutive MS spectra with a signal-to-noise (S/N) ratio of at least three. Peak areas and peak heights of metabolites were assessed using extracted-ion chromatograms (HILIC–MS) or electropherograms (CE–MS).

4.3 Results and discussion

4.3.1 HILIC–MS method

For the optimization of the HILIC–MS method for anionic metabolic profiling, the composition of the mobile phase was studied. Ammonium acetate, sometimes in combination with acetic or formic acid, is often used as mobile phase additive in HILIC–MS for urinary metabolic profiling studies [28]. In a previous CE–MS study, we found triethylamine and piperidine to be more favorable salts with respect to attainable signal intensities for anionic metabolites [3]. Infusion experiments were performed to study the influence of several mobile phase additives on signal intensities of the test metabolites glutaric acid, hippuric acid, proline, pyroglutamic acid and uridine. These metabolites represent the diverse compound classes present in urine. The metabolites (100 μM each) were dissolved in acetonitrile-water (1:1, v/v) with and without 10 mM ammonium acetate, 10 mM ammonium formate, 0.1% formic acid, 0.1% triethylamine or 0.1% piperidine. Using ammonium formate and formic acid, test metabolite signals were lower as obtained with ammonium acetate. With triethylamine, the signal intensities of the tested metabolites were on average 6.5 times higher compared to ammonium acetate. Similar results were obtained using piperidine with 6.3 times higher signals. However, the use of triethylamine and piperidine as mobile phase additives in HILIC–MS resulted in broad and asymmetric peaks, thereby strongly reducing separation efficiency. Furthermore, proline and pyroglutamic acid could not be detected. Therefore, ammonium acetate was selected and the influence of its concentration (0-10 mM) in a mobile phase of acetonitrile-water was further evaluated. The separation, peak shapes and signal intensities of the five test metabolites obtained with HILIC–MS were taken into consideration. A concentration of 10 mM ammonium acetate in the eluent yielded more symmetric and narrow peaks as compared to 0 and 5 mM ammonium acetate. This was especially apparent for the compounds uridine and hippuric acid of which the peak widths were about 40% smaller at 10 mM ammonium acetate. Furthermore, only at 10 mM ammonium acetate, baseline separation of proline and pyroglutamic acid

was achieved. Still, the increase of ammonium acetate concentration from 0 to 10 mM resulted in up to 59% lower signal intensities, except for proline which showed a remarkable signal increase of 67%. Adjusting the pH of the mobile phase to 9.0 with ammonium hydroxide did not affect the separation of the test metabolites, but decreased their signal intensities up to 60% as compared to pH 6.8 (not adjusted) due to ion suppression.

The acetonitrile-water gradient of the mobile phase containing 10 mM ammonium acetate (pH 6.8) was optimized to obtain a large separation window without deteriorating the peak shape and detection limits (LODs) of metabolites. A high percentage of acetonitrile in the mobile phase at the start of the analysis is desirable in order to maximize the separation of metabolites. Therefore, the start of the gradient was set at 90% acetonitrile and held at 90% for 5 min to enhance the separation of metabolites eluting in the beginning of the chromatogram. Then, the percentage acetonitrile was gradually decreased to 50% and different slopes of the gradient were tested to maximize peak separation and minimize the peak widths. A gradient of 4% acetonitrile per min appeared to be optimum by providing the best resolution of metabolite peaks within a relatively short analysis time. The mobile phase was held at 50% acetonitrile for another 5 min in order to elute all compounds from the column. Thereafter, the percentage acetonitrile in the mobile phase was switched back to 90% and hold for ten min to allow reequilibration of the HILIC–MS system.

Because of the high percentage of acetonitrile at the start of the gradient, the sample injection solvent had to contain a large amount of acetonitrile to prevent peak splitting and to achieve narrow and symmetric peaks. The maximum allowable acetonitrile percentage in the sample is, however, limited by the solubility of polar and charged compounds. More than 80% acetonitrile in the sample solvent resulted in precipitation of metabolites. Moreover, dilution of the sample with acetonitrile will reduce the concentration sensitivity. The use of a sample injection volume of 5 μL containing 80% acetonitrile in combination with a gradient starting at 90% acetonitrile resulted in narrow and symmetric peaks. Injection volumes of 7.5 μL and larger gave deformed and splitted peaks.

The optimized HILIC-MS method comprised gradient elution employing 10 mM ammonium acetate (pH 6.8) in acetonitrile-water, an injection solvent containing 80% acetonitrile, and an injection volume of 5 μL.

4.3.2 Comparison of CE–MS and HILIC–MS

4.3.2.1 Reproducibility, sensitivity and metabolite coverage

Using the optimized HILIC–MS method, the test metabolites were baseline separated with retention times ranging from 3.1 to 13.5 min (Figure 4.1). The retention times were highly reproducible for all metabolites with relative standard deviations (RSDs) below 0.2%. Also peak area repeatability was acceptable with RSD values below 8% for ten consecutive measurements (Table 4.1). Mixtures of the five test metabolites in the concentration range 0.25-80 μM were analyzed to determine the linearity of the method. For all compounds a good linearity was observed with coefficients of determination (R^2) above 0.99. LODs (S/N ratio of 3) for the test metabolites were in the range of 0.04-2.21 μM (Table 4.2).

The anionic test metabolites have previously been analyzed with an optimized CE–MS method employing a triethylamine-containing BGE and sheath liquid [3]. In CE–MS, all metabolites were baseline separated with migration times from 7.0 to 17.5 min. RSDs for elution/migration time of the test compounds were lower with HILIC–MS, but repeatability for peak areas was similar for most metabolites (Table 4.1).

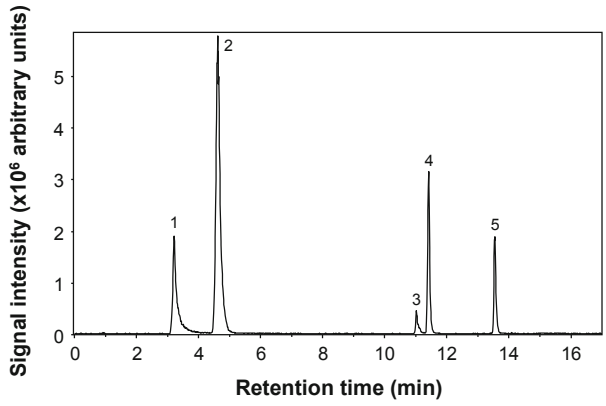

Figure 4.1 Multiple extracted-ion chromatogram obtained during HILIC–MS of a test metabolite mixture (50 μM each). Metabolites: 1. uridine (*m/z* 243.06); 2. hippuric acid (*m/z* 178.05); 3. proline (*m/z* 114.06); 4. pyroglutamic acid (*m/z* 128.04); 5. glutaric acid (*m/z* 131.04). Gradient elution with (a) 10 mM ammonium acetate in water-acetonitrile (1:1, v/v) and (b) 10 mM ammonium acetate in water-acetonitrile (1:9, v/v): 0-5 min, 100% b; 5-15 min, from 100% b to 100% a; 15-20 min, 100% a. See section 4.2 for other experimental conditions.

Using CE–MS, LODs for the five test metabolites ranged from 0.92 to 9.08 μM [3]. Thus, the LODs obtained with HILIC–MS were up to 80 times lower for these compounds (Table 4.2). Most likely, this can be explained by the larger sample injection volume employed in HILIC (5 μL) as compared to CE (16 nL). Notably, the sensitivity difference is not as severe as predicted from the difference in injection volume (factor 300). The ionization efficiencies of the test metabolites may be relatively higher under the applied CE–MS conditions. Moreover, plate numbers in CE–MS are in general higher compared to HILIC–MS, which can contribute to higher peak signals. The observed difference in S/N ratios between HILIC–MS and CE–MS was not the same in magnitude for each test metabolite. Noise levels varied extensively throughout the HILIC chromatograms and CE electropherograms. Furthermore, ionization conditions in HILIC–MS vary due to the gradient elution leading to different ionization efficiencies.

Table 4.1 Retention/migration time and peak area repeatability (RSD (%), n=10) for five test metabolites analyzed using HILIC–MS and CE–MS

Compound	Retention/migration time		Peak area	
	HILIC–MS	*CE–MS*	*HILIC–MS*	*CE–MS*
Glutaric acid	0.03	0.33	7.70	10.83
Hippuric acid	0.13	0.38	5.26	6.57
Proline	0.03	0.34	2.42	6.08
Pyroglutamic acid	0.04	0.42	6.14	7.25
Uridine	0.16	0.36	4.56	3.97

Table 4.2 Limits of detection (μM) for five test metabolites applying HILIC–MS and CE–MS

Compound	HILIC–MS	CE–MS[a]	Factor difference
Glutaric acid	0.75	9.08	12.1
Hippuric acid	0.05	0.92	18.4
Proline	2.21	3.99	1.81
Pyroglutamic acid	0.04	3.21	80.3
Uridine	0.06	1.61	26.8

Metabolites were dissolved in acetonitrile-water (4:1, v/v) and water using HILIC–MS and CE–MS, respectively

[a] Previously reported by Kok *et al.* [3].

In order to examine the applicability of the optimized HILIC–MS and CE–MS method for the analysis of biological samples, a rat urine sample was repetitively analyzed. Notably, in CE–MS urine samples were diluted with BGE in a proportion of 1:1 (v/v), whereas for HILIC–MS a dilution of 1:4 (v/v) with acetonitrile was required for proper HILIC separation. Based on the analysis of rat urine, the CE–MS and HILIC–MS method were compared in terms of number of observed molecular features, analyte elution/migration order and repeatability. The base-peak chromatograms and electropherograms obtained with the optimum HILIC–MS and CE–MS method showed large numbers of peaks, which were widely spread over the elution/migration time window (Figures 4.2A and 4.2C). Overall, the absolute signal intensities were higher with HILIC–MS. HILIC–MS provided almost 4 times more molecular features than CE–MS (1360 versus 347 features). Five molecular features, which were detected with both systems (same m/z value) and which were spread over the elution/migration window (Figures 4.2B and 4.2D), were selected to assess repeatability. Ten consecutive measurements of the urine sample showed that the elution time repeatabilities of these urine metabolites were better for HILIC–MS (0.17-0.55%) with respect to CE–MS (5.93-11.8%), whereas peak area repeatabilities were similar (Table 4.3). As demonstrated before [4], CE–MS migration time repeatability for the rat urine components could be reduced to <0.4% by applying a proper alignment procedure.

Taken all molecular features into account, proportionately more molecular features with m/z values below 200 were detected with CE–MS, whereas the relative number of features with m/z values above 400 was higher in HILIC–MS (Figure 4.3).

Table 4.3 Retention/migration time and peak area repeatability (RSD (%), n=10) for five metabolites in rat urine detected with HILIC–MS and CE–MS

***m/z* value**	**Retention/migration time**		**Peak area**	
	HILIC–MS	*CE–MS*[a]	*HILIC–MS*	*CE–MS*[a]
212.00	0.17	10.8	7.89	13.3
112.05	0.29	5.93	11.6	8.58
124.99	0.55	10.8	13.6	13.2
243.06	0.39	8.09	10.5	13.5
167.02	0.23	11.8	7.68	7.85

[a] Previously reported by Kok *et al.* [4].

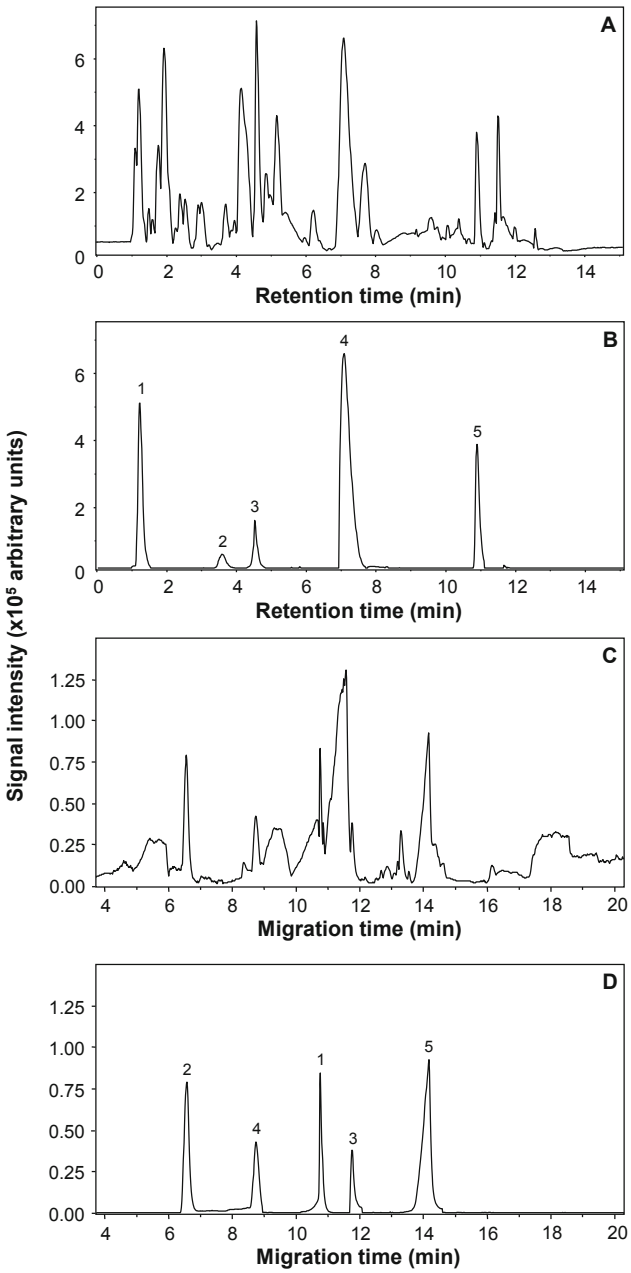

Figure 4.2 Base-peak and multiple extracted-ion chromatograms and electropherograms obtained when analyzing a rat urine sample by HILIC–MS (A, B) and CE–MS (C, D).
Peaks: 1. *m/z* 212.00; 2. *m/z* 112.05; 3. *m/z* 124.99; 4. *m/z* 243.06; 5. *m/z* 167.02.
See section 4.2 for experimental conditions.

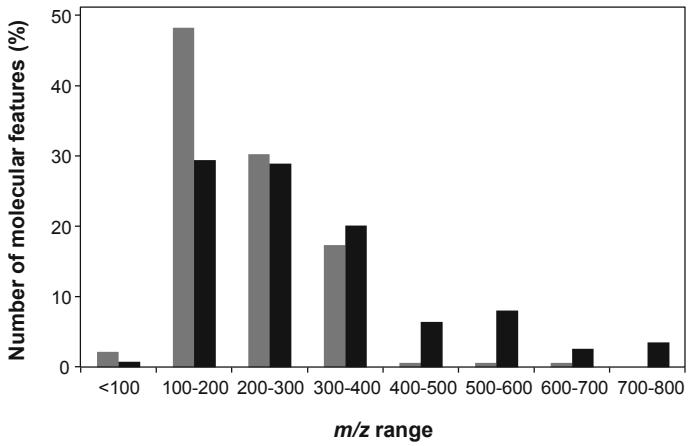

Figure 4.3 Distribution of *m/z* values of molecular features detected during CE–MS (grey bars) and HILIC–MS (black bars) of a rat urine sample. The relative number of features (%) in the various *m/z* ranges is depicted taking the total number of molecular features detected with the respective analytical method as 100%.

To compare the two analytical methodologies more extensively, molecular features which were detected with both CE–MS and HILIC–MS (common molecular features) were determined. Molecular features were considered to be common if the difference of the respective *m/z* values was less than 10 mDa. An *m/z* value only detected with one of the two methods was regarded as a unique molecular feature for the respective method. Based on these criteria, HILIC–MS and CE–MS revealed 1216 and 203 unique features, respectively, and 144 common molecular features were found with *m/z* values ranging from 108 to 503 of which most had *m/z* values below 300. There were 28 isobaric compounds among the 144 common molecular features. These features could not be assigned unambiguously based on mass only and were therefore dismissed during further evaluation. The ratios of peak areas and peak heights of the remaining 116 common molecular features were calculated to compare the signal intensities obtained with HILIC–MS and CE–MS (Figure 4.4). The ratios of peak area and height ranged from 0.20-63.9 and 0.59-130.5, and were on average 10.1 and 16.3, respectively. For eleven compounds, peak abundances in CE–MS were even higher than obtained with HILIC–MS. The large variance in intensity ratio between HILIC–MS and CE–MS might be caused by varying degrees of ionization suppression. As the separation principles of HILIC and CE are so manifestly different, the co-migration of urinary compounds, and thus, ionization conditions may vary strongly along the elution/migration window.

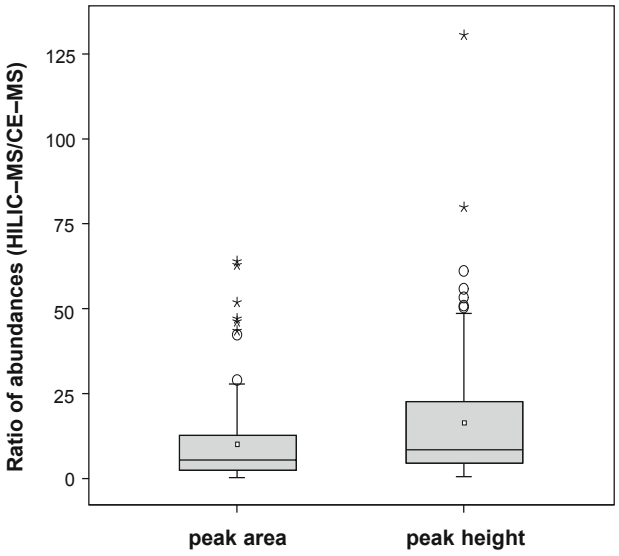

Figure 4.4 Box plot of the ratio of peak areas and peak heights of 116 common molecular features in a rat urine sample detected with both HILIC–MS and CE–MS. The 25th percentile, median and 75th percentile are represented by the boxes. The whiskers indicate the 5th and 95th percentile and the squares represent the average ratio of the peak areas and peak heights, respectively. Circles indicate ratios higher than 95% of the observed ratios and asterisks symbolize outliers.

4.3.2.2 Separation selectivity

In order to appreciate the observed differences in selectivity between CE–MS and HILIC–MS, the elution and migration behavior of the test metabolites and common features in rat urine were evaluated, involving – when possible – the molecular structure of the compounds. The observed elution order of the test metabolites in HILIC roughly follows compound polarity (Figure 4.1). Uridine is the only test metabolite that is uncharged at the applied pH of 6.8, which may explain its fast elution. The aromatic ring of hippuric acid reduces its overall polarity leading to modest retention in HILIC. Proline and pyroglutamic acid have a rather polar structure providing significant retention. The dicarboxylic glutaric acid is highly polar and strongly retained in HILIC. In CE–MS, proline migrated first, followed by successively uridine, hippuric acid, pyroglutamic acid and glutaric acid [3], exhibiting a different order than observed during HILIC–MS. The CE migration order largely follows the overall negative charge of the compounds at

pH 11.7. The amino group of proline is partly protonated, whereas glutaric acid is doubly charged at pH 11.7.

The distinctive separation selectivity of CE and HILIC was strongly underlined by plotting the CE–MS migration times and the HILIC–MS retention times of the 116 common features observed during the analysis of rat urine (Figure 4.5). Compounds are scattered over the entire plot, indicating a clear lack of correlation between CE migration and HILIC retention. To explore the observed differences in separation selectivity of CE and HILIC in more molecular detail, the m/z values of the common features were searched against metabolomics databases [29-31]. This way, sixteen molecular features could be assigned based on accurate mass. These features included typical urinary compounds, such as allantoin, glucuronic acid, hippuric acid, indoxyl sulfate, phenol and uric acid (Table 4.4, red diamonds in Figure 4.5). As can be expected, all identified compounds, except creatinine, have an acidic group allowing their negative ionization.

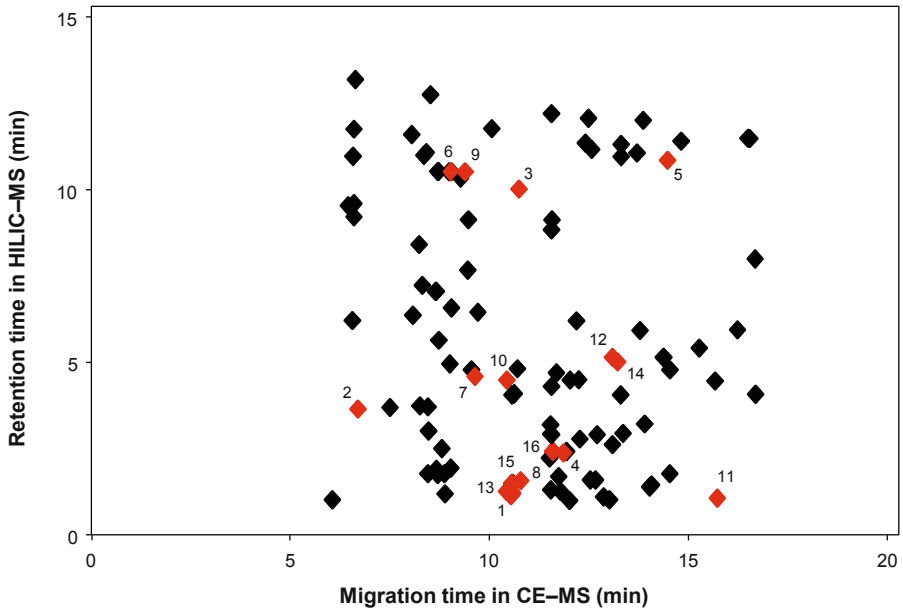

Figure 4.5 Relation between migration times and retention times of common molecular features obtained during CE–MS and HILIC–MS of a rat urine sample. The red diamonds with numbers represent metabolites which were putatively identified based on accurate mass. The identities together with the corresponding numbers are given in Table 4.4.

The CE and HILIC migration and retention data of the identified metabolites in Table 4.4 confirm the different selectivity of the methods. For example, the sulfonic acids cresol sulfate, benzenediol sulfate, methoxycatechol sulfate and indoxyl sulfate were hardly retained and not separated in HILIC–MS, whereas in CE–MS these compounds showed considerable migration times. Moreover, benzenediol sulfate could be separated from the other sulfonic acids in CE, most probably because it carries relatively more negative charge due to deprotonation of its phenol group. In contrast, taurine, also comprising a sulfonic acid group, could only be separated from the other sulfonic acids in HILIC. In CE–MS, uric acid (diprotic) and its oxidation product allantoin (monoprotic) were separated based on their charge-to-size ratios. For these compounds, a large difference in retention times was observed in HILIC–MS, although the molecular structures of allantoin and uric acid are highly similar.

Table 4.4 Retention times (RT) and migration times (MT) of putatively identified urinary metabolites detected with both HILIC–MS and CE–MS

#	m/z value	RT (min) HILIC	MT (min) CE	Putative identity
1	93.03	1.14	10.67	phenol
2	112.05	3.59	6.51	creatinine
3	124.01	10.05	10.73	taurine
4	157.09	2.39	11.86	allantoin
5	167.02	10.89	14.47	uric acid
6	168.08	10.55	8.99	methylhistidine
7	178.05	4.61	9.64	hippuric acid
8	187.01	1.48	10.78	cresol sulfate
9	187.07	10.55	9.38	glycylhydroxyproline
10	188.03	4.50	10.43	kynurenic acid
11	188.99	1.08	15.72	benzenediol sulfate
12	193.05	5.17	13.09	glucuronic acid
13	203.00	1.20	10.67	methoxycatechol sulfate
14	204.03	5.03	13.21	xanthurenic acid
15	212.00	1.23	10.70	indoxyl sulfate
16	276.14	2.43	11.58	methionine-lysine

Another example is the separation of compounds having a carboxylic group. Methylhistidine, hippuric acid, glycylhydroxyproline, kynurenic acid, glucuronic acid, xanthurenic acid and methionine-lysine had retention times between 2.43 and 10.55 min, and migration times ranged from 8.99-13.21 min. The elution order of these metabolites in the CE system differed completely from their elution order in HILIC. Furthermore, despite the apparent larger separation window in the optimized HILIC method, methylhistidine and glycylhydroxyproline co-eluted, whereas separation of these compounds was achieved in CE.

4.4 Concluding remarks

In order to assess the added value of CE–MS for anionic metabolic profiling of urine, a previously developed CE–MS method was compared to an optimized HILIC–MS method using ammonium acetate as mobile phase additive. In general, higher signal intensities were obtained with HILIC–MS compared to CE–MS. This is mainly due to the considerably larger injection volume applied in HILIC–MS. Still, the average difference in analyte response between CE–MS and HILIC–MS is much less than estimated from injection volume only, indicating overall higher absolute sensitivity in CE–MS. Notably, CE–MS can be particularly useful when volume-limited samples have to be analyzed. The separation selectivities of CE–MS and HILIC–MS appeared to be considerably different, as demonstrated by the low correlation between CE migration and HILIC retention of urinary metabolites. The unique detection of 203 and 1216 molecular features in rat urine by CE–MS and HILIC-MS, respectively, further demonstrated the different selectivity of the methods. Hence, although HILIC–MS revealed significantly more molecular features than CE–MS, there is a clear added value for CE–MS. In metabolomics studies, CE–MS could aid in finding discriminatory compounds (e.g. potential biomarkers) and help to elucidate metabolic pathways.

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V

Anionic metabolic profiling of urine from antibiotic-treated rats by capillary electrophoresis–mass spectrometry

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Analytical and Bioanalytical Chemistry, 405 (2013) 2585-2594

Abstract

A recently developed capillary electrophoresis (CE)–negative-ionization mass spectrometry (MS) method was used to profile anionic metabolites in a microbial-host co-metabolism study. Urine samples from rats receiving antibiotics (penicillin and streptomycin sulfate) for zero, four or eight days were analyzed. A quality control sample was measured repeatedly to monitor the performance of the applied CE–MS method. After peak alignment, relative standard deviations (RSDs) for migration time of five representative compounds were below 0.4%, whereas RSDs for peak area were 7.9-13.5%. Using univariate and principal component analysis of obtained urinary metabolic profiles, groups of rats receiving different antibiotic treatment could be distinguished based on seventeen discriminatory compounds, of which fifteen were down-regulated and two were up-regulated upon treatment. Eleven compounds remained down- or up-regulated after discontinuation of the antibiotics administration, whereas a recovery effect was observed for others. Based on accurate mass, nine compounds were putatively identified, these included the microbial-mammalian co-metabolites hippuric acid and indoxyl sulfate. Some discriminatory compounds were also observed by other analytical techniques, but CE–MS uniquely revealed ten metabolites modulated by antibiotic exposure, including aconitic acid and an oxocholic acid. This clearly demonstrates the added value of CE–MS for non-targeted profiling of small anionic metabolites in biological samples.

5.1 Introduction

The gut microbiota, the population of microorganisms that resides in the gastrointestinal tract, contributes significantly to the metabolic phenotype of the host via interactive microbial-host co-metabolism [1, 2]. Global metabolic profiling is the comprehensive analysis of metabolites in a biological system providing a snapshot of the biochemical status of a complex organism. This metabolic profile contains information from endogenous processes encoded in the host genome and from environmental inputs such as nutritional factors and gut microbial activity. Furthermore, information relating to trans-genomic interactions between the host genome and gut microbiome is also captured in the metabolic signature. Metabolic profiling strategies therefore provide a window into the host metabolic system and permit the influence of the gut microbiome and trans-genomic interactions on the host to be studied [3-5]. Germ-free or antibiotic-treated animal models are typically used to study the influence of the gut microbiota on host metabolism [1, 2]. Several analytical techniques have been applied to profile urine and fecal samples of rats and mice after administration of antibiotics, including proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, gas chromatography (GC) and reversed-phase (RP) liquid chromatography (LC) [6-11]. In all reported studies, antibiotic exposure modulated the biochemical signatures of animals. In the studies applying ^{1}H NMR spectroscopy, the discrimination was based on a range of compounds, including microbially derived metabolites, amino acids, organic acids and short-chain fatty acids [6, 8-10]. Using GC and LC approaches, oligosaccharides, pyridines and purines were also found to be discriminatory urinary compounds [7].

Capillary electrophoresis (CE) coupled with mass spectrometry (MS) using a sheath-liquid interface shows good potential for profiling highly polar and charged metabolites, and therefore, is expected to give complementary information about alterations in polar metabolites after antibiotic treatment with respect to the other applied methodologies [12]. Most of the reported CE–MS methods for global metabolic profiling have been conducted using positive electrospray ionization (ESI), because of the relatively higher analyte responses as compared to negative ionization mode CE–MS. However, a significant proportion of the metabolites present in biological samples is acidic in nature and can only be ionized effectively using negative ESI. Therefore, the application of negative ionization mode CE–MS in metabolic profiling studies may capture additional metabolic information and expand metabolome coverage. So far, CE–MS based studies performed in negative ionization mode, have generally followed a targeted approach [13]. In these studies, compound classes are preselected based upon *a priori* knowledge and their

abundance in biological samples is assessed. However, the application of this approach in an untargeted manner may allow the unbiased profiling of a class of metabolites not measured by other metabolomics approaches. Therefore, negative mode CE–MS based metabolic profiling offers a complementary alternative to NMR and LC–MS.

Recently, we have developed a CE–MS method with improved signal intensities for metabolites in negative ionization mode [14]. The optimized method employed a background electrolyte (BGE) of 25 mM triethylamine and a sheath liquid of 5 mM triethylamine in water-methanol (1:1, v/v). It exhibited a considerable increase in coverage of the polar human urine metabolome when compared to commonly used CE–MS methods applying ammonium acetate. Moreover, signal intensities were significantly higher with gain factors of up to seven [14]. In the present study, we evaluated the applicability of this negative mode CE–MS method for global anionic metabolic profiling of urine samples from rats receiving the antibiotics penicillin G and streptomycin sulfate for zero, four or eight days. The resulting profiles were aligned and the compounds present in the various urine samples were determined with a peak picking procedure. Peak areas of the compounds were assessed and individually normalized to the sum of the areas of all compounds. Average peak areas for the compounds detected before and after antibiotic treatment were compared, and principal component analysis (PCA) was also performed to discriminate between the various treatment groups based on differences between the obtained metabolic profiles. In addition, the metabolites with discriminating power were putatively identified. The same rat urine samples have previously been analyzed with high-resolution ^{1}H NMR spectroscopy [6]. The ^{1}H NMR spectroscopic urinary profiles revealed metabolic differences following antibiotic treatment. This offered the opportunity to compare the results obtained from the two analytical platforms and appreciate the value of CE–MS for anionic metabolic profiling. The CE–MS results were related to other studies on microbial-host co-metabolism as well.

5.2 Materials and methods

5.2.1 Chemicals

Methanol and formic acid were obtained from Fluka (Steinheim, Germany). Sodium hydroxide (NaOH) and creatinine were purchased from Sigma Aldrich (Steinheim, Germany), and triethylamine was from Fisher Scientific

(Loughborough, UK). Water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA) prior to use.

5.2.2 Rat urine samples

Urine samples from 18 rats were provided by AstraZeneca (Dept. of Drug Metabolism and Pharmacokinetics, Macclesfield, United Kingdom) and stored at -80°C. Rats were divided into three groups (n=6 each), receiving different regimens of antibiotic treatment, as extensively described elsewhere [6]. Briefly, one group (AB4) received antibiotics from day 0 to day 4, whereas another group of rats (AB8) received antibiotics for eight days. AB4 animals allowed the metabolic consequences of short-term bacterial recolonization to be studied; the period of four days was not sufficient for complete recolonization of the gut microbiota [6]. A control group (AB0) did not receive any antibiotics. The antibiotics, penicillin G (2 mg/mL) and streptomycin sulfate (4 mg/mL), were provided *ad libitum* in the rats' drinking water. These antibiotics are effective against both Gram-positive and Gram-negative bacteria, and complementary contribute to depletion of the microbiota. Sample collection was performed overnight for 16 hours on days -1 to 0 (D0), days 3-4 (D4) and days 7-8 (D8). Aliquots (10 μL) of all urine samples were pooled and used as quality control (QC) sample. Prior to analysis, urine samples were mixed with BGE (1:1, v/v). In total, 54 rat urine samples were randomly analyzed and the QC sample was measured after every fourth run to assess the stability and repeatability of the CE–MS method.

5.2.3 CE–MS

CE–MS experiments were performed on a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton, USA) coupled to a time-of-flight (TOF) mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) using a sheath-liquid electrospray interface from Agilent Technologies (Waldbronn, Germany). Separations were carried out in a fused-silica capillary (Polymicro Technologies, Phoenix, USA) with an internal diameter of 50 μm and a total length of 100 cm. New fused-silica capillaries were rinsed with 1 M NaOH for 20 min and water for 10 min at 20 psi. Between runs, capillaries were flushed with acetic acid (10% solution) for 4 min, water for 3 min and BGE for 1 min, applying a pressure of 50 psi. The BGE was 25 mM triethylamine (pH 11.7). The sheath liquid consisted of 5 mM triethylamine in water-methanol (1:1, v/v) and was delivered at 5 μL/min using a 10 mL syringe (Hamilton, Reno, USA) and a syringe pump of KD Scientific (Holliston, USA). The use of triethylamine, which is highly volatile,

did not affect the performance of the mass spectrometer and additional cleaning procedures were not needed. Rat urine samples were injected hydrodynamically using a pressure of 0.5 psi for 30 s. The separation voltage was 30 kV for 30 min and the capillary temperature was set at 20°C. The following interface conditions were used: dry gas temperature, 180°C; dry gas flow, 4 L/min; nebulizer pressure, 10 psi; ESI voltage, 4 kV. Data were acquired in negative ionization mode in the mass range 50 to 800 m/z with a repetition rate of 1 Hz. At the end of every run, a 10 mM sodium formate plug was injected (1 psi, 30 s), flushed through the capillary and the sodium formate clusters detected by ESI-MS were subsequently used for internal mass calibration of the recorded mass spectra.

5.2.4 Data analysis

CE–MS data were processed using MsXelerator software from MsMetrix (Maarssen, The Netherlands). The metabolite profiles were aligned based on a reference sample using the reference peak warping (RPW) function with the m/z values 112.05, 243.06, 124.99, 160.04 and 167.02 used as reference compounds allowing a maximum difference in m/z between the various metabolic profiles of 0.01 Da. Using the high resolution peak picking procedure of the MsXelerator software, compounds present in the QC sample having a migration time between 5 and 30 min and with a signal-to-noise (S/N) ratio of three for at least ten successive spectra were determined. After removal of isotope peaks, peak matching was carried out to determine peak areas of the common compounds in the rat urine samples. Compounds were considered matching (i.e. the same) when their mass difference and migration-time difference was smaller than 0.01 Da and 0.1 min, respectively. Per injected sample, peak areas were normalized to the total peak area obtained in the electropherogram. Average peak areas of detected compounds for each treatment group were calculated and area ratios between the treatment groups were determined to assess differences in metabolite profiles. Paired t-tests (AB4-D0 versus AB4-D4 & AB4-D8, and AB8-D0 versus AB8-D4 & AB8-D8) were conducted to determine significant changes among individual metabolite concentrations in rat urine upon antibiotic treatment. Independent samples t-tests were performed to assess differences in metabolite concentrations between AB4 and AB8 rats at both day 0 and day 4. P-values ≤ 0.05 were considered to indicate a statistically significant difference. Furthermore, the resulting table of normalized peak areas for detected compounds was used for PCA. The PCA loading plot was used to determine the variables that were responsible for the separation of the different rat urine samples. Discriminatory compounds between the various treatment groups were putatively

identified by comparing the observed masses with masses recorded in the metabolomics databases HMDB, METLIN and MassBank [15-17].

5.3 Results and discussion

5.3.1 CE–MS performance

To assess the reproducibility of the applied CE–MS method, the QC sample was measured repetitively throughout the entire two-week period in which the urine samples were analyzed. A typical base-peak electropherogram of rat urine is shown in Figure 5.1A. Peak areas, migration times and effective mobilities for five representative rat-urine compounds detected in the QC sample (Figure 5.1B) were determined and relative standard deviations (RSDs) were calculated. These five compounds were selected based on their migration times (covering the complete metabolite profile) and abundance (low and high intensities). The RSDs of the obtained peak areas of each of the selected compounds ranged from 7.9 to 13.5% (Table 5.1). This is considered acceptable for an ESI-MS based bioanalytical method. The peak areas showed a random variation in time and no significant difference was observed between the first and the last day, indicating good system robustness. The migration times of the selected QC compounds showed a relatively large variability (RSDs 8.1-11.8%, Table 5.1). Therefore, effective electrophoretic mobilities were calculated using creatinine (m/z 112.05; detected at circa 6.5 min) as neutral electroosmotic flow (EOF) marker. The identity of the EOF marker was confirmed by addition of creatinine to the sample. RSD values of the effective electrophoretic mobilities were considerably smaller (factor 2.4-4.7) than the RSD values for migration time (Table 5.1), indicating that a part of the variability was caused by EOF variation between runs.

The reported RSDs represent a day-to-day repeatability over fourteen days using two different capillaries. The observed variability in migration times is quite commonly observed in CE, especially when bare fused-silica (BFS) capillaries are used [18, 19]. Still, the observed RSDs for electrophoretic mobility were too large for a reliable comparison of metabolic profiles, indicating the need for an alignment procedure (see Section 5.3.2). The observed migration-time variation might be caused by adsorption of low abundant proteins or other matrix compounds to the capillary wall. Non-covalent coating of the capillary wall with charged polymers may in principle be used to substantially improve the repeatability of migration times of metabolites [20]. However, such a capillary coating appeared to be unstable

in combination with a BGE containing triethylamine [14]. This might be due to a potential displacement of the charged-polymer coating by triethylamine.

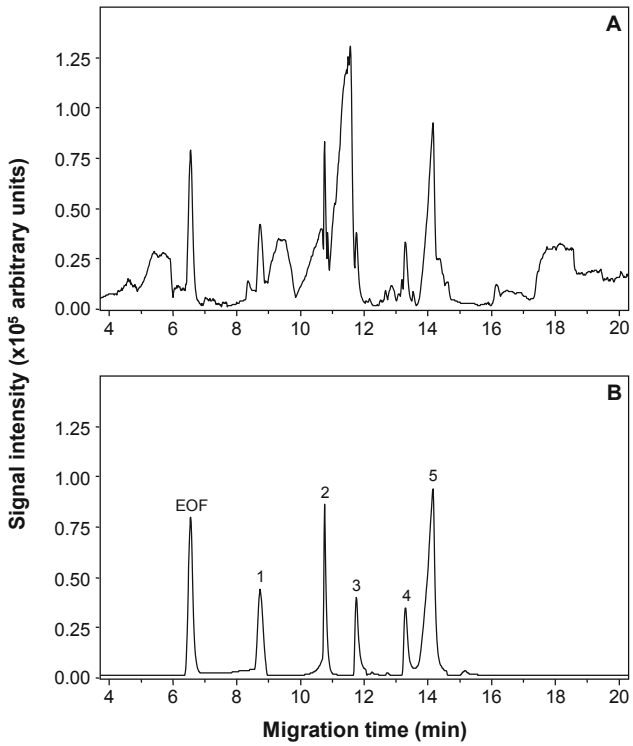

Figure 5.1 Base-peak electropherogram (A) and multiple extracted-ion electropherogram (B) obtained during CE–MS of the rat urine QC sample.
Compound *m/z* values: EOF marker, 112.05; 1. 243.06; 2. 212.00; 3. 124.99; 4. 160.04; 5. 167.02. Experimental conditions, see Section 5.2.

Table 5.1 Reproducibility of peak areas, migration times and effective electrophoretic mobilities (μ_{eff}) of five representative urinary compounds (*m/z*) detected during repetitive CE–MS analysis of the QC sample (n=15, two-week period)

m/z	Peak area RSD (%)	Migration time RSD (%)	μ_{eff} RSD (%)
243.06	13.5	8.1	3.4
212.00	13.3	10.8	2.3
124.99	13.2	10.8	2.3
160.04	8.9	11.4	2.8
167.02	7.9	11.8	4.6

5.3.2 Data preprocessing

Because of the variability in the migration times of compounds in the different samples, peak alignment was necessary in order to reliably compare the metabolite profiles after the various antibiotic treatments. Various peak alignment procedures have been used in CE for metabolomics [18, 19]. In this study, peak alignment was performed with the RPW function of the MsXelerator software. RPW is a three-dimensional alignment method that is based on representative peaks well spread across the migration time axis. Mass was taken into account for the alignment, because a two-dimensional (migration time and intensity) alignment procedure, using base-peak electropherograms, was insufficient for aligning the metabolic profiles properly.

Creatinine and the compounds listed in Table 5.1 (except m/z 212.00) were selected as reference compounds (see Section 5.2.4) to which all metabolite profiles were aligned. The compound with an m/z value of 212.00 was used as control to assess the variability in migration time after peak alignment (*vide infra*). The alignment is based on a spline function to fit a non-linear curve between the observed migration times of the reference compounds in the various samples. Despite the variations in analyte migration times between analyzed samples, the reference compounds could be aligned properly (Figure 5.2A and 5.2B). In order to further check the effectiveness of the profile alignment, the migration-time RSDs of three arbitrary compounds – migrating in the beginning, in the middle and at the end of the profile – were determined. As can be seen in Figure 5.2C, only small variations in the migration times were observed after peak alignment. The largest RSD was observed for m/z value 212.00 with a value of only 0.4%. This is a tremendous improvement compared to the variability before alignment (10.8%; Table 5.1), and will allow the proper application of algorithms used for multivariate analysis.

After alignment, peak picking was performed to determine which compounds were present in the rat urine samples (see Section 5.2.4 for procedure). This resulted in the detection of 347 compounds with S/N ratios above three in the QC sample. Then, peak matching was carried out to reveal compounds present in all rat urine samples and the respective peak areas were determined. For a proper comparison, peak area normalization is necessary due to considerable variation in individual rat urine volumes. Normalization to creatinine concentration is often used in MS-based urinary metabolomics [21, 22]. However, the ^{1}H NMR spectroscopic study of the same urine samples indicated that creatinine clearance varied with antibiotic treatment and was therefore not considered a suitable normalization method [6].

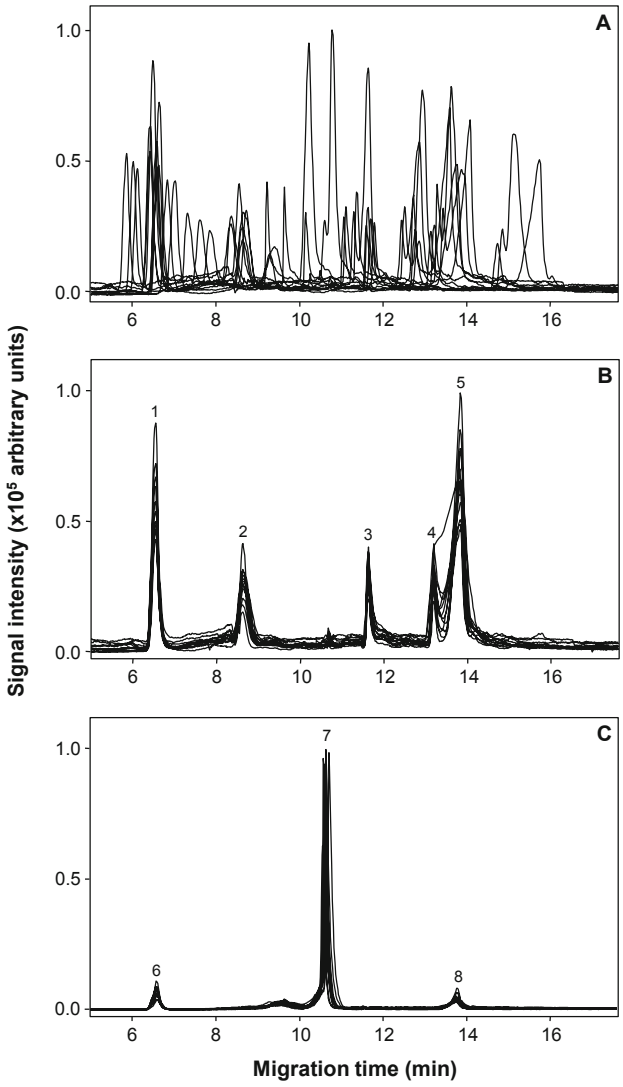

Figure 5.2 Multiple extracted-ion electropherograms obtained during repeated CE–MS analysis (n=15) of the QC sample before (A) and after (B, C) peak alignment using the reference peak warping function with peaks 1-5 as reference compounds and peaks 6-8 as verification compounds.

Compound *m/z* values: 1. 112.05; 2. 243.06; 3. 124.99; 4. 160.04; 5. 167.02; 6. 503.16; 7. 212.00; 8. 335.05.

Normalization based on total ion current was also dismissed, because of the relatively high background signals observed in the CE–MS electropherograms. Therefore, we decided to normalize individual peak areas with respect to total peak area, an approach commonly used in NMR-based metabolomics. This was found to improve the peak area variability between urine samples from individual rats. For each treatment group of rats the common and normalized peak area RSDs of the compounds mentioned in Table 5.1 were determined. The RSDs of normalized peak areas were up to a factor 4 lower.

5.3.3 Exploring metabolite profiles

Before performing multivariate data analysis, the urinary metabolic profiles were investigated to assess the quality of the data after the various preprocessing steps and to evaluate whether useful information about changes in metabolite levels after antibiotic treatment can be obtained. Average peak areas of corresponding compounds in urine samples from rats in the same treatment group were calculated for the three different time points (day 0, 4 and 8). Taking a criterion of an average peak area ratio of ≥ 3 or ≤ 0.33, compounds were considered to be potentially significantly up- or down-regulated upon antibiotic treatment.

Some compounds showed striking differences in peak areas before and after the administration of antibiotics. None of those observed compounds could be linked to the administered penicillin G and streptomycin sulfate or metabolites derived from those antibiotics. Both penicillin G and streptomycin sulfate are acidic, and could potentially be detected by CE–MS in negative ionization mode. However, penicillin G and streptomycin sulfate are poorly absorbed from the gastrointestinal tract and hardly enter the systemic circulation [6]. Therefore, one would indeed not expect to detect these compounds and their metabolites in urine samples after antibiotic treatment. In the ^{1}H NMR study of Swann *et al.*, antibiotic-related metabolites were also not found in urine samples, but only in fecal samples of treated rats [6].

At identical study conditions (i.e. at day 0 and 4) for the groups of rats receiving four (AB4) and eight days of antibiotic treatment (AB8), average peak areas of urinary compounds were similar ($p>0.05$), indicating a proper normalization procedure. This is depicted for three metabolites in Figure 5.3, where the grey (AB4 rats) and black bars (AB8 rats) are equal at day 0 and 4. Furthermore, these three metabolites show a large fluctuation upon antibiotic treatment.

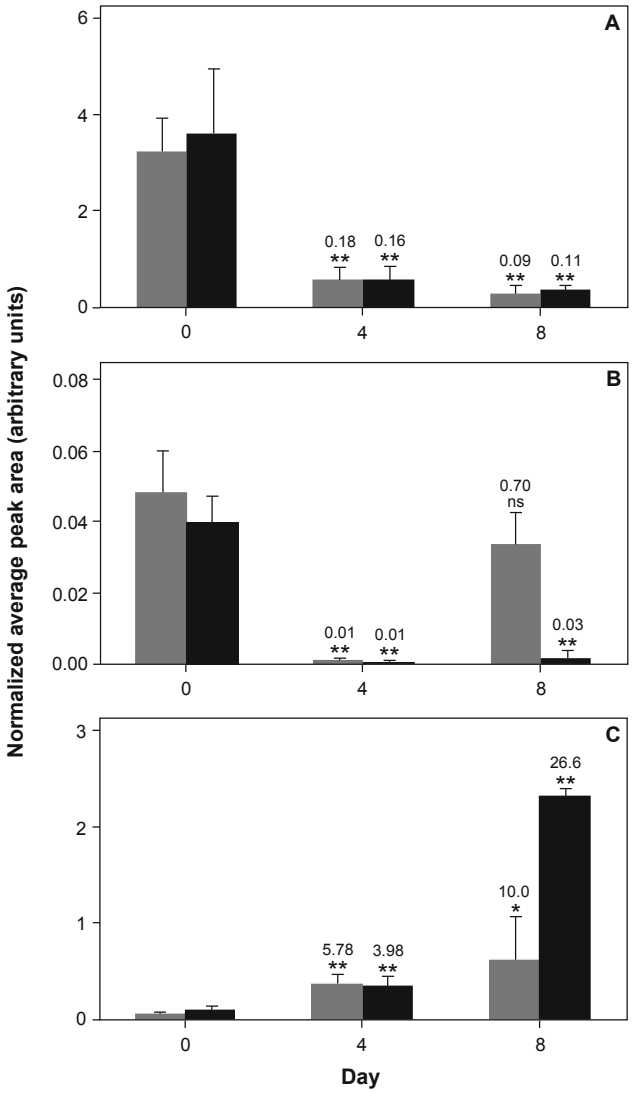

Figure 5.3 Normalized average peak areas (± standard deviation) at day 0, 4 and 8 of three urinary compounds with (A) *m/z* 178.05, (B) *m/z* 462.97 and (C) *m/z* 503.16 upon antibiotic treatment. The grey bars represent rats (n=6) receiving four days of antibiotic treatment (AB4) and the black bars represent rats (n=6) receiving eight days of antibiotics (AB8). Peak areas of the respective compounds at day 0 were compared with the areas at day 4 and 8 for both the AB4 and AB8 animals. Fold changes in peak area are indicated above the bars including the statistical significance of the observed differences: *, $p \leq 0.05$; **, $p \leq 0.01$; ns, $p > 0.05$.

The compounds with *m/z* values 178.05 and 462.97 show a large decrease of a factor of 5.9 and 87.3, respectively after four days, whereas the peak area for *m/z* 503.16 was a factor 4.9 higher. After discontinuation of the antibiotic treatment, two different effects were observed for the initially decreased compounds. Peak areas for *m/z* 178.05 remained significantly decreased at day eight with respect to day zero ($p \leq 0.01$), whereas a recovery effect (i.e. increasing peak areas) was observed for *m/z* 462.97 ($p > 0.05$) (Figure 5.3, grey bars). Particularly apparent after the use of antibiotics for eight days was a significant increase (factor of 26.6) of the metabolite with an *m/z* of 503.16 (Figure 5.3, black bars). Overall, the results indicated that the quality of the processed data allowed the determination of differences in compound abundances among the various treatment groups.

5.3.4 PCA of metabolite profiles

PCA of the urinary metabolic profiles was performed to observe whether the differently treated rats could be discerned on the basis of CE–MS and to reveal discriminating compounds in an unsupervised way. The PCA score plot of the metabolic profiles of all rat urine samples is shown in Figure 5.4A. The first principal component (PC) accounted for 33% of the variance, whereas PC2 explained 21% of the variance. The QC replicates are closely clustered in the center of the PCA score plot. This confirms the stability and reproducibility of the CE–MS method (including alignment), and thus also its suitability for metabolic profiling studies. Samples of untreated rats (AB0-D0, AB0-D4, AB0-D8, AB4-D0 and AB8-D0) are more widespread, with four samples as outliers. This spread represents the variation of the composition of urine originating from various individual rats and collected at different days. However, as indicated by PCA, the urinary metabolic profiles from non-treated rats and antibiotic-treated rats were markedly different (Figure 5.4A). Swann *et al.* also distinguished samples originating from non-treated and treated rats, using ^{1}H NMR spectroscopy [6]. The samples after four (AB4-D4 and AB8-D4) and eight days (AB8-D8) of continuous intake of the antibiotics showed no separation in the overall PCA plot. PCA performed on only the metabolic profiles of urine samples after continuous antibiotic treatment for four (AB4-D4+AB8-D4) and eight days (AB8-D8) also showed no distinction (Figure 5.4B). This result suggests that the duration of antibiotic treatment does not lead to major differences in the metabolic profiles measured by CE–MS. It should be noted that during exploration of the metabolic profiles (Section 5.3.3) the compound with *m/z* 503.16 showed a higher increase in signal after eight days than after four days of antibiotic treatment (Figure 5.3C). The subtle variation in the

concentration of m/z 503.16 may be too small to cause discrimination of the two groups by PCA.

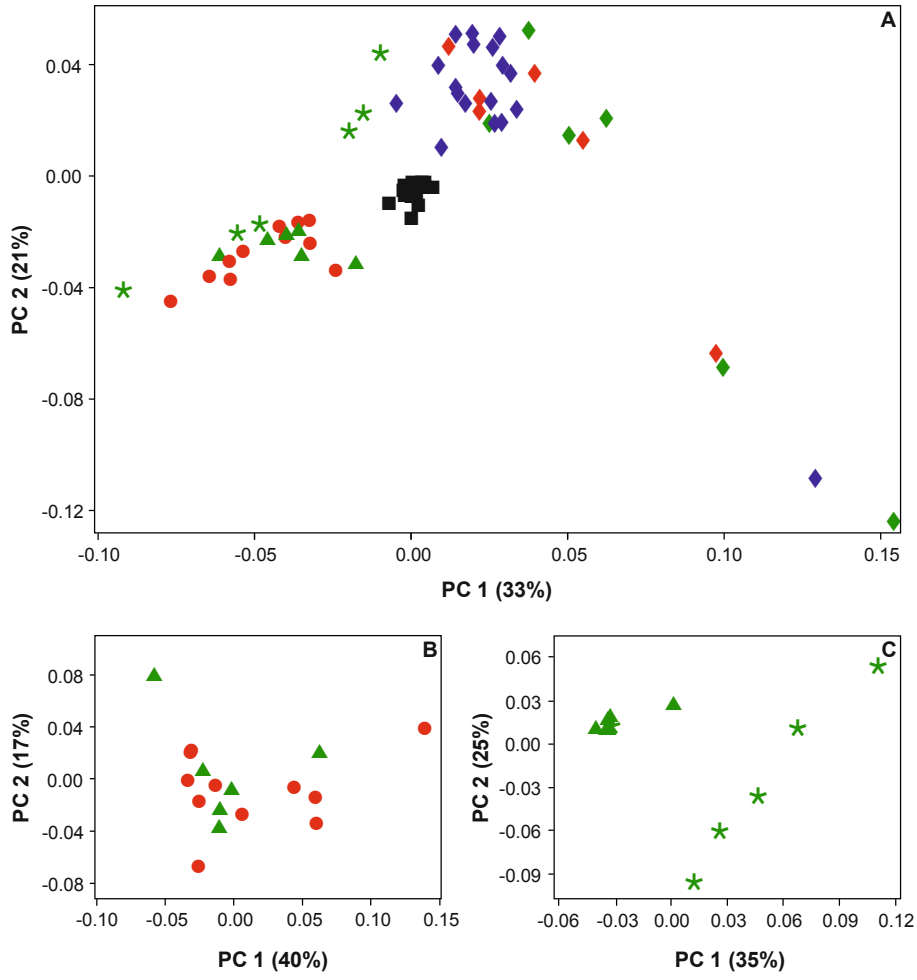

Figure 5.4 PCA score plots of metabolic profiles of rat urine samples analyzed by CE–MS. PCA was performed on all samples (A), on urine samples of rats receiving continuous antibiotic treatment for four and eight days (B) and on urine samples at day eight after four and eight days of antibiotic treatment (C).

Squares represent QC samples; diamonds represent untreated rats (AB0-D0, AB0-D4, AB0-D8, AB4-D0 and AB8-D0); dots represent rats after four days of continuous antibiotic treatment (AB4-D4 and AB8-D4); stars represent rats at day eight after four days of antibiotic treatment (AB4-D8); triangles represent rats after eight days of continuous administration of antibiotics (AB8-D8). The colors indicate the various time points: blue represents the samples at day zero, red the samples at day four and green the samples at day eight.

Discrimination of the samples from the AB4-D8 and AB8-D8 groups was less pronounced in the overall PCA plot (Figure 5.4A). However, when PCA was performed on the metabolic profiles of only these samples, there was a much better separation with noticeable differences in urinary metabolites (Figure 5.4C), except for one sample (sitting in the cluster of triangles). Similar results were obtained within the NMR study [6]. PCA of samples from the groups AB8-D4 and AB8-D8 showed a similar separation as in Figure 5.4C. This could be expected as no differences were observed between AB4-D4 and AB8-D4.

Interestingly, PCA of all metabolic profiles revealed the emergence of two subgroups within the recolonizing AB4-D8 group consistent with animal housing. The metabolic profiles of three rats showed near resemblance to untreated rats, whereas the profiles of the other three rats with the same antibiotic regimen were similar to the profiles of rat urine samples during treatment (Figure 5.4A). The same subgroups emerged in the study by Swann *et al.* when these urinary metabolic profiles were measured by NMR [6]. In addition, the microbial populations were also enumerated in the fecal samples of these animals and clear differences were observed in the bacterial profiles of these two AB4-D8 subgroups. The subgroup with the closest resemblance to non-treated rats had a substantially higher bacterial load compared to the other subgroup [6].

5.3.5 Discriminating compounds related to antibiotic treatment

The PCA loading plot indicated the variables (metabolites) responsible for the discrimination of the various rat urine samples (see Figure 5.5 for m/z values). After antibiotic treatment for four days (AB4-D4 and AB8-D4), fifteen compounds appeared to be down-regulated and two compounds were up-regulated. The three compounds that showed large ratios of peak areas before and after four-day treatment (Section 5.3.3) were also indicated by PCA as discriminatory between the various treatment groups. The abundances of the compounds in the different urine samples, relative to the average peak areas observed for non-treated rats are depicted in a heat map (Figure 5.5). The red color indicates decreased urine concentrations for the particular compounds upon antibiotic treatment, whereas green represents increased abundances. The cells in the heat map representing urine samples from non-treated rats are predominantly very dark or black, which indicates that the concentration of the seventeen discriminatory compounds did not vary significantly over time when rats were not exposed to antibiotic treatment. Establishing reference values by averaging the peak areas of untreated rats per m/z value appears valid.

Following cessation of antibiotic administration at day four, some metabolic modulations persisted at day eight, whilst in some compounds a recovery effect occurred (Figure 5.5). Ambiguous results were obtained for the *m/z* values 93.03, 132.04, 172.99, 212.00 and 446.99, leading to the appearance of two subgroups. For subgroup A, the peak areas at day eight were similar to non-treated rats, whereas for subgroup B, the effect of the antibiotics on the metabolite profile was still present at day eight (Figure 5.5). As indicated above, this probably reflects the cage-related differences in bacterial recolonization [6].

Based on the exact masses, nine of the discriminatory compounds could be putatively identified (Table 5.2). The compounds with *m/z* value 178.05 and 212.00 are most probably hippuric acid and indoxyl sulfate, respectively. The appearance of ESI fragments at *m/z* values 134.05 and 132.04 in the mass spectra [15-17], underlines the presence of hippuric acid and indoxyl sulfate, respectively.

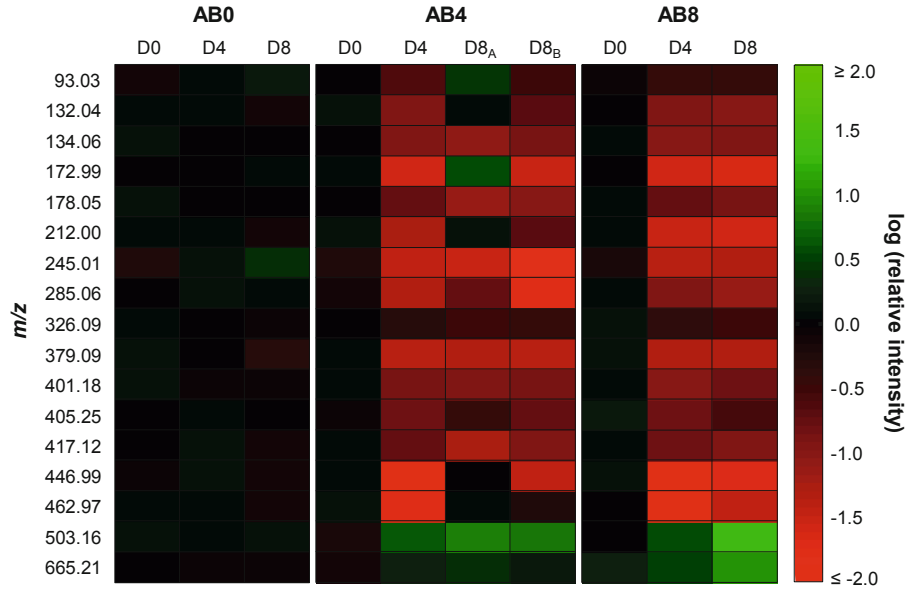

Figure 5.5 Heat map of average peak areas in time (D0, D4 and D8) for discriminating compounds in urine from rats receiving zero (AB0), four (AB4) or eight days (AB8) of antibiotic treatment. The two subgroups observed in the AB4-D8 group are indicated with A en B.

Each block represents the average peak area of the indicated *m/z* value for a specific rat group, relative to the average peak area observed for the indicated *m/z* value for non-treated rats. Peak areas are depicted as color-coded log values with red indicating decreased and green increased peak areas. Black represents no changes in urine concentration.

Putative identities of the *m/z* values are listed in Table 5.2.

Table 5.2 Putatively identified discriminatory compounds (up- or down-regulated) revealed by PCA of CE–MS data obtained for urine of non-treated and antibiotic treated rats

Putative identity	MT (min)[a]	Obs. *m/z*[b]	Calc. *m/z*[b]	Error (mDa)
Phenol	11.49	93.032	93.034	-1.84
Indoxyl	10.70	132.044	132.045	-0.55
Fragment hippuric acid	9.40	134.060	134.061	-1.60
Aconitic acid	11.65	172.991	173.009	-17.7
Hippuric acid	9.40	178.050	178.050	-0.02
Indoxyl sulfate	10.70	212.002	212.002	0.24
Oxocholic acid	7.22	405.246	405.264	-17.9
Trisaccharide	6.69	503.157	503.161	-3.92
Tetrasaccharide	6.62	665.207	665.214	-6.74

[a] Migration time after alignment

[b] Observed and calculated *m/z* values

Both hippuric acid and indoxyl sulfate were down-regulated upon antibiotic treatment. The concentration of hippuric acid remained significantly lower post-treatment, whereas a recovery effect was observed for indoxyl sulfate in one subgroup of AB4. This is consistent with the findings of Swann *et al.* with ^{1}H NMR spectroscopic analysis of the same urine samples [6]. Moreover, the decrease in concentration of hippuric acid and indoxyl sulfate after gut microbial depletion has been observed in other studies as well [7, 9, 10]. Hippuric acid and indoxyl sulfate have found to be mammalian-microbial co-metabolites, since their production is dependent on the presence of microorganisms in the gastrointestinal tract [1, 6, 7]. It is expected therefore, that administration of antibiotics will lead to down-regulation of both hippuric acid and indoxyl sulfate.

The mass of 93.03 could be attributed to phenol, a compound that is synthesized from tyrosine by some bacteria. The concentration of phenol in urine was decreased after the use of antibiotics for four days. As mentioned above, two subgroups of rats were observed based on the obtained metabolic profiles after discontinuation of the antibiotics. The phenol concentration increased for three rats in the AB4-D8 group; the concentration for the other three rats in the same group remained lower compared to day zero (Figure 5.5). Zheng *et al.* also found a decrease in phenol in rat urine after the oral administration of the antibiotics imipenem and cilastatin. Moreover, a recovery effect was observed after discontinuation of treatment [7].

The *m/z* values 172.99 and 405.25 were putatively identified as aconitic acid and an oxocholic acid, respectively. Aconitic acid plays a role in the tricarboxylic acid

(TCA) cycle, being the intermediate in the conversion of citric acid to isocitric acid. Aconitic acid has not been found before as a discriminative compound for urinary metabolic profiles of untreated and antibiotic-treated rats. However, other compounds of the TCA cycle were altered upon antibiotic treatment. Swann and co-workers found an up-regulation of citric acid and fumaric acid [6]. In contrast, Zheng *et al.* observed a decrease in the urinary concentration of citric acid and fumaric acid after the intake of antibiotics [7]. In the present study, citric acid was also detected with the applied CE–MS method, but PCA did not reveal this compound to differentiate between the various rat urine samples. Because of the relatively high urinary concentration of citric acid and the multiple carboxylic acid groups, this compound showed distorted peaks in the CE–MS profiles, which complicated the comparison of peak areas between the urine samples.

3-Oxocholic acid, 3,7-dihydroxy-12-oxocholanoic acid and 7-ketodeoxycholic acid are dihydroxy bile acids with a molecular weight of 406.27, which are secreted via the urinary tract. These bile acids differ in the position (3, 7 and 12) of two hydroxyl groups. To the best of our knowledge, this is the first time that such a bile acid has been found to be elevated in rat urine after gut microbial depletion with antibiotic treatment. Other bile acids have shown to be altered in various tissue samples of rats and mice after microbial manipulation [23, 24].

The compounds detected with m/z 503.16 and 665.21 are most probably a tri- and a tetrasaccharide, respectively. These compounds were up-regulated in rat urine after the administration of penicillin G and streptomycin sulfate (Figure 5.5). In the ^{1}H NMR study of Swann *et al.* on the same sample set, there was an increase in urine concentration of glycoproteins and an increase in oligosaccharides in analyzed fecal samples [6]. Degradation of glycoproteins will lead to more oligosaccharides, and thereby, results in elevated concentrations of oligosaccharides, which was also observed in fecal samples of mice after vancomycin-induced depletion of gut microorganisms [9].

Eight of the detected compounds responsible for discrimination of the various groups of rats could not be assigned based on accurate mass only, because there were multiple metabolite candidates in the databases with equal masses. These compounds all had different migration times, indicating they are unique discriminatory compounds (i.e. no ESI-induced adducts or fragments). Interestingly, compounds that correspond with these m/z values were not reported in other antibiotic treatment studies using ^{1}H NMR, GC and LC methodologies.

This indicates that the CE–MS method in negative ionization mode provides complementary information to the other metabolite profiling platforms, thereby

increasing the coverage of the urinary metabolome, which is important in global metabolic profiling studies. In order to extend the knowledge of microbial-host co-metabolism, identification of the unknown compounds will be required. This could be achieved, for instance, by conducting MS/MS experiments using a quadrupole (Q)-TOF-MS instrument and/or by spiking the urine samples with reference standards.

5.4 Concluding remarks

A new CE–MS method for the global profiling of anionic metabolites, requiring minimal sample pretreatment (only 1:1 v/v dilution with BGE), was applied to urine samples from rats receiving various antibiotic treatments. Repeated analysis of a QC sample throughout the study showed that the method enabled acquisition of reproducible peak areas for representative urinary compounds over a two-week period. Moreover, a dedicated peak alignment procedure resulted in CE–MS profiles with low migration-time variability, allowing reliable comparison of the urinary sample data. Rats differentially treated with antibiotics could be distinguished based on their urinary metabolic profiles obtained with CE–MS. Seventeen discriminatory compounds were revealed, including several acidic or neutral compounds such as indoxyl sulfate, phenol, an oxocholic acid, oligosaccharides and aconitic acid. The latter cannot be assessed by CE–MS applying a low-pH BGE and positive ESI. Employment of a CE–MS method particularly suited to profile cationogenic metabolites [20] for the analysis of the same rat urine samples, revealed eleven discriminating compounds. Based on m/z values, only two of the observed discriminatory compounds could be linked to compounds found with negative mode CE–MS. This indicates the added value of methods allowing global profiling of biological samples in negative ionization mode. Some of the putatively identified compounds were also observed in other metabolic profiling studies on gut microbial depletion, including a ^{1}H NMR spectroscopic study performed on the same sample set. However, CE–MS also indicated at least ten discriminating compounds which were not detected in other related studies, thereby clearly demonstrating the potential of CE–MS in negative ionization mode for metabolic profiling. Identification of the observed discriminatory compounds by MS/MS and standard addition is still required to increase insights in the related metabolic processes. Currently, we are comparing the presented CE–MS method to ion-pair reversed-phase LC–MS and hydrophilic interaction chromatography (HILIC)–MS for the detection of anionic metabolites.

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VI

Hydrophilic interaction chromatography–mass spectrometry for anionic metabolic profiling of urine from antibiotic-treated rats

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Journal of Pharmaceutical and Biomedical Analysis, 92 (2014) 98-104

Abstract

Hydrophilic interaction chromatography–mass spectrometry (HILIC–MS) was used for anionic metabolic profiling of urine from antibiotic-treated rats to study microbial-host co-metabolism. Rats were treated with the antibiotics penicillin G and streptomycin sulfate for four or eight days and compared to a control group. Urine samples were collected at day zero, four and eight, and analyzed by HILIC–MS. Multivariate data analysis was applied to the urinary metabolic profiles to identify biochemical variation between the treatment groups. Principal component analysis (PCA) found a clear distinction between those animals receiving antibiotics and the control animals, with twenty-nine discriminatory compounds of which twenty were down-regulated and nine up-regulated upon treatment. In the treatment group receiving antibiotics for four days, a recovery effect was observed for seven compounds after cessation of antibiotic administration. Thirteen discriminatory compounds could be putatively identified based on their accurate mass, including aconitic acid, benzenediol sulfate, ferulic acid sulfate, hippuric acid, indoxyl sulfate, penicillin G, phenol and vanillin 4-sulfate. The rat urine samples had previously been analyzed by capillary electrophoresis (CE) with MS detection and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Using CE–MS and ^{1}H NMR spectroscopy seventeen and twenty-five discriminatory compounds were found, respectively. Both hippuric acid and indoxyl sulfate were detected across all three platforms. Additionally, eight compounds were observed with both HILIC–MS and CE–MS. Overall, HILIC–MS appears to be highly complementary to CE–MS and ^{1}H NMR spectroscopy, identifying additional compounds that discriminate the urine samples from antibiotic-treated and control rats.

6.1 Introduction

The gut microbiota, the population of microorganisms that inhabit the gastrointestinal tract, is involved in the regulation of various metabolic pathways of the host via interactive and symbiotic microbial-host co-metabolism. Such interactions can be modulated by the diet, disease and the use of antibiotics, and contribute significantly to the metabolic phenotype of the host. The gut microbiota is a highly dynamic and expansive variable and there are several ways to study the interactions between these microbes and the host. One approach that has provided great insight into these trans-genomic interactions is the application of metabolic profiling to study biological samples from animal models of modified gut microbial states. Metabolic profiling (metabonomics/metabolomics) is the comprehensive analysis of metabolites in a biological system, providing information about the physiology and biochemical pathways of an organism [1-7]. Germ-free animal models or animals of which the microbiota is depleted through oral antibiotic treatment are typically used to study the influence of the gut microbiota on host metabolism. Urine and/or fecal samples of these animals have been analyzed using various analytical techniques, including proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, gas chromatography (GC), reversed-phase (RP) liquid chromatography (LC) and capillary electrophoresis (CE) [8-15]. Most often, GC, LC and CE are coupled to mass spectrometry (MS) in order to obtain both sensitive detection and information on metabolite identification. None of the applied analytical techniques is individually capable of measuring the complete set of metabolites within a sample, since metabolites have diverse physicochemical properties and are present at concentrations that span over nine orders of magnitude [16]. However, various methodologies can provide complementary information about biochemical processes and when used in combination can increase the coverage of the metabolome, improving the understanding of microbial-host co-metabolism. All reported studies exploring the metabolic impact of orally administered antibiotics have found significant variation in urinary metabolic profiles pre- and post-exposure [8-15]. The observed differences were based on metabolites from various compound classes, detected with different analytical techniques. For instance, using ^{1}H NMR spectroscopy differences in concentration levels of amino acids and organic acids before and after treatment were found [8, 10, 12, 15]. In studies applying GC and LC, the discrimination was mainly based on fluctuating levels of oligosaccharides, pyridines and purines [9]. When CE was employed, a variety of polar compounds was found to be discriminatory [14].

Hydrophilic interaction chromatography (HILIC)–MS is a metabolic profiling approach that is effective for measuring polar metabolites. Such metabolites are separated based on a combination of partitioning and electrostatic interactions with a polar stationary phase [17-19]. HILIC–MS can provide additional biochemical information following antibiotic treatment, complimentary to previously used techniques, thereby enhancing the understanding of host-microbiome co-metabolism. CE–MS has also been used to gain information about the polar components of the metabolome, but as shown before, HILIC–MS can be highly complementary [20, 21]. Ibáñez and coworkers used both HILIC–MS and CE–MS in positive ionization mode to study the effect of polyphenols on the proliferation of colon cancer cells. Using HILIC–MS, 1077 features were observed, of which 214 were differentially expressed in the colon cancer cells after treatment with polyphenols. With CE–MS 2890 features were detected, of which 212 compounds were altered significantly. Of these compounds, 12 and 22 metabolites were putatively identified with HILIC–MS and CE–MS, respectively. There was no overlap in identified metabolites showing the complementarity of HILIC–MS and CE–MS [20]. Saric *et al.* analyzed the *Fasciola hepatica* metabolome and identified 37 and 90 metabolites when applying CE–MS and HILIC–MS in positive ionization mode, respectively. In total, 29 of these identified metabolites were found with both techniques. This implies that employing HILIC–MS in addition to CE–MS resulted in the identification of an additional 61 compounds [21].

In many metabolic profiling studies, the focus is often primarily on measuring cationogenic metabolites using positive electrospray ionization (ESI). However, a significant number of metabolites of interest is acidic and can only be ionized efficiently using negative ESI. In this study, we evaluated the applicability of HILIC–MS for anionic metabolic profiling of urine samples from control rats and those receiving the antibiotics penicillin G and streptomycin sulfate for four or eight days. Urine samples were collected at the beginning of the study (day 0) and on days four and eight. Following HILIC–MS analysis, the resulting metabolic profiles were compared using principal component analysis (PCA). Metabolites that appeared to be up- or down-regulated upon antibiotic treatment were putatively identified based on observed masses. The same rat urine samples had previously been analyzed with CE–MS and ^{1}H NMR spectroscopy to study microbial-host co-metabolism [8, 14]. This gave an excellent opportunity to compare the diverse analytical techniques and to determine the complementarity and added value of HILIC–MS for metabolic profiling studies. The comparison was based on the number, identity and relative abundance of discriminatory compounds.

6.2 Materials and methods

6.2.1 Chemicals

Acetonitrile was supplied by Fluka (Steinheim, Germany) and ammonium acetate was obtained from Merck (Darmstadt, Germany). Prior to use, water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA).

6.2.2 Rat urine samples

Urine samples were previously analyzed by CE–MS and ^{1}H NMR spectroscopy, and therefore have been extensively described elsewhere [8, 14]. Briefly, urine samples were taken from eighteen male Wistar derived AlpkHsdRccHan:WIST rats, which were divided into three treatment groups of six rats each. The antibiotics penicillin G (2 mg/mL) and streptomycin sulfate (4 mg/mL) were provided *ad libitum* for 0 (AB0), 4 (AB4) or 8 days (AB8) in the rats' drinking water. Urine samples were collected overnight for 16 hours on days -1 to 0 (D0), 3-4 (D4) and 7-8 (D8). All urine samples (n=54) were randomly analyzed after dilution with acetonitrile in a proportion of 1:4, v/v and centrifugation at 10,000 rcf for 10 min. A mixture of aliquots of all urine samples was used as quality control (QC) sample and measured after every fifth run to assess the stability of the HILIC–MS system.

6.2.3 HILIC–MS

All rat urine samples (5 μL) were analyzed on a Waters XBridge Amide column (3.0 x 100 mm) with 3.5 μm particles, maintained at 45°C during separation. HILIC–MS analyses were performed on a Shimadzu LC system (Kyoto, Japan) coupled to a time-of-flight (TOF) mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany). Urine samples were analyzed under gradient elution with (A) 10 mM ammonium acetate (pH 6.8) in water-acetonitrile (1:1, v/v) and (B) 10 mM ammonium acetate (pH 6.8) in water-acetonitrile (1:9, v/v) at a flow rate of 0.5 mL/min. The gradient started at 100% B for 5 min, followed by a gradual decrease to 0% B in 10 min. Thereafter, the system was maintained at 100% A for 5 min, after which it was immediately switched to 100% B for reequilibration of the HILIC–MS system. A new measurement was started after 10 min at 100% B. The mass spectrometer was operated with negative ESI, applying the following conditions: dry gas temperature, 180°C; dry gas flow, 4 L/min, nebulizer pressure, 50 psi; ESI voltage, 2 kV. Data were acquired in the mass range m/z 50-800 with a repetition rate of 1 Hz. Recorded mass spectra were internally calibrated using sodium acetate clusters.

6.2.4 Data analysis

The obtained metabolic profiles were aligned using the reference peak warping (RPW) function of MsXelerator software (MsMetrix, Maarssen, The Netherlands) in order to allow correct comparison of the various profiles. Alignment was based on a reference sample using eleven compounds as reference compounds with a maximum allowed difference in *m/z* between the various profiles of 0.01 Da. The compounds used for the alignment had the following of *m/z* values and retention times: *m/z* 203.00 (1.20 min), *m/z* 225.93 (1.61 min), *m/z* 112.05 (3.59 min), *m/z* 157.04 (4.15 min), *m/z* 124.99 (4.52 min), *m/z* 273.01 (5.17 min), *m/z* 311.12 (6.22 min), *m/z* 243.06 (7.09 min), *m/z* 167.02 (10.89 min), *m/z* 332.99 (11.49 min) and *m/z* 96.97 (12.57 min).

After alignment, peak picking was carried out to determine molecular features present in the QC sample (signal-to-noise (S/N) ratio of at least 3, ≥10 consecutive spectra) and peak matching was performed to determine common compounds (mass difference ≤0.01 Da, retention time difference ≤0.1 min) in the various rat urine samples. Peak areas of the compounds were normalized based on the total peak area obtained in the chromatograms to correct for the considerable variation in individual rat urine volumes. The normalized peak areas were used for PCA. Compounds that showed differences between the various treatment groups were tentatively identified by consulting the online databases HMDB, METLIN and Massbank [22-24], and by comparing obtained and theoretical isotope patterns.

6.3 Results and discussion

6.3.1 HILIC–MS

A previously optimized HILIC–MS system for anionic metabolic profiling using gradient elution with a mobile phase containing 10 mM ammonium acetate (pH 6.8) and acetonitrile was applied, providing good resolution and sensitivity of test metabolites with limits of detection (LODs) in the range of 0.04-2.2 μM [25]. The performance of the HILIC–MS system for urine analysis was assessed using a repetitively analyzed QC sample, revealing in total 1360 molecular features. Relative standard deviations (RSDs) for retention time and peak area of five of these features which were distributed over the complete metabolic profile (i.e. eluting in the beginning, middle and end) were in the range of 0.17-0.55% and 7.7-13.6%, respectively. As an example, the urinary profiles obtained at the various time points of one rat receiving antibiotics for four days are shown in Figure 6.1.

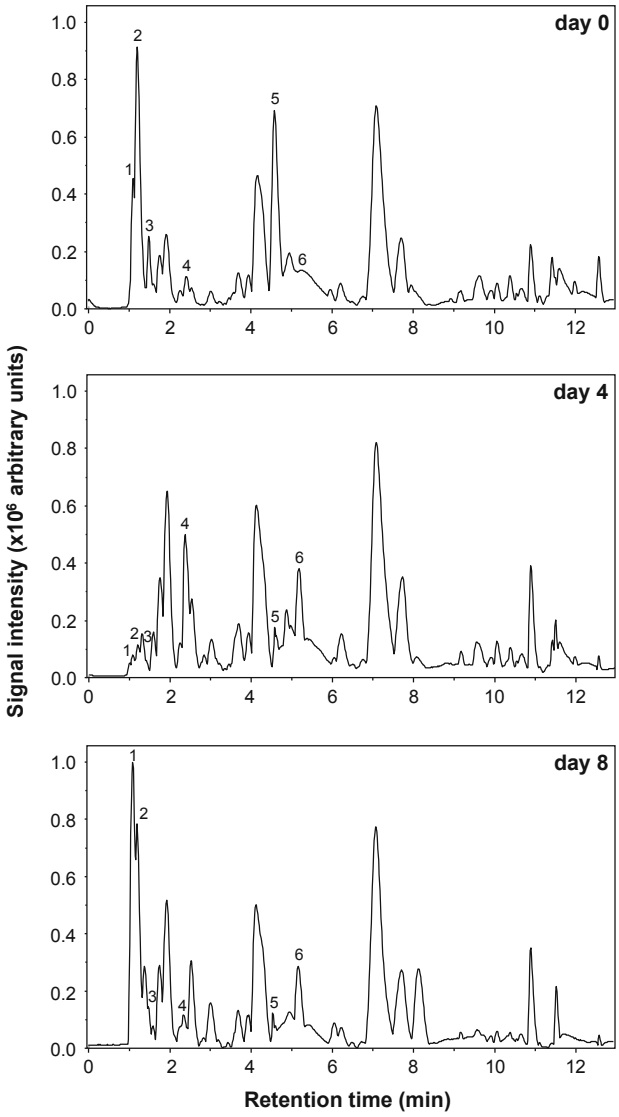

Figure 6.1 Base-peak chromatograms obtained with HILIC–MS of urine samples collected at day 0, 4 and 8 from a rat receiving antibiotic treatment for four days. Peaks that visually seem to be altered upon antibiotic treatment: 1. *m/z* 172.99; 2. *m/z* 212.00; 3. *m/z* 228.00; 4. *m/z* 192.05; 5. *m/z* 178.05; 6. *m/z* 273.01.

Differences upon antibiotic treatment could be observed from the resulting base-peak chromatograms obtained by HILIC–MS analysis. For example, two

compounds showed an increase in peak area after antibiotic treatment (peaks 4 and 6), whereas four others showed a decrease (peaks 1, 2, 3 and 5). After cessation of antibiotic treatment, a recovery effect was observed for peaks 1-4, whereas the variation in the other peaks persisted (Figure 6.1, day 8).

6.3.2 PCA and discriminatory compounds

PCA of the urinary metabolic profiles (see Section 6.2.4 for procedure) was performed to visualize the variance present between the various treatment groups of rats and to reveal discriminatory compounds in an unsupervised way. Figure 6.2A shows the PCA score plot of all samples. The replicates of the QC sample are in the center, showing that the analytical variation was much less than the biological changes seen on antibiotic administration. The PCA score plot indicates that the urine samples from control and antibiotic-treated rats were significantly different, with the first and second principal component (PC) accounting for 35% and 25% of the variance, respectively. In the overall PCA plot, the samples after four and eight days of antibiotic treatment could not be discerned. There was also no distinction when PCA was performed on only the metabolic profiles of urine samples after four and eight days of treatment (Figure 6.2B). These observations indicate that the duration of treatment does not result in significant differences in the urinary profiles obtained with HILIC–MS. Discontinuation of the antibiotic treatment at day four, therefore allowing the gut microbiota to re-establish for four days, resulted in the metabolic profiles of AB4 rats to differ from those of AB8 rats at day eight. Discrimination between rat urine samples obtained during treatment and obtained after stopping antibiotic treatment for four days was more pronounced when only these samples were used for PCA (Figure 6.2C).

The PCA loading plot indicated the discriminatory compounds, which were responsible for the distinction between the various urinary metabolite profiles. Twenty compounds were found to be down-regulated upon antibiotic treatment for four and eight days, whereas nine were up-regulated (Table 6.1). To determine the changes in concentration during the study, the peak areas of the discriminatory compounds in the various rat urine samples, relative to the abundances observed for untreated rats, are depicted in Figure 6.3. Red indicates a decreased concentration for that particular discriminatory compound, whereas green indicates an increased concentration. After stopping the administration of antibiotics, some compounds remained up- or down-regulated, whereas a recovery effect occurred for other compounds. Ambiguous results were observed for seven compounds with the m/z values 93.04, 132.05, 176.99, 200.13, 212.00, 228.00 and 446.99. A subgroup of

three rats showed a recovery effect (AB4-D8$_A$), whereas the metabolic modulations upon antibiotic treatment persisted for the other subgroup (AB4-D8$_B$). These variations reflect cage-related differences in bacterial load after cessation of antibiotic treatment, as previously observed [8, 14].

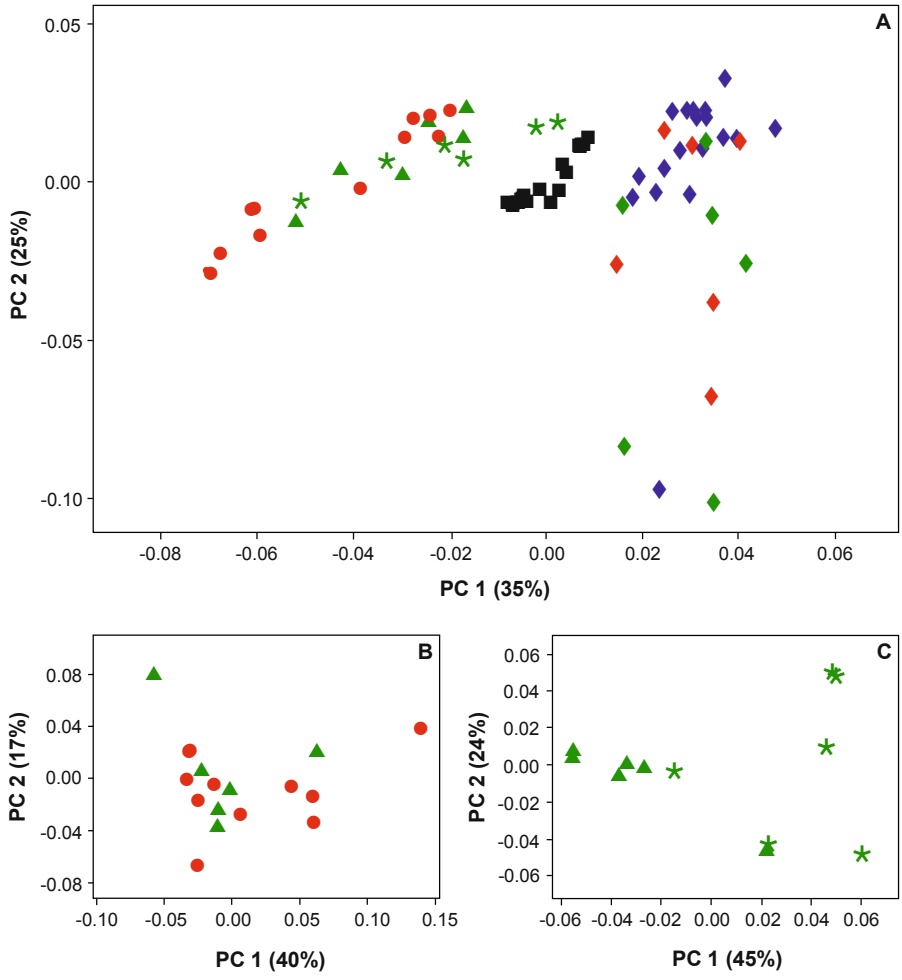

Figure 6.2 PCA score plots of metabolic urinary profiles obtained with HILIC–MS. PCA was performed on all samples (A), on urine samples of rats continuously treated with antibiotics for four and eight days (B), and on urine samples at day 8 after four and eight days of antibiotics administration (C). Legend: squares, QC samples; diamonds, control rats; dots, urine samples after four days of continuous treatment; stars, rats at day 8 after four days of treatment; triangles, rats after 8 days of continuous antibiotic treatment. Colors indicate the different days of the study: blue, day 0; red, day 4; green, day 8.

Based on the obtained accurate masses, thirteen compounds were putatively identified (see Table 6.1), including penicillin G, one of the administered antibiotics. Two compounds were identified as hippuric acid and indoxyl sulfate, which have been found before to be urinary mammalian-microbial co-metabolites since their production is dependent on microorganisms present in the gastrointestinal tract [8, 9, 11, 12, 14, 15, 26-28]. Phenol (m/z 93.04) and aconitic acid (m/z 172.99) were also previously observed to be down-regulated in urine upon oral antibiotic treatment [9, 14]. The compound with m/z 188.99 is most probably a sulfate conjugate of benzenediol which can be present in three isomeric forms, viz. catechol (*ortho*-dihydroxybenzene), resorcinol (*meta*-dihydroxybenzene) and hydroquinone (*para*-dihydroxybenzene). Benzenediol can be derived from dietary intake, but it can also be generated by the degradation of tyrosine and some other compounds by gut bacteria [22, 29]. Therefore, depletion of the gut microbiota can result in a decreased urinary concentration of benzenediol. Since the various isomeric forms cannot be distinguished based on mass only, the question remains which isomeric form or which forms are decreased after antibiotic treatment. Constructing an extracted-ion chromatogram of m/z 188.99 showed two closely yet baseline separated eluting compounds, of which one was decreased. Most probably, the benzenediols are at least partly separated and one or more could possibly be excluded as being discriminatory by comparing the obtained retention times with retention times after the analysis of standard solutions of reference compounds. The decrease in the urinary concentration of benzenediols after the administration of antibiotics was also observed by Zheng *et al.* using GC–MS [9] and by Sun *et al.* using RPLC–MS [15].

Other putatively identified compounds were ferulic acid, ferulic acid sulfate and vanillin 4-sulfate. Ferulic acid might be a fragment of ferulic acid sulfate arising from the ESI process, since the retention times of these two compounds are identical. Ferulic acid is a food derived compound which can be metabolized into vanillin by various bacterial strains [30, 31]. In the present study, the concentration of ferulic acid sulfate was increased, whereas the abundance of vanillin 4-sulfate in urine was decreased upon antibiotic treatment. These results suggest that the conversion of ferulic acid sulfate was partially inhibited by the depletion of the gut microbiota.

The other discriminatory compounds could not be tentatively identified based on recorded masses as a metabolomics database-search resulted in multiple hits. The m/z values 132.05 and 165.06 and the m/z values 163.04 and 243.00 have identical retention times (see Table 6.1) and peak shapes, as observed for ferulic acid and ferulic acid sulfate, and therefore are most probably adducts or fragments. In

contrast, the *m/z* values 187.04 and 353.10 exhibit the same retention time, but have different peak shapes. Therefore, these molecular features are likely to originate from different compounds.

Table 6.1 Down-regulated (-) and up-regulated (+) compounds upon antibiotic treatment for four and eight days

m/z	Retention time (min)	+/-	Putative identity
188.986	1.08	-	benzenediol sulfate
172.992[a]	1.13	-	aconitic acid
93.040[a]	1.14	-	phenol
132.047[a]	1.23	-	indoxyl
212.001[ab]	1.23	-	indoxyl sulfate
446.990[a]	1.23	-	
242.011	1.45	-	
227.997	1.51	-	
187.037	2.92	-	
353.101	2.92	-	
132.047	4.59	-	
165.055	4.59	-	
245.013[a]	4.59	-	vanillin 4-sulfate
134.062[a]	4.61	-	fragment hippuric acid
178.050[ab]	4.61	-	hippuric acid
266.994	4.62	-	sodium adduct vanillin 4-sulfate
379.093[a]	4.65	-	
119.052	5.26	-	
285.169	5.58	-	
326.089[a]	9.11	-	
176.986	1.13	+	
192.048	2.40	+	fragment penicillin G
333.093	2.40	+	penicillin G
200.128	2.57	+	
163.039	4.87	+	
242.996	4.87	+	
193.050	5.17	+	ferulic acid
273.008	5.17	+	ferulic acid sulfate
307.091	6.68	+	

[a] also observed with CE–MS

[b] also observed with ^{1}H NMR spectroscopy

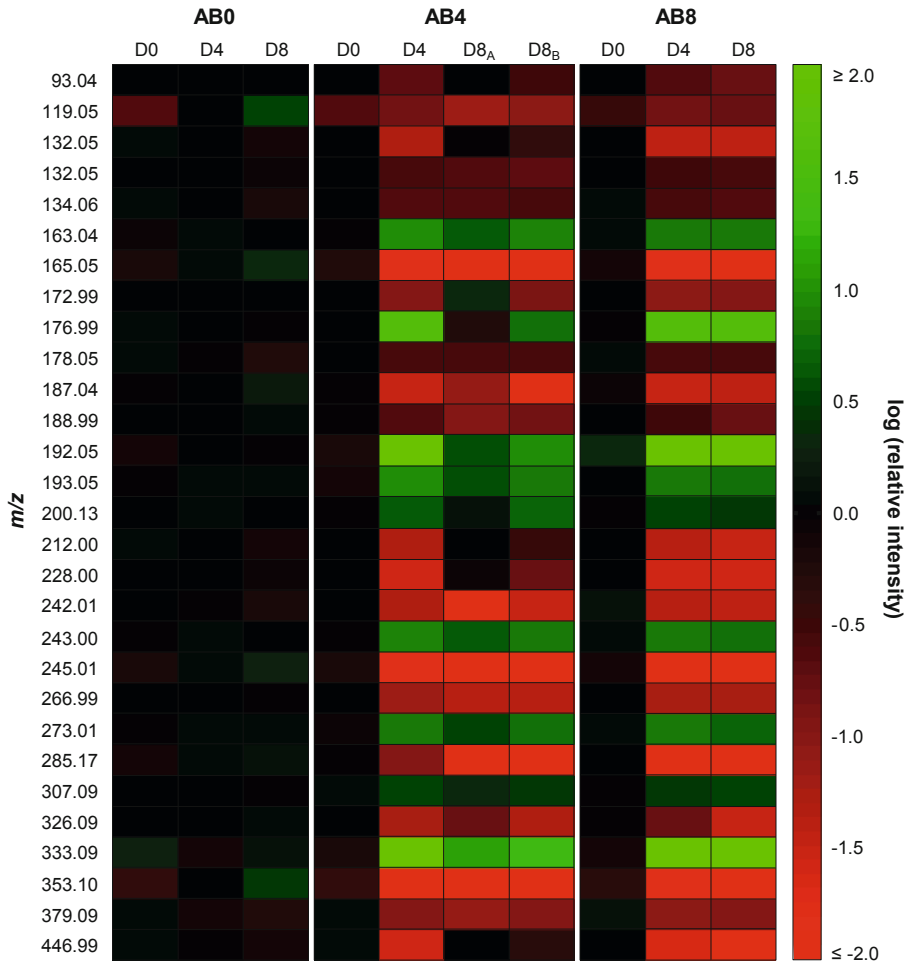

Figure 6.3 Heat map of average peak areas of discriminatory compounds in urine from rats receiving 0 (AB0), 4 (AB4) or 8 (AB8) days of antibiotic treatment. Peak areas relative to the average peak area observed in urine from untreated rats are depicted using a logarithmic scale and a color scheme.

6.3.3 Comparison with CE–MS

The rat urine samples have previously been analyzed with CE–MS. Treated and control rats could be distinguished based on seventeen discriminatory compounds [14]. Ten of these compounds were also found with HILIC–MS to be down-regulated after the administration of antibiotics (Table 6.1). The observed variation in abundance in time of these common compounds upon antibiotic treatment was compared quantitatively. Therefore, the obtained peak intensities in both

HILIC–MS and CE–MS of the respective compounds at day 0 were set at 100% for each individual rat. Relative peak areas were calculated for the corresponding rat urine samples collected at day 4 and 8. The average of these relative metabolite decreases and respective standard deviations were calculated. The values for hippuric acid (*m/z* 178.05) and indoxyl sulfate (*m/z* 212.00) are depicted in Figure 6.4. In general, the relative peak intensities obtained with HILIC–MS and CE–MS are similar. The relative peak areas for hippuric acid at day 8 after four days of antibiotic treatment were lower when analyzed with CE–MS compared to HILIC–MS. However, this difference was not statistically significant ($p>0.05$). Large but similar RSDs were found with HILIC–MS and CE–MS for indoxyl sulfate at day 8, because of the two observed subgroups (see above).

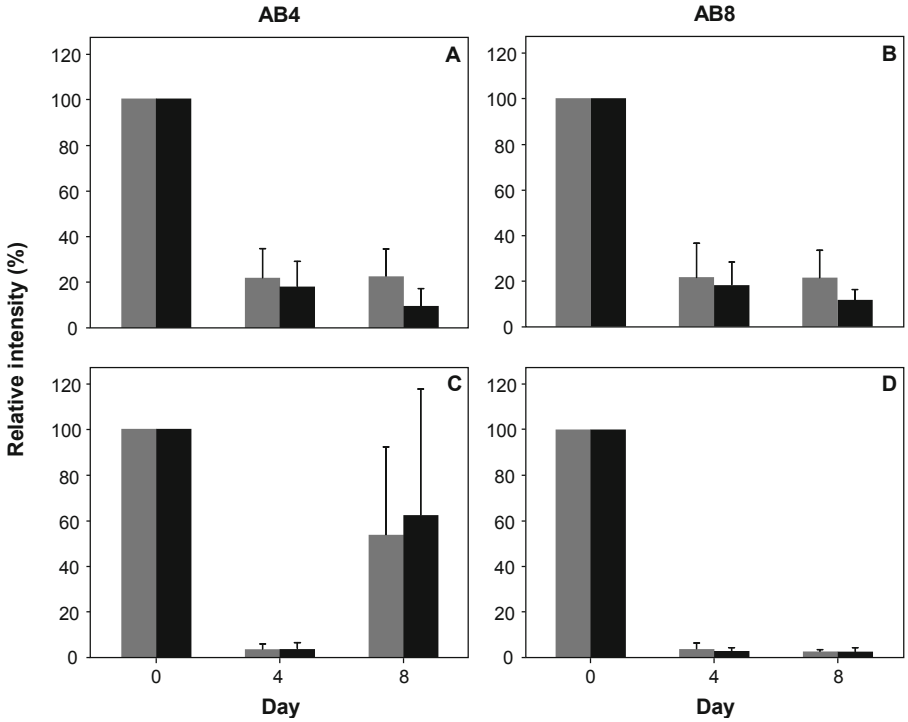

Figure 6.4 Relative peak intensities of the discriminatory compounds *m/z* 178.05 (A-B) and *m/z* 212.00 (C-D) obtained with HILIC–MS (grey bars) and CE–MS (black bars) in urine of rats treated for 4 (AB4) or 8 (AB8) days with antibiotics. Peak areas of the discriminatory compounds observed at day 0 were set at 100% for each individual rat urine sample. Relative intensities were determined for the corresponding samples taken at day 4 and 8 of the study, and averages (n=6 each) and standard deviations were calculated.

The detection of twenty-nine discriminatory compounds with HILIC–MS of which ten were also found with CE–MS implies that nineteen of these compounds were unique to the HILIC–MS acquired profiles. This included the compounds putatively identified as benzenediol sulfate, (fragment of) penicillin G, ferulic acid and ferulic acid sulfate, which are negatively charged at high pH and should theoretically be detectable in CE–MS. CE–MS also indicated seven compounds that were not detected with HILIC–MS. These seven compounds include five down-regulated compounds (m/z 285.06, 401.18, 405.25, 417.12 and 462.97) and two up-regulated compounds (m/z 503.16 and 665.21). Three of the compounds were identified as an oxocholic acid (m/z 405.25), a trisaccharide (m/z 503.16) and a tetrasaccharide (m/z 665.21). Extracted-ion chromatograms/electropherograms of m/z values uniquely detected with either HILIC–MS or CE–MS were constructed to determine whether the compounds were not detected with the other analytical technique or whether PCA did not reveal the compounds to be discriminatory. Remarkably, no significant peaks were found by the other technique for the seven and nineteen discriminatory compounds observed with CE–MS and HILIC–MS, respectively. This does not necessarily imply that the analytical techniques are not capable of measuring the respective compounds. In CE–MS, small and multiple charged anionic metabolites may not be observed within the applied detection window. In HILIC–MS, all compounds will elute by sufficiently increasing the percentage of water in the mobile phase. However, analyte signals may also suffer from matrix effects. For instance, neutral metabolites will in CE–MS migrate with the electroosmotic flow (EOF) with many other neutral compounds, which will affect their ESI efficiencies. Ion suppression due to co-elution can also occur in HILIC–MS. Next to matrix effects, ionization efficiencies between CE–MS and HILIC–MS can also differ due to different ionization conditions. The discriminatory compounds that were not detected with CE–MS showed relatively high peak intensities in HILIC–MS. These compounds eluted in the start of the chromatogram in a high percentage of acetonitrile. A high concentration of organic solvent can be beneficial for evaporation in the ESI process. In CE–MS applying a sheath-liquid interface, the sheath liquid which contributes most to the electrospray, has a constant composition throughout the analysis. Furthermore, formation of adducts and fragmentation of compounds can be different under the ionization conditions used in HILIC–MS and CE–MS, respectively. This can result in the detection of other molecular features and discriminatory compounds. Another reason for the observed differences between HILIC–MS and CE–MS is the difference in overall sensitivity, mainly caused by the difference in injection volume

(a factor 300). For example, this might be the reason why penicillin G is only detected with HILIC–MS. Penicillin G is expected to be absent or present in very low abundance in the rat urine samples, because its poorly absorbed from the gastrointestinal tract when orally administered. These results indicate that the HILIC–MS method is sufficiently sensitive to detect low concentrations of penicillin G, that were not visible with the applied CE–MS method.

6.3.4 Comparison with ^{1}H NMR spectroscopy

Using ^{1}H NMR spectroscopy, there was a clear differentiation in the urinary metabolic profiles of control and treated rats [8]. This was based on twenty-five compounds, of which eighteen were up-regulated after the administration of antibiotics. A comparison of these results with the HILIC–MS results is more complicated than a comparison between HILIC–MS and CE–MS since it can only be based on identity and not on observed masses. Two of the discriminatory compounds found with HILIC–MS, putatively identified as hippuric acid and indoxyl sulfate, were also found with ^{1}H NMR spectroscopy to be down-regulated upon antibiotic treatment. Also the microbially derived 3-hydroxyphenylpropionic acid (3-HPPA) and 4-HPPA were observed with ^{1}H NMR spectroscopy to be lowered after the administration of antibiotics [8]. Based on molecular weight, the m/z value 165.06 found with HILIC–MS could correspond to these compounds, however, other candidate metabolites share the same mass. Despite an accurate mass, unambiguous identification based on m/z only may not be possible. The number of possible identities could be decreased by addition of other structural information such as tandem MS data [32].

The other with HILIC–MS observed m/z values could not be related to discriminatory compounds found with ^{1}H NMR spectroscopy. With ^{1}H NMR spectroscopy many discriminatory compounds with a low molecular weight (<150 Da) were detected, whereas with HILIC–MS relatively more molecular features with higher masses were observed. So, HILIC–MS and ^{1}H NMR spectroscopy were found to be highly complementary.

6.4 Concluding remarks

HILIC–MS in negative ionization mode for the analysis of anionic metabolites is capable of discriminating metabolite profiles obtained from control and antibiotic-treated rats. Using HILIC–MS, the concentration of twenty-nine compounds were found to be altered upon the administration of antibiotics, of which thirteen could be putatively identified. Previously, a CE–MS and ^{1}H NMR spectroscopy study on

the same rat urine sample set revealed seventeen and twenty-five discriminatory compounds, respectively. Of these, hippuric acid and indoxyl sulfate, known to be microbial-mammalian co-metabolites, were observed with all three analytical techniques. Furthermore, eight other compounds were found with both HILIC–MS and CE–MS. The combined use of HILIC–MS and ^{1}H NMR spectroscopy provides the largest number of discriminatory compounds with a narrow window of metabolic overlap. The additional application of CE–MS in negative mode extends the biochemical resolution of the metabolome, providing a further dimension of metabolic information regarding highly polar and negatively charged compounds. The number of detected metabolites might even be further increased by applying HILIC–MS and CE–MS in positive ionization mode or by using another analytical technique like RPLC–MS. In conclusion, the applied analytical techniques are highly complementary and therefore a multiplatform approach with HILIC–MS, CE–MS and ^{1}H NMR spectroscopy will reveal more discriminatory compounds in metabolic profiling studies, thereby enhancing the coverage of the anionic metabolome and eventually the unraveling of biochemical pathways.

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VII

Feasibility of capillary electrophoresis–mass spectrometry and hydrophilic interaction chromatography–mass spectrometry for the profiling of urinary anionic metabolites involved in inborn errors of metabolism

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In preparation

Abstract

Capillary electrophoresis (CE) and hydrophilic interaction chromatography (HILIC) coupled to mass spectrometry (MS) were used for anionic metabolic profiling of urine from patients suffering from inborn errors of metabolism. The goal was to test the capability of CE–MS and HILIC–MS in negative mode for revealing known and new potential biomarkers by comparison of urine from patients with urine from controls. The examined metabolic disorders were citrullinemia, medium chain acyl-coenzyme A dehydrogenase (MCAD) deficiency and 3-methylcrotonyl-coenzyme A carboxylase (MCC) deficiency. Principal component analysis (PCA) on urinary metabolic profiles obtained with CE–MS and HILIC–MS revealed a distinction between patients and controls. Concentrations of various compounds were significantly higher (factor 2 to >100) or lower (factor 2 to 50) in the urine samples of patients compared to the control samples. The accurate masses of a few discriminatory compounds matched the masses of some known biomarkers for the different inborn errors of metabolism. Concentrations of citrulline and orotic acid were indeed found with both CE–MS and HILIC–MS to be altered in patients with citrullinemia. Two known biomarkers for MCAD deficiency (hexanoylglycine and suberylglycine) were indicated by PCA of the HILIC–MS data, as were 3-methylcrotonylglycine and 3-hydroxyisovaleric acid in the case of MCC deficiency. In order to identify unknown discriminatory compounds, HILIC–MS/MS was performed on the MCAD deficiency samples. Different collision energies were employed to optimize the metabolite fragmentation. Based on the obtained MS/MS data, four discriminatory compounds could be assigned to asparagine-valine, glutamine, phosphotyrosine and saccharine. In addition, the identity of the earlier assigned hexanoylglycine and suberylglycine could be confirmed. In conclusion, untargeted CE–MS and HILIC–MS appears useful for characterizing anionic metabolites in urine which are relevant to metabolic disorders.

7.1 Introduction

Inborn errors of metabolism comprise a group of disorders caused by an inherited defect in genes that code for enzymes responsible for the conversion of metabolites. The defect results in a disruption or an abnormality in specific metabolic pathways and is characterized by the accumulation or deficiency of one or more metabolites. The inherited metabolic diseases can be subdivided in disorders affecting amino acid metabolism, carbohydrate metabolism, organic acid metabolism, lysosomal storage, the urea cycle and the oxidation of fatty acids. Diagnosis of these diseases is made by analyzing one or more affected metabolites in various matrices. Applied analytical methodologies, include immunoassays, thin layer chromatography, gas chromatography (GC), liquid chromatography (LC) and, more recently, tandem mass spectrometry (MS). In addition, affected genes and enzymes are examined [1].

A large number of metabolites is highly polar and ionogenic, and therefore, capillary electrophoresis (CE) and hydrophilic interaction chromatography (HILIC) might be eminently suited for the analysis of many relevant metabolites in inborn errors of metabolism. In CE, analytes are separated based on their charge-to-size ratios [2]. In HILIC, compounds are separated based on a combination of partitioning and electrostatic interactions with a polar stationary phase [3-5]. CE and HILIC can be readily hyphenated to MS for the selective detection and identification of the metabolites of interest.

An essential part of human metabolites are anionogenic and can only be analyzed efficiently using MS in negative ionization mode. Recently, we have developed a CE–MS method for untargeted analysis of anionic metabolites. We have demonstrated its usefulness for anionic metabolic profiling of urine and compared it with an optimized HILIC–MS method [6-8]. In the present study, we investigated whether these untargeted methodologies are capable of probing established biomarkers of inborn errors of metabolism. It is also interesting to see if the untargeted approaches can reveal additional compounds of which the concentrations are significantly decreased or increased due to metabolic diseases. If successful, ideally one could envisage a multipurpose method capable of characterizing and diagnosing multiple errors of metabolism, thereby also saving money and time.

The goal of this study was to evaluate the feasibility of CE–MS and HILIC–MS using negative ionization mode for the detection of known and new potential biomarkers of metabolic diseases. For this purpose, samples of patients of three inherited metabolic disorders were selected: citrullinemia [9-11], medium chain acyl-coenzyme A dehydrogenase (MCAD) deficiency [12-14] and 3-methylcrotonyl-coenzyme A carboxylase (MCC) deficiency [15, 16]. These

represent disorders of the amino acid metabolism, the urea cycle and the fatty acid oxidation. Urine samples were taken from three patients of each respective disorder. In addition, urine samples from controls were collected. Previously optimized CE–MS and HILIC–MS methods for anionic metabolites were used to analyze all urine samples [6, 8]. Following CE–MS and HILIC–MS analysis, the metabolic profiles were compared using multivariate data analysis. Compounds discriminating patient and control samples were assessed and differences in their concentration were calculated. The possibility to identify potentially new biomarkers was demonstrated for one inborn error of metabolism by HILIC–MS/MS.

7.2 Materials and methods

7.2.1 Chemicals

Ammonium hydroxide (25% solution), acetonitrile, formic acid and methanol were obtained from Fluka (Steinheim, Germany). Ammonium acetate was supplied by Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was from Sigma Aldrich (Steinheim, Germany) and triethylamine from Fisher Scientific (Loughborough, United Kingdom). Prior to use, water was deionized and purified with a Milli-Q purification system (Millipore, Bedford, USA).

7.2.2 Urine samples

Urine samples were collected from nine patients suffering from the inborn errors of metabolism citrullinemia, MCAD deficiency and MCC deficiency (n=3 each) after informed consent. Samples were stored at -80°C until analysis. Also control samples were analyzed, which were matched to the patient samples based on age and storage time. Prior to CE–MS analysis, urine samples were diluted with the background electrolyte (BGE) in a proportion of 1:1, v/v. For HILIC–MS, urine samples were mixed with acetonitrile in a proportion of 1:4, v/v and centrifuged at 10,000 rcf for 10 min. A mixture of all urine samples was employed as quality control (QC) sample. This QC sample was measured after every fifth run to determine the repeatability and stability of the CE–MS and HILIC–MS systems.

7.2.3 CE–MS

CE–MS was carried out on a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton, USA) coupled to a time-of-flight (TOF) mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) using a sheath-liquid electrospray interface from Agilent Technologies (Waldbronn, Germany). A BGE of

25 mM triethylamine (pH 11.7) and a sheath liquid of 5 mM triethylamine in water-methanol (1:1, v/v) were employed. Fused-silica capillaries (Polymicro Technologies, Phoenix, USA) had a total length of 100 cm and an internal diameter of 50 μm. Hydrodynamic injections were performed using a pressure of 0.5 psi for 30 s. The separation voltage was 30 kV and the capillary temperature was set at 20°C. The sheath liquid was delivered with a flow rate of 5 μL/min using a syringe pump (KD Scientific, Holliston, USA) and a 10 mL gas-tight syringe of Hamilton (Reno, USA). The following CE–MS interface conditions were applied: dry gas temperature, 180°C; dry gas flow, 4 L/min; nebulizer pressure, 10 psi; electrospray ionization (ESI) voltage, 4 kV. Data were acquired in negative ionization mode in the mass range 50 to 800 *m/z* with a repetition rate of 1 Hz. Sodium formate clusters were used for internal calibration.

7.2.4 HILIC–MS

All urine samples were analyzed on an LC system (Shimadzu, Kyoto, Japan) coupled to a micrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) via an electrospray interface from Agilent (Waldbronn, Germany). Separations were carried out on a Waters XBridge™ Amide column (3.5 μm, 3.0 x 100 mm) and the column temperature was set at 45°C.

Urine samples (5 μL) were analyzed under gradient elution with solvent A containing 10 mM ammonium acetate (pH 6.8) in water-acetonitrile (1:1, v/v) and solvent B containing 10 mM ammonium acetate (pH 6.8) in water-acetonitrile (1:9, v/v). The gradient was as follows: 0-5 min, 100% B; 5-15 min, from 100% B to 100% A; 15-20 min, 100% A. Thereafter, the HILIC–MS system was switched back to 100% B and maintained for 10 min to allow for reequilibration of the system.

The mass spectrometer was operated in negative ionization mode using the following conditions: dry gas temperature, 180°C; dry gas flow, 4 L/min; nebulizer pressure, 50 psi; ESI voltage, 2 kV. Data were recorded in the mass range *m/z* 50-800 with an acquisition rate of 1 Hz. Internal calibration of recorded masses was applied using detected sodium acetate clusters.

7.2.5 MS/MS experiments

The samples of the patients with MCAD deficiency and the matched control samples were analyzed with HILIC–MS/MS. The HILIC–MS/MS experiments were carried out using a LC system (Agilent Technologies) consisting of a G1379B vacuum degasser, a G1312B binary pump, a G1367C autosampler and a G1316B

thermostatted column compartment. The LC system was coupled to a 6460 triple quadrupole (Q) mass spectrometer (Agilent Technologies), which was operated in negative mode. LC conditions were as described above (Section 7.2.4). The MS conditions were as follows: dry gas temperature, 180°C; dry gas flow, 4 L/min; nebulizer pressure, 50 psi; ESI voltage, 2 kV; collision energy, 10, 20, 30 and 40 eV. Data were recorded using the Agilent MassHunter Workstation software.

7.2.6 Data analysis

The metabolite profiles obtained with CE–MS were aligned based on a reference sample using the reference peak warping function of the MsXelerator software (MsMetrix, Maarssen). Peak picking was performed on metabolite profiles obtained with CE–MS and HILIC–MS to determine the molecular features present in the QC sample with a signal-to-noise (S/N) ratio of at least three for ten consecutive spectra. Spikes were removed and only monoisotopic peaks were used for further analysis. Peak matching was carried out to determine common compounds present in the various patient and control samples. Urinary compounds were considered common with a mass difference of maximum 0.01 Da and a migration or retention time difference of lower than 0.1 min. Normalization of peak areas was based on the total peak area obtained in the different electropherograms/chromatograms to correct for differences in individual urine volumes. Principal component analysis (PCA) was applied using the normalized peak areas to determine differences between patient and control samples. Independent samples t-tests were performed to assess differences in metabolite concentrations between patients and controls. A p-value ≤ 0.05 was considered to indicate a statistically significant difference. Furthermore, fold changes in concentrations of these discriminatory compounds were calculated using the average normalized peak areas observed in patients and controls. Compounds that showed differences were putatively identified. The obtained accurate masses were compared to masses recorded in the online databases HMDB, METLIN and Massbank. The observed fragments using HILIC–MS/MS were interpreted and obtained MS/MS spectra were compared with available MS/MS spectra in online databases [17-19].

7.3 Results and discussion

7.3.1 Systems performance and PCA

The applied CE–MS method employed a BGE and sheath liquid containing triethylamine for optimum metabolite responses in negative mode. HILIC–MS

involved gradient elution employing a mobile phase containing 10 mM ammonium acetate (pH 6.8) and acetonitrile. These systems have shown be reproducible and suitable for anionic metabolic profiling studies [6-8, 20].

Analysis of the QC sample by CE–MS and HILIC–MS resulted in the detection of approximately 220 and 700 molecular features within 20 and 15 min, respectively (Figure 7.1). The stability of the CE–MS and HILIC–MS systems was assessed based on the electropherograms and chromatograms obtained when repetitively analyzing the QC sample. Six molecular features were selected spread across the peak profiles, and relative standard deviations (RSDs) of retention time and peak area were calculated.

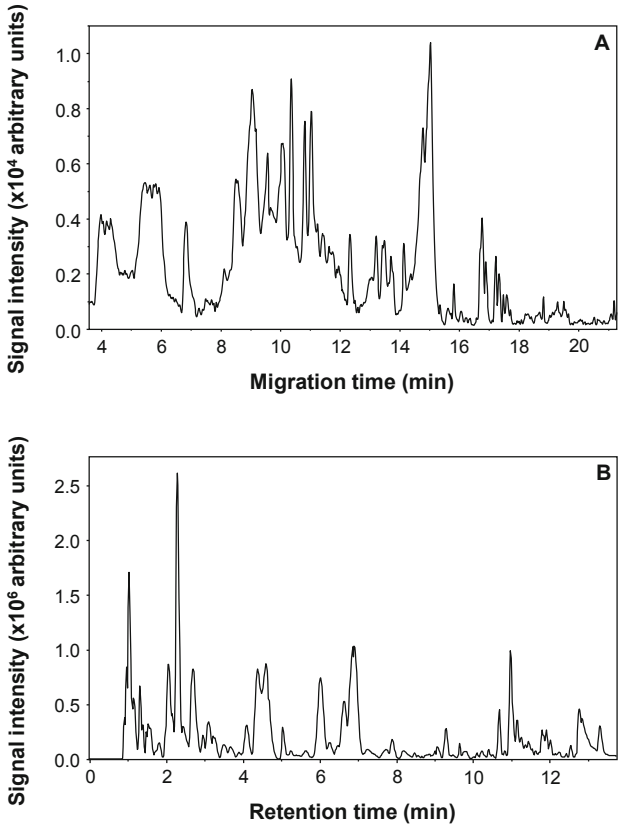

Figure 7.1 Typical base-peak electropherogram (A) and chromatogram (B) obtained with CE–MS and HILIC–MS, respectively, for a mixture of urine samples from patients suffering from an inborn error of metabolism and matched controls (QC sample).

Before alignment, RSDs for CE migration times and HILIC retention times were below 5.4% and 0.5%, respectively. RSDs for peak area were lower than 12.1% and 7.9% for CE–MS and HILIC–MS, respectively. The metabolic profiles from all urine samples of patients and controls were acquired using CE–MS and HILIC–MS, and the obtained data were used for further analysis.

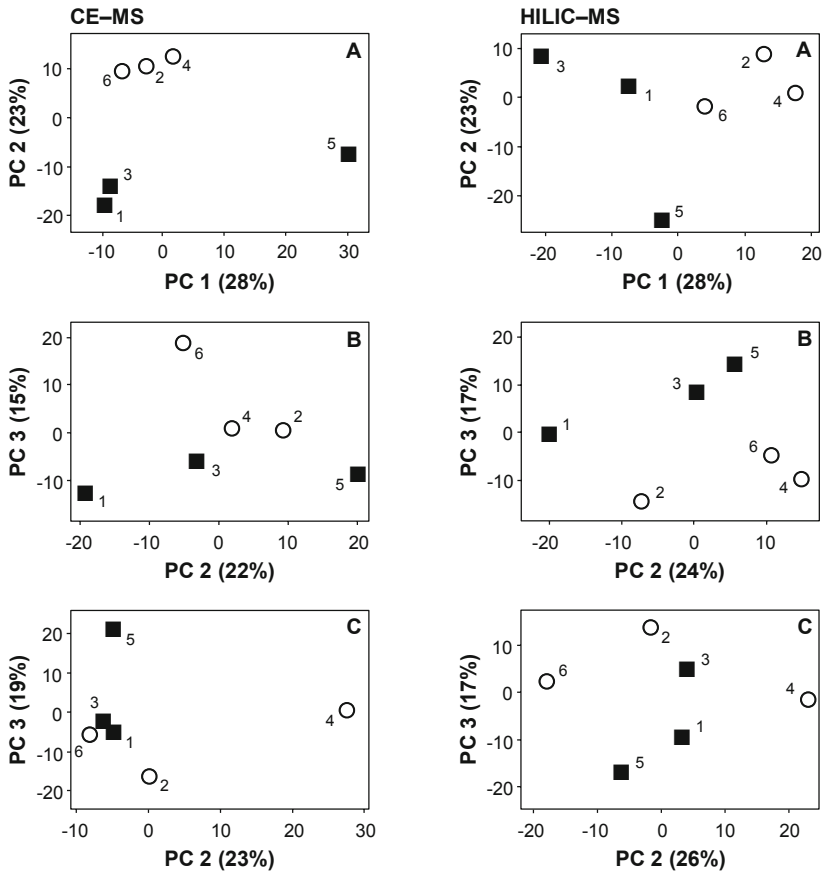

Figure 7.2 PCA score plots of metabolic urinary profiles of patients with an inborn error of metabolism (squares 1, 3 and 5) and matched controls (circles 2, 4 and 6) obtained with CE–MS and HILIC–MS. The studied inborn errors of metabolism are (A) citrullinemia, (B) MCAD deficiency and (C) MCC deficiency.

In order to correct for variation in individual urine volumes, peak areas of detected features were normalized against the total peak area obtained in the respective CE–MS electropherogram and HILIC–MS chromatogram. Tables containing m/z values and normalized peak areas of each detected molecular feature were constructed and used for PCA. In overall PCA score plots using metabolite profiles of all samples analyzed with CE–MS and HILIC–MS, the repetitive analyses of the QC sample clearly clustered in the center of the score plots, demonstrating the stability of the analytical systems.

To explore more detailed differences between urine samples of patients and controls, PCA was performed on CE–MS and HILIC–MS data obtained for each inborn errors of metabolism (Figure 7.2). Although the number of samples was limited, patient and control samples could be discerned in the PCA plots obtained for both CE–MS and HILIC–MS. The discrimination between controls and patients suffering from citrullinemia seems to be better in the PCA plots obtained with CE–MS data (Figure 7.2A), whereas patients with MCAD deficiency might be better distinguished based on HILIC–MS data (Figure 7.2B). The PCA score plots of CE–MS and HILIC–MS metabolite profiles of urine from MCC deficiency patients and from controls were similar (Figure 7.2C).

7.3.2 Discriminatory compounds

PCA provided various discriminatory compounds by which patient samples were discerned from control samples. Extracted-ion electropherograms and chromatograms of each discriminatory compound were constructed. For each inborn error of metabolism, different discriminatory compounds were found by CE–MS and HILIC–MS (Tables 7.1-7.3). Peak areas corresponding to these urinary compounds were significantly higher or lower ($p \leq 0.05$) in patient samples compared to the control samples. Fold differences in urinary concentrations of the discriminatory compounds ranged from 2 to even more than 100, indicating a very low abundant or absent compound in the patient or control samples.

The number of revealed discriminatory compounds was similar for CE–MS and HILIC–MS, whereas with HILIC–MS more than three times as many molecular features were found. The diagnosis of the metabolic disorders is normally based on altered concentrations of one or more specific metabolites. The m/z values of the discriminatory compounds found by PCA were compared with the masses of the metabolites known to be present with an altered concentration in the respective disorders. Furthermore, extracted-ion electropherograms and chromatograms for masses of these known metabolites were made.

Table 7.1 Discriminatory compounds of which the concentrations were found to be higher or lower ($p \leq 0.05$) in urine of patients with citrullinemia as compared to matched controls

Higher concentrated (m/z)		**Lower concentrated (m/z)**	
CE–MS	*HILIC–MS*	*CE–MS*	*HILIC–MS*
131.08	132.06	107.04	107.05
136.17	155.02	119.04	167.03
141.01	174.10	121.02	187.01
155.02	175.07	148.31	214.05
173.05	188.10	157.11	222.10
173.09	230.11	187.07	303.07
174.10	374.12	212.00	324.07
216.10	390.16	305.45	346.05
366.37	455.19	358.62	352.09
371.16	632.15		355.04
552.68	694.20		372.07
571.59			394.13
573.43			541.21
628.92			569.13

Citrullinemia is typically characterized by an increased level of citrulline and a down-regulated concentration of ornithine. Elevated concentrations of glutamine and orotic acid have also been found in patients with citrullinemia [11]. In the present study, concentrations of twenty-three and twenty-five compounds were found to differ between patients and controls as observed with CE–MS and HILIC–MS, respectively (Table 7.1). Compounds with m/z values 155.02 and 174.10, corresponding to masses of orotic acid and citrulline, respectively, were found with both CE–MS and HILIC–MS. The normalized peak areas obtained when analyzing urine samples of patients were more than 100 times higher compared to peak abundances in control samples. These factors are in accordance with concentration differences normally observed between controls and patients with citrullinemia.

In MCAD deficiency, the break-down of medium-chain fatty acids into acetyl-coenzyme A is impaired resulting in the accumulation of medium-chain fatty acids, glycine esters, carnitine esters and dicarboxylic acids. Furthermore, plasma glucose levels may drop substantially [12-14]. Three discriminatory compounds found with HILIC–MS (Table 7.2) having m/z 145.06, 172.10 and 230.10 correspond to masses of adipic acid, hexanoylglycine and suberylglycine, respectively, which are known to be biomarkers for MCAD deficiency. The urinary concentrations of these

biomarkers were on average respectively 6, 23 and 53 times higher in patients compared to controls. These fold changes are consistent with concentration differences found with conventional analytical methodologies. CE–MS revealed six discriminatory compounds, which could not be related to metabolites known to be altered in MCAD deficiency. The peak areas of two compounds were 8 and 26 times higher, whereas the peak areas of the other four compounds were significantly lower in urine samples of patients (factor 2-4). Using extracted-ion electropherograms, a peak of m/z 172.10 (hexanoylglycine) with a relatively low intensity was observed in urine of patients and not detected in the urine of the matched controls. This peak remained unnoticed during the applied peak picking procedure (see Section 7.2.6), due to its low signal intensity. Carnitine esters were not among the observed discriminating compounds found with CE–MS and HILIC–MS in negative ionization mode. Carnitine esters require positive ESI for optimum detection.

Table 7.2 Discriminatory compounds of which the concentrations were found to be higher or lower ($p \leq 0.05$) in urine of patients with MCAD deficiency as compared to matched controls

Higher concentrated (m/z)		**Lower concentrated (m/z)**	
CE–MS	*HILIC–MS*	*CE–MS*	*HILIC–MS*
141.08	145.06	323.15	184.00
181.13	172.10	358.62	230.11
	181.99	399.16	258.05
	230.10	548.84	260.02
	243.13		289.04
	447.00		305.06
	507.25		399.17

Table 7.3 Discriminatory compounds of which the concentrations were found to be higher or lower ($p \leq 0.05$) in urine of patients with MCC deficiency as compared to matched controls

Higher concentrated (m/z)		**Lower concentrated (m/z)**	
CE–MS	*HILIC–MS*	*CE–MS*	*HILIC–MS*
355.75	117.06	150.01	145.06
520.14	156.07	157.01	197.07
		176.04	302.11
		190.01	360.08
		212.00	
		352.09	

In MCC deficiency, elevated abundances of the metabolites 3-methylcrotonylglycine, 3-hydroxyisovaleric acid and 3-hydroxyisovalerylcarnitine have been found [15, 16]. CE–MS revealed elevated abundances of two compounds in patients compared to controls. The difference in peak area was relatively low (factor 2-3). In addition, concentrations of six compounds were a factor 3-14 lower in patients compared to controls (Table 7.3). The *m/z* values of these compounds do not correspond to the three metabolites known to be up-regulated in patients with MCC deficiency. Construction of extracted-ion electropherograms of *m/z* values of these three metabolites revealed the detection of a compound with *m/z* 156.06 in the urine of patients, corresponding to 3-methylcrotonylglycine, whereas this compound was not observed in the urine samples of the matched controls. However, the peak intensities of this *m/z* value were not high enough to be selected in the peak picking procedure. Using HILIC–MS, six urinary compounds were found to be discriminatory (Table 7.3). Higher concentrations of *m/z* 117.06 and 156.07 were present in patients suffering from MCC deficiency. Peak areas for these compounds were 11 and 29 times higher, respectively. Based on accurate mass, these compounds were putatively identified as 3-hydroxyisovaleric acid and 3-methylcrotonylglycine, respectively. Extracted-ion chromatograms of *m/z* 261.17 (3-hydroxyisovalerylcarnitine) revealed multiple peaks, however, no differences in peak abundances were found between patient and control samples.

7.3.3 HILIC–MS/MS

Untargeted metabolic profiling of urine samples of patients with an inborn error of metabolism resulted in several discriminatory compounds. Among these differentiating compounds, some known biomarkers for the various inherited diseases were found, however, other compounds were observed as well of which the concentrations in patient and control samples differed significantly. In order to explore the potential role of these compounds in biochemical pathways, their identification is indicated. Therefore, as a proof-of-principle, HILIC–MS/MS experiments were carried out using a triple Q mass spectrometer, taking the samples of patients with MCAD deficiency, the most common of the three studied inborn errors of metabolism, as test case.

Several collision energies (10, 20, 30 and 40 eV) were intermittently applied during HILIC–MS/MS resulting in various degrees of fragmentation of the probed metabolites. The observed fragments and proposed identities of the metabolites are shown in Table 7.4. As examples, the obtained fragmentation patterns of *m/z* 230

(in HILIC–TOF-MS observed as m/z 230.10) and m/z 258 (m/z 258.05 in HILIC–TOF-MS) are shown in Figure 7.3.

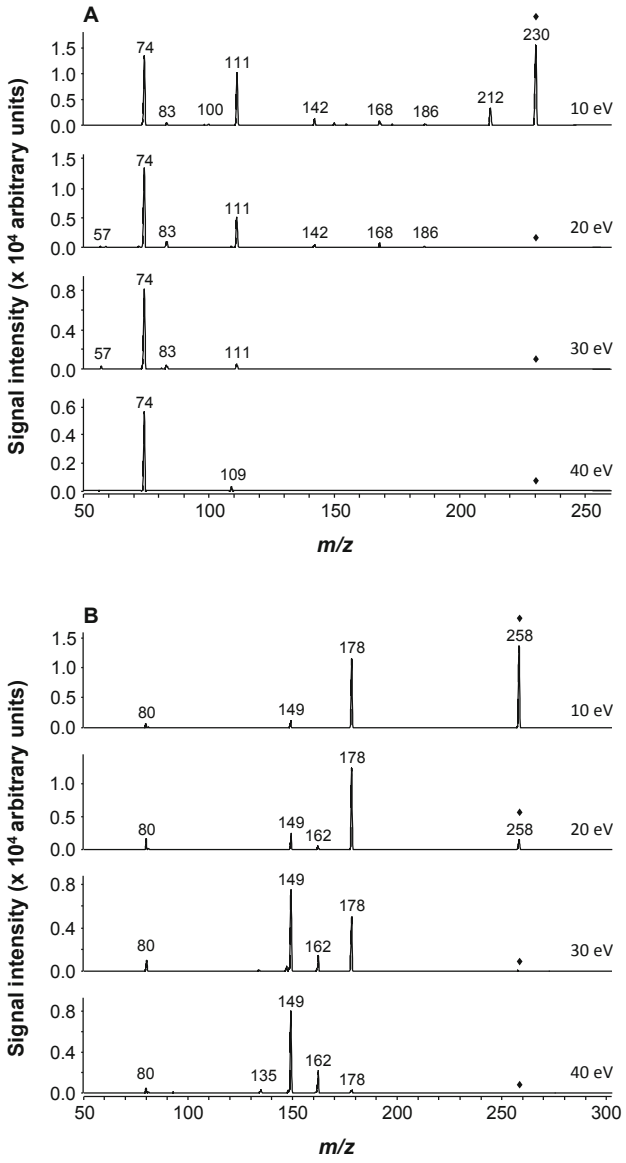

Figure 7.3 Spectra of two compounds observed with HILIC–MS/MS, of which the concentrations were up-regulated (m/z 230.10, A) and down-regulated (m/z 258.05, B) in patients suffering from MCAD deficiency. Collision energies of 10, 20, 30 and 40 eV were applied to obtain as many fragments as possible enabling identification of the compounds.

The largest number of fragments of m/z 230 was obtained employing a collision energy of only 10 eV. In contrast, a higher energy is required for m/z 258 to induce as many fragments as possible. Increasing the collision energy to 20 and 40 eV resulted in the detection of fragments with m/z values of 135 and 162. More fragments increase the possibility to identify compounds of interest. If only one fixed collision energy is used, essential structural information might be missed. Furthermore, the detection of the same fragments at various collision energies increases the reliability of the analysis.

Two discriminatory compounds were found with HILIC–MS having similar masses (m/z 230.10 and 230.11). Due to limited mass resolution, these masses cannot be distinguished in a triple Q mass spectrometer. However, these compounds exhibit a substantial difference in retention time and MS/MS spectra (Table 7.4). Based on the observed fragments, the compounds were identified as suberylglycine (m/z 230.10) and a dipeptide of asparagine and valine (m/z 230.11). Suberylglycine is a known biomarker for MCAD deficiency, whereas asparagine-valine has not found before to be altered in MCAD deficiency.

Table 7.4 Mass fragments and proposed identities of compounds discriminating between patients with MCAD deficiency and matched controls

m/z	Mass fragments	Proposed identity
145	58; 74; 84; 109; 127	glutamine
172	74; 128	hexanoylglycine
182	62; 106	saccharine
230	57; 74; 83; 111; 142; 168; 186; 212	suberylglycine
230	145; 171; 187; 213	asparagine-valine
258	80; 135; 149; 162; 178	amino sugar phosphate
260	74; 80; 93; 106; 119; 135; 163; 180; 199; 215	phosphotyrosine

The compound with m/z 258.05 could be assigned to an amino sugar phosphate. Unfortunately, the exact identity could not be identified despite the MS/MS information. Possible identities are glucosamine-phosphate, galactosamine-phosphate, aminofructose-phosphate and kanosamine-phosphate. However, this type of metabolites is normally not present or detected in urine, and the relevance of these findings needs to be investigated. The identity could be further confirmed by analyzing reference samples of these metabolites with the same system. The compound with m/z 260.02 showed a fragment with a mass of 80, indicating a phosphate group, and was identified as phosphotyrosine. In contrast to the amino

sugar phosphates, phosphotyrosine has been previously detected in urine, but altered urinary concentrations have not yet been associated with MCAD deficiency. Hexanoylglycine (m/z 172.10) and saccharine (m/z 181.99) could also be identified based on MS/MS detection. Hexanoylglycine was already putatively identified based on its accurate mass and is commonly observed as a marking metabolite in MCAD deficiency. The m/z value 145.06 was tentatively identified as adipic acid, which is also a biomarker of MCAD deficiency. However, this was not confirmed by the results of HILIC–MS/MS. The appearance of saccharine is not expected since it is a food additive and not an endogenous metabolite. Coincidentally, patients could have ingested food products containing more saccharine compared to the controls. The discriminatory compounds with m/z value 243.13 and 289.04 could not be identified based on obtained spectra. Also the other discriminatory compounds observed with HILIC–MS could not be assigned, because good-quality MS/MS spectra were not obtained.

7.4 Concluding remarks

Previously optimized CE–MS and HILIC–MS methods were applied for the analysis of urine samples from patients with inborn errors of metabolism and matched controls. Based on PCA of the CE–MS and HILIC–MS data, patient samples could be distinguished from control samples for all studied metabolic diseases, demonstrating the capability of CE–MS and HILIC–MS for the characterization and diagnosis of multiple errors of metabolism. A similar number of discriminatory compounds was found with CE–MS and HILIC–MS, whereas HILIC–MS provided substantially more molecular features. Based on accurate mass, various discriminatory compounds could be related to metabolites known to be down- or up-regulated in the different disorders. Not all known biomarkers of the inborn errors of metabolism were found with the CE–MS and HILIC–MS methods. Almost all biomarkers of the studied inborn errors of metabolism have acidic groups and are expected to be detectable using negative ionization mode. Since CE–MS and HILIC–MS conditions were not optimized for specific metabolites, ionization efficiencies might be suboptimal for the undetected biomarkers of the studied metabolic diseases. Besides the known biomarkers of the inborn errors of metabolism, untargeted analysis of urine samples from patients and controls using CE–MS and HILIC–MS also revealed other discriminatory compounds. MS/MS experiments can be performed to identify unknown discriminatory compounds, as shown in this study with HILIC–MS/MS. The biological relevance of identified compounds still needs to be investigated.

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VIII

Conclusions and future perspectives

In this thesis, capillary electrophoresis–mass spectrometry (CE–MS) and hydrophilic interaction chromatography–mass spectrometry (HILIC–MS) methods have been developed and optimized for the analysis of anionic metabolites. The characteristics of the methods have been compared and their utility for anionic metabolic profiling of urine has been evaluated. Particular attention was given to sensitivity, reproducibility, separation selectivity and metabolite coverage. Based on the data obtained and the practical experience gained, in this final chapter, several particular aspects regarding the performance and applicability of CE–MS and HILIC–MS will be discussed in an attempt to draw some general conclusions. Perspectives and recommendations for further research will be provided as well.

8.1 CE–MS performance

At the start of the research project described in this thesis, CE–MS in negative ionization mode was mainly carried out using ammonium acetate as both background electrolyte (BGE) and sheath liquid additive. Metabolite responses in negative ionization mode were often relatively low, resulting in poor sensitivity in metabolomics applications. Enhancement of metabolite responses in CE–MS using negative electrospray ionization (ESI) was required in order to bring CE–MS to a competitive level in anionic metabolic profiling. It was shown in *Chapter 3* that the use of triethylamine in the BGE and sheath liquid instead of ammonium acetate is an effective strategy to improve the limits of detection (LODs) of anionic metabolites significantly. The achieved signal enhancement allows detection of low-abundant metabolites in biological samples, which were missed with an earlier CE–MS method using ammonium acetate. Triethylamine appeared to be fully compatible with MS detection causing no adverse effects or source contamination.

A low reproducibility of analyte migration times is often mentioned as a drawback of CE–MS. Nevertheless, relative standard deviations (RSDs) for migration times below 1% were commonly achieved for aqueous standard solutions of metabolites when using a triethylamine-containing BGE. When analyzing biological samples, however, migration-time reproducibilities can become less favorable and too low to reliably compare electrophoretic metabolic profiles. As has been previously demonstrated, the use of capillary coatings of (multiple) layers of charged polymers could provide stable and repeatable analyses of metabolites in biological samples. However, such capillary coatings appeared to be incompatible with the triethylamine-containing BGE, probably due to the displacement of the polymer coating by triethylamine. Still, the migration-time variability observed during analysis of urine samples can be substantially and satisfactory diminished by

employing an appropriate alignment procedure, as was outlined in *Chapter 5*. In MS-based metabolic profiling studies (whether liquid chromatography (LC) or gas chromatography (GC)), time alignment is a general requirement. Hence, the modest migration-time stability of CE–MS does not lead to increased data handling time or reduced comparison performance.

In untargeted metabolomics studies, the coverage of metabolites present in biological samples should be as high as possible. The capacity to detect a large number of metabolite classes is of great importance for finding relevant compounds that can distinguish samples originating from various groups (e.g. healthy versus diseased). A large separation window and efficient ionization conditions are prerequisites for obtaining an extensive coverage of the metabolome. Depending on the overall charge (positive/negative) of the analytes and the polarity (normal/reversed) of the applied voltage, in CE, analytes migrate faster or slower than the electroosmotic flow (EOF). When using positively charged capillary coatings in combination with reversed polarity (as e.g. the group of Soga), the effective electrophoretic mobility of anionic compounds is in the same direction as the EOF, resulting in relatively short analysis times and a separation window of limited size. Furthermore, electrostatic interactions of negatively charged metabolites with the positively charged capillary wall may result in broadened and distorted peaks. Moreover, the reversal of the applied voltage can lead to corrosion of the steel needle of the co-axial sheath-liquid interface, potentially leading to current drops and capillary clogging. Basic CE theory indicates that optimum resolution and a large separation window for anionic metabolites is obtained when the effective mobility of the analytes is in opposite direction of the EOF. Enhanced analyte separation and a larger separation window will result in less co-migration of metabolites and matrix components, and thereby, in less analyte ionization suppression. Indeed, a large separation window for anionic metabolites was obtained using bare fused-silica (BFS) capillaries using normal polarity, and triethylamine in the BGE. As compared to the reversed-polarity system employing the positively charged coating and an ammonium acetate BGE, the urinary metabolite coverage was substantially increased by revealing more than twice as much molecular features. Furthermore, signal intensities of urinary metabolites were significantly higher using triethylamine. This improvement is the result of both more efficient analyte ionization and less ionization suppression due to less co-migration of compounds.

Another aspect adding to the overall CE–MS sensitivity is the minimal sample pretreatment required for urine samples. Just mixing with BGE in a proportion of

1:1 (v/v) is sufficient, preventing metabolite losses and minimizing sample dilution, thereby contributing to enhanced metabolite coverage.

8.2 HILIC–MS performance

In HILIC–MS, the attainable analyte responses – and thus sensitivity – depend on the composition of the mobile phase. The mobile phase employed in HILIC–MS contains most frequently acetonitrile. A high percentage acetonitrile is beneficial for an efficient ionization process. As gradient elution is commonly employed, ionization efficiencies may vary along the chromatogram. Mobile phase additives, such as acetate or formate buffers, may affect ionization efficiencies. Some additives in principle might improve signal intensities, however, their effect on separation has to be taken into account as well. For example, as shown in *Chapter 4*, optimum metabolite signals were obtained with the addition of triethylamine to the mobile phase, but unfortunately, triethylamine also caused broad and asymmetric peaks.

In HILIC–MS, a high concentration of acetonitrile at the start of the gradient is often desirable for the efficient separation of less polar compounds. However, the composition at the start of the gradient also affects the tolerable composition of the injection solvent. Adaptation of this solvent to the acetonitrile content of the mobile phase might be necessary to prevent peak broadening and splitting. However, a high percentage of acetonitrile may result in precipitation of polar and charged compounds, due to their limited solubility in acetonitrile. Furthermore, high acetonitrile percentages may cause phase separation in the sample, complicating analysis. Obviously, dilution of the samples with acetonitrile also reduces the metabolite concentrations.

Retention-time and peak-area reproducibility are quite favorable in HILIC–MS. In the studies described in this thesis, the variability in retention time metabolites was below 0.6%. A relatively long equilibration time of the HILIC–MS system is required in order to obtain this repeatability. For instance, in this research project an equilibration time of ten minutes was employed. This is long compared to the actually applied gradient of twenty minutes and increases the total analysis time substantially.

8.3 Data handling and metabolite identification

After analyzing biological samples, preprocessing of the obtained data is needed to allow a correct comparison of metabolite profiles. An important step is the alignment of the obtained electrophoretic or chromatographic profiles. Appropriate alignment can be challenging, especially when the shifts in migration/retention

times are substantial, non-linear and/or discontinuous. A dedicated alignment algorithm based on peak warping appeared to be very effective for the treatment of CE–MS data.

Another important data preprocessing step is the normalization of measured metabolite abundances to correct for inter-sample differences, especially when urine samples are analyzed. Normalization to creatinine concentration is often used in MS-based urinary metabolomics in order to correct for subject connected differences in produced urine volume. However, this is not a suitable method if the amount of excreted creatinine varies among the subjects of investigation. Metabolite profiles can also be normalized based on the total peak area, taking all detected metabolites into consideration and potentially also correcting for an overall drift in system detection response.

After data preprocessing, multivariate data analysis can be employed to find significant differences in metabolite concentration between groups of samples. Unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA) are most commonly applied. The dimensionality of metabolomics data can be reduced for an easier interpretation of obtained data, with the objective to reveal discriminatory compounds.

In order to verify whether differences between groups of samples do not originate solely from analytical variation, it is recommendable to analyze a quality control (QC) sample at regular intervals during a metabolic profiling study. A pooled mixture of sample aliquots is highly useful for this purpose as it represents all metabolites present in the analyzed samples. The QC results can aid in monitoring the overall separation and signal intensities in time. Furthermore, by including repetitive analyses of the QC sample in multivariate data analysis procedures, clustering of the QC replicates are a measure for stable system performance.

The identification of revealed discriminatory compounds is indicated in order to explore their role in biochemical pathways. Time-of-flight (TOF)-MS provides accurate mass information, which may aid the identification of discriminatory compounds by comparison with metabolite masses recorded in online databases. Still, many metabolites exhibit identical or highly similar masses, which cannot be assigned by accurate mass only. Therefore, MS/MS experiments can be performed to confirm putative identities, as was demonstrated in *Chapter* 7. The putative identity could also be confirmed by the addition of chemical reference standards to the biological samples of interest. Unfortunately, for many metabolites there are no reference compounds commercially available.

8.4 Applicability of CE–MS and HILIC–MS

In this thesis, CE–MS and HILIC–MS were used for the analysis of urine samples. The sample pretreatment required for urine samples is quite simple for CE–MS. Dilution of urine with BGE in a proportion of 1:1 (v/v) suffices. Particular attention should be paid to sample pretreatment in HILIC–MS in order to assure good separation performance. The sample preparation depends on the metabolite concentrations in the sample, the applied mobile phase, column, and injection volume. In the studies described in this thesis, urine samples had to be diluted with acetonitrile in a proportion of 1:4 (v/v). As discussed above, solubility problems may occur when mixing aqueous biological samples with relatively large volumes of acetonitrile. For the CE–MS and HILIC–MS analysis of protein-containing samples, deproteinization of e.g. blood and plasma should be carried out to prevent adsorption of proteins to the capillary wall or HILIC column.

In general, higher signal intensities were obtained for urinary metabolites with HILIC–MS compared to CE–MS, mostly due to the larger injection volume applied in HILIC–MS. Still, the average difference in signal intensities between CE–MS and HILIC–MS was shown to be smaller as could be expected from the injection-volume difference only. This indicates an overall higher absolute sensitivity in CE–MS, which may be related to more narrow peaks and more favorable ionization conditions.

Analysis of the same urine samples by CE–MS and HILIC–MS showed that some metabolites are detected with both techniques. The CE–MS and HILIC–MS methods also appeared to be highly complementary by uniquely detecting specific metabolites in biological samples. Separation selectivities of CE–MS and HILIC–MS were found to be considerably different, as was demonstrated by a lack of correlation between migration and retention times of metabolites detected by both techniques. CE appeared to be capable to separate compounds, which could not be separated with HILIC, and vice versa.

In *Chapters 5*-7, it was shown that urine samples from antibiotic-treated rats and from patients with inborn errors of metabolism could be distinguished from control samples with both CE–MS and HILIC–MS. This discrimination was partly based on discriminatory compounds uniquely found by the applied technique, again demonstrating the complementarity of CE–MS and HILIC–MS. In HILIC–MS commonly considerably more molecular features are detected than in CE–MS, but often the actual number of discriminatory compounds found with PCA is quite similar with CE–MS and HILIC–MS. This might be explained by the fact that due to the higher concentration sensitivity in HILIC–MS a large part of the detected

molecular features correspond to minor metabolite adducts and fragments. The detection of adducts and metabolite fragments complicates the data interpretation and does not necessarily increase the coverage of metabolites.

As CE–MS and HILIC–MS are complementary, employment of both analytical techniques will lead to an enhanced coverage of the metabolome, thereby increasing the probability of finding distinctive metabolites which could aid in the elucidation and understanding of metabolic pathways. Considering highly polar metabolites, there is no clear cut in suitability for certain classes of compounds for either of the techniques. However, as found in the comparative study described in this thesis, CE–MS seems to be more appropriate than HILIC–MS for metabolites with a relatively small molecular weight. HILIC–MS might be a first choice when low-abundant polar metabolites need to be analyzed, because of the generally higher observed analyte signals compared to CE–MS. When only limited amounts of samples are available, CE–MS is the preferred analytical technology due to the small applied injection volume. Another potential advantage of CE–MS is the in principle better predictability of migration times of metabolites. Involvement of migration-time information may aid in the identification of unknown metabolites found to be discriminatory between groups of samples. For instance, if based on accurate mass there are multiple candidates, some might be eliminated as their migration times do not match the theoretical migration time as predicted from their charge-to-size ratio. The separation mechanism in HILIC is complex which makes it very difficult to predict retention times of compounds based on their molecular structure.

8.5 Future perspectives

Having effective CE–MS and HILIC–MS methods for the analysis of anionic metabolites available now, it would be interesting to combine these techniques with their positive ion mode counterparts in order to establish an optimal coverage of the polar metabolome. This would allow detection of all metabolites as long as they are ionogenic (acidic, basic or zwitterionic) and/or can be ionized in positive or negative mode.

Further enhancement of the sensitivity of CE–MS is desirable and feasible. Implementation of electrophoretic and chromatographic preconcentration approaches in principle can lower concentration detection limits considerably, and it would be beneficial to develop dedicated preconcentration strategies for metabolites. It would be recommendable to evaluate ion-exchange and/or mixed-mode materials for metabolite preconcentration by solid-phase extraction.

Sensitivity improvements can also be expected from developments of new MS interfaces, especially for CE–MS. For instance, sheathless CE–MS interfacing provides good opportunities for metabolic profiling. In this interfacing approach, the dilution of the CE effluent by sheath liquid is avoided and nano-electrospray sensitivities can be obtained. It has already been shown that signal intensities of metabolites are significantly higher using sheathless CE–MS interfaces compared to sheath-liquid interfaces. However, sheathless CE–MS has mainly been carried out using positive ESI. The usefulness of sheathless interfacing in negative ionization mode still has to be shown. Furthermore, the robustness of sheathless interfaces still needs to be demonstrated in large-scale metabolic profiling studies.

In HILIC–MS, a reduction in column diameter could increase the sensitivity. Using nano-LC, lower flow rates will result in more efficient and optimal ESI. Another approach for down-scaling and efficient coupling to MS is the use of CE and LC on a microchip. Microfluidic systems might provide good separation efficiencies in a short analysis time, but the peak capacities may be too low for metabolic profiling.

Identification of discriminatory compounds is crucial in order to elucidate metabolic pathways. In many metabolic profiling studies, a large number of discriminatory compounds is observed. These metabolites of interest often remain unidentified. Online metabolomics databases could be extended to further facilitate metabolite identification. For instance, more MS/MS spectra and migration/retention data for various commonly applied methods could be included. TOF mass spectrometers are most often used in metabolic profiling studies and provide accurate mass information. However, many metabolites can still not be distinguished based on these accurate masses. Tentative identities should be more confirmed employing MS/MS experiments. Another possibility to improve compound identification is the use of mass spectrometers with an even higher mass resolution than a TOF mass spectrometer, such as Orbitrap and Fourier transform ion cyclotron (FT-ICR) mass spectrometers.

After compound identification, validation and quantification of these metabolites of interest should be pursued in large cohort-studies. This is pivotal to prove the power of metabolic profiling studies and to produce diagnostic biomarkers for daily clinical practice using one or more analytical methodologies, where CE–MS and HILIC–MS play a key role for the analysis of the highly polar part of the metabolome.

IX

Appendices

Nederlandse samenvatting

Endogene metabolieten zijn de tussen- en eindproducten van metabolisme en zijn via verschillende reactiepaden met elkaar verbonden. Deze metabolieten en hun concentraties zijn het gecombineerde resultaat van genexpressie, eiwitexpressie en omgevingsfactoren (bijvoorbeeld voeding, geneesmiddelen en bacteriën), die tezamen het fenotype van een organisme bepalen. Door het bestuderen van metabolieten in biologische monsters kan de fysiologische toestand (bijvoorbeeld ziek of gezond) van een organisme vastgesteld worden en kunnen biochemische processen opgehelderd worden. Het meten van metabolieten en het bestuderen van hun onderlinge samenhang wordt metabolomics genoemd. Metabolomics kan uitgevoerd worden door één of meerdere vooraf geselecteerde metabolieten te analyseren (*targeted approach*) of door ongericht zoveel mogelijk metabolieten tegelijkertijd te analyseren om biochemisch en/of pathologisch relevante – maar vooraf onbekende – metabolieten te identificeren (*untargeted approach*). Laatstgenoemde aanpak is uitdagend, aangezien metabolieten verschillende fysisch-chemische eigenschappen kunnen hebben en in sterk uiteenlopende concentraties voorkomen.

Onderzoek waarin het profileren van alle aanwezige metabolieten nagestreefd wordt om nieuwe mogelijke biomarkers voor bijvoorbeeld ziektes of geneesmiddelinteracties op te sporen, beslaat doorgaans een aantal opeenvolgende stappen. Het proces begint met een biologische of medische vraagstelling en het experimentele ontwerp van monstername en metingen dat leidt tot relevante resultaten, die een accuraat antwoord op de probleemstelling mogelijk maken. Biologische monsters, zoals urine, plasma of weefsel, van verschillende groepen mensen of proefdieren (bijvoorbeeld ziek/gezond, behandeld/niet behandeld, blootgesteld/niet blootgesteld) worden verzameld en dienen daarna eerst voorbehandeld te worden om op de juiste wijze geanalyseerd te kunnen worden. De monsters kunnen vervolgens met één of meer analysetechnieken geanalyseerd worden met als doel gedetailleerde metabolietprofielen te verkrijgen. De verkregen profielen worden vervolgens met elkaar vergeleken om te bepalen of er verschillen zijn tussen de monsters behorend bij verschillende groepen. Voordat de profielen op de juiste wijze met elkaar vergeleken kunnen worden, is een aantal datavoorbewerkingsstappen essentieel, waaronder het uitlijnen van profielen in de tijd en de normalisatie van verkregen signalen. Om signalen van metabolieten te vinden, die onderscheidend zijn voor onderzochte monsters uit verschillende groepen, wordt over het algemeen multivariate data-analyse toegepast, zoals principale-componentenanalyse (PCA). De op deze wijze gevonden kenmerken van

onderscheidende componenten (bijvoorbeeld massa, identiteit, migratie/retentietijd) dienen vervolgens toegekend te worden aan reële metabolieten (identificatie) om daarna zo mogelijk hun rol in de bestudeerde biochemische processen op te helderen en te begrijpen. Uiteindelijk kunnen de geïdentificeerde componenten mogelijk als biomarkers dienen voor specifieke pathologie of interventies, en gebruikt worden voor het stellen van diagnoses.

Diverse analysetechnieken kunnen toegepast worden voor de profilering van metabolieten. Kernspinresonantie (NMR), gaschromatografie (GC) en vloeistofchromatografie (LC) zijn veel gebruikte technieken in metabolomics. NMR geeft veel structuurinformatie, maar is relatief ongevoelig, waardoor metabolieten met lage concentraties mogelijk niet gedetecteerd worden. Voor metabolomics-doeleinden worden GC en LC doorgaans gekoppeld aan massaspectrometrie (MS) om massaspecifieke en dus selectieve detectie van metabolieten te bewerkstelligen. Met GC–MS kan een efficiënte scheiding en detectie van diverse componenten behaald worden. Het nadeel van GC is echter dat een derivatiseringsprocedure vaak noodzakelijk is voor de analyse van polaire en niet-vluchtige verbindingen. *Reversed-phase* LC–MS is een frequent toegepaste analysemethode in metabolomics, maar is minder geschikt voor het profileren van zeer polaire metabolieten, omdat deze verbindingen weinig of geen retentie ondervinden op de kolom.

Om de meer polaire metabolieten te analyseren zijn capillaire elektroforese (CE) en hydrofiele interactie chromatografie (HILIC) als scheidingsmethoden geïntroduceerd in metabolomics. Met CE worden analieten onder invloed van een elektrisch veld gescheiden op basis van elektroforetische mobiliteit, dat een functie is van hun lading-grootte-verhouding. De scheiding vindt veelal plaats in *fused*-silica capillairen gevuld met een achtergrondelektroliet (BGE). CE heeft een hoge scheidingsefficiëntie in een relatief korte analysetijd. Tevens is het gebruik van organische oplosmiddelen minimaal en is CE geschikt voor zeer kleine monsterhoeveelheden. Bij HILIC wordt er gebruik gemaakt van hydrofiele stationaire fasen. De meest toegepaste fasen zijn gebaseerd op silica waaraan functionele groepen gebonden kunnen zijn, zoals amino-, amide- en diolgroepen. Als mobiele fase wordt over het algemeen een mengsel van acetonitril en een waterige buffer gebruikt. Het scheidingsmechanisme van HILIC is nog niet volledig opgehelderd. Componenten worden vaak hoofdzakelijk gescheiden op basis van hun verdeling tussen de mobiele fase en een waterlaag op het oppervlak van de stationaire fase. Afhankelijk van het type stationaire fase spelen daarnaast

elektrostatische interacties, vorming van waterstofbruggen en hydrofobe interacties een rol bij de scheiding.
CE en HILIC kunnen gekoppeld worden aan MS, waarbij doorgaans gebruik gemaakt wordt van electrospray-ionisatie (ESI). De BGE of mobiele fase wordt zo onder invloed van een elektrisch veld verneveld. Bij CE wordt meestal een *sheath liquid* gebruikt om het noodzakelijke elektrische contact mogelijk te maken. Tevens wordt een voor ESI geschikt debiet gecreëerd en wordt het vernevelingsproces bevorderd. Door verdamping en elektrostatische afstoting tijdens ESI komen analieten in de gasfase terecht, waarna hun massa-ladingverhoudingen in de massaspectrometer gemeten worden. Indien er gebruik gemaakt wordt van massaspectrometers met een hoge resolutie en massanauwkeurigheid, kunnen metabolieten mogelijk geïdentificeerd worden op basis van accurate massa.
Veel metabolieten in biologische monsters zijn zeer polair en daarom lijken CE–MS en HILIC–MS uitermate geschikt voor het profileren van deze verbindingen. Bij aanvang van het in dit proefschrift beschreven onderzoek waren de mogelijkheden van CE–MS voor het analyseren van metabolieten reeds beschreven. Deze analysetechniek werd echter met name toegepast voor de profilering van kationische metabolieten door gebruik te maken van positieve ESI. De scheiding van metabolieten werd gerealiseerd met een zure BGE in een *bare fused*-silica capillair of in een capillair waar op de binnenwand een laag van een positief-geladen polymeer was aangebracht. Deze coating van het capillair voorkomt de eventuele adsorptie van analieten en vergroot het tijdsvenster waarin kationische componenten gescheiden kunnen worden.
Een groot deel van de in biologische monsters aanwezige metabolieten is echter anionisch en kan met een hogere efficiëntie geioniseerd worden indien negatieve ESI gebruikt wordt. In een beperkt aantal onderzoeken is CE–MS in negatieve modus toegepast, waarbij gebruik gemaakt werd van een BGE met ammoniumacetaat in combinatie met gecoate of ongecoate capillairen. De reproduceerbaarheid van en de verkregen signalen met deze CE–MS systemen waren vaak relatief laag. Aangezien anionische metabolieten een belangrijk deel uitmaken van het totaal aan metabolieten, is de optimalisering en toepassing van CE–MS in negatieve modus belangrijk. Het onderzoek beschreven in dit proefschrift was daarom in eerste instantie gericht op de ontwikkeling van een verbeterde CE–MS methode voor het profileren van anionische metabolieten. De invloed van de samenstelling van de BGE en *sheath liquid* op de signalen van anionen is onderzocht om de methode te optimaliseren. Bovendien is een HILIC–MS methode in negatieve modus ontwikkeld. De samenstelling van de

mobiele fase, de toegepaste gradiënt en de samenstelling van de monsteroplossing zijn daarvoor geoptimaliseerd om een goede scheiding en relatief lage detectielimieten voor anionen te verkrijgen. Daarnaast is de toepasbaarheid van de ontwikkelde CE–MS methode en HILIC–MS methode geëvalueerd door urinemonsters te analyseren. Het toepassen van beide analysetechnieken bood de mogelijkheid om de methoden te vergelijken en de toegevoegde waarde van zowel CE–MS als HILIC–MS voor het profileren van metabolieten te bepalen.

Hoofdstuk 1 is de introductie van dit proefschrift, waarin achtergrondinformatie over metabolomics, CE, HILIC en MS wordt gegeven. Daarnaast worden bovenstaande uitgangspunten van het uitgevoerde onderzoek beschreven. Een uitgebreid literatuuroverzicht van studies waarin meerdere analysetechnieken, waaronder CE, toegepast en met elkaar vergeleken zijn, wordt gegeven in ***Hoofdstuk 2***. In het eerste gedeelte komen algemene aspecten aan de orde, zoals monstervoorbewerking, de opzet van de analysemethoden, data-analyse en welke metabolieten geanalyseerd kunnen worden. In het tweede gedeelte worden uitgebreide tabellen gegeven met de experimentele condities van de verschillende vergelijkingsonderzoeken. Tevens worden de vergelijkingsstudies nader behandeld om de toegevoegde waarde van CE ten opzichte van de andere technieken te beoordelen.

Hoofdstuk 3 beschrijft de ontwikkeling van een CE–MS methode voor de profilering van anionische metabolieten. Om de signalen van anionen te verbeteren, zijn diverse samenstellingen van BGEs en *sheath liquids* onderzocht met behulp van infusie-experimenten. Door toevoeging van triethylamine aan de BGE en *sheath liquid* bleken de signalen van anionische metabolieten vergroot te worden. De toepasbaarheid hiervan in CE–MS is verder onderzocht aan de hand van een testmengsel van representatieve metabolieten. De CE–MS methode met triethylamine is daarbij geoptimaliseerd op basis van de scheiding, detectielimieten en de reproduceerbaarheid van migratietijden en piekoppervlakten van de testmetabolieten. Tevens wordt een vergelijking gemaakt met twee frequent gebruikte CE–MS methoden, waarbij gebruik gemaakt is van ammoniumacetaat voor de analyse van anionen. Een BGE met 25 mM triethylamine (pH 11,7) en een *sheath liquid* met 5 mM triethylamine bleken geschikt om tien testmetabolieten reproduceerbaar te scheiden. De met deze methode verkregen detectielimieten (0,7-9,1 μM) waren gemiddeld substantieel lager dan detectielimieten van CE–MS methoden, waarbij ammoniumacetaat toegepast is. De analyse van humane urinemonsters liet ook een verschil zien tussen de CE–MS methoden met triethylamine en ammoniumacetaat. Door gebruik te maken van triethylamine

werden 231 verbindingen in urine gedetecteerd, terwijl met ammoniumacetaat in de BGE en *sheath liquid* 115 en 102 verbindingen werden gevonden met respectievelijk een ongecoat en gecoat *fused*-silica capillair. De signalen van de componenten, die zowel met de triethylamine als met de ammoniumacetaat methoden werden gedetecteerd, waren tot zeven keer hoger indien triethylamine werd gebruikt. Deze resultaten demonstreren de geschiktheid van een BGE en *sheath liquid* met triethylamine voor het analyseren van anionen.

In ***Hoofdstuk 4*** wordt een vergelijking tussen de geoptimaliseerde CE–MS methode en een HILIC–MS methode gepresenteerd voor het profileren van anionische metabolieten. Voor de vergelijking zijn de reproduceerbaarheid, gevoeligheid, het aantal gedetecteerde metabolieten en de selectiviteit van de analysemethoden in beschouwing genomen. De concentratie en pH van de waterige bufferoplossing van de mobiele fase toegepast in HILIC–MS zijn geoptimaliseerd voor een reproduceerbare scheiding en maximale gevoeligheid van anionen. Tevens is de invloed van de gradiënt, het injectievolume en de samenstelling van de monsteroplossing bepaald. Op basis van de optimaliseringsresultaten zijn een mobiele fase met 10 mM ammoniumacetaat (pH 6,8) in acetonitril-water, een monsteroplossing bestaande uit 80% acetonitril en een injectievolume van 5 μL geselecteerd. De gradiënt is gedurende vijf minuten op 90% acetonitril gehouden. Daarna is in tien minuten het percentage acetonitril geleidelijk verlaagd naar 50%. Na vijf minuten op 50% acetonitril is het percentage direct verhoogd tot 90% en tien minuten daarop gehouden alvorens een nieuwe meting gestart werd. Aan de hand van vijf testmetabolieten zijn de reproduceerbaarheid en gevoeligheid van de geoptimaliseerde HILIC–MS methode vastgesteld. Detectielimieten voor de testmetabolieten waren 0,04-2,21 μM. Deze detectielimieten bleken tot een factor tachtig lager te zijn dan de detectielimieten verkregen met CE–MS. Dit verschil wordt met name veroorzaakt door het verschil in injectievolume tussen CE–MS (16 nL) en HILIC–MS (5 μL). De toepassing van CE–MS en HILIC–MS voor de analyse van urinemonsters is ook geëvalueerd. Er werden met CE–MS en HILIC–MS gemiddeld respectievelijk 347 en 1360 verbindingen in rattenurine gedetecteerd. In totaal werden 144 verbindingen gevonden met zowel CE–MS als HILIC–MS. De piekoppervlakten van deze urinecomponenten waren gemiddeld tien keer hoger in HILIC–MS. Verder was er een lage correlatie tussen de migratietijden in CE en retentietijden in HILIC van deze verbindingen. Dit gevonden verschil in selectiviteit tussen CE–MS en HILIC–MS is tevens aangetoond met behulp van een aantal geïdentificeerde urinecomponenten. De selectiviteit van de analysemethoden is gedeeltelijk verklaard

aan de hand van fysisch-chemische eigenschappen van de geïdentificeerde metabolieten en de scheidingsprincipes van CE en HILIC.
De bruikbaarheid van CE–MS en HILIC–MS voor het profileren van anionen in biologische monsters wordt geëvalueerd in ***Hoofdstuk 5 en 6***. Urinemonsters van ratten, die gedurende 0, 4 of 8 dagen de antibiotica penicilline G en streptomycine sulfaat toegediend hebben gekregen, zijn geanalyseerd om interacties tussen het metabolisme van de gastheer en darmbacteriën te bestuderen. Een mengsel van alle urinemonsters is herhaald geanalyseerd als kwaliteitscontrolemonster om de reproduceerbaarheid van de analysemethoden tijdens de metingen te bewaken. De migratietijd-reproduceerbaarheid van urinecomponenten was te laag om de verkregen CE–MS profielen met elkaar te vergelijken. Het uitlijnen van deze profielen was daarom noodzakelijk. Daarvoor is een specifieke methode ontwikkeld, die in staat bleek te zijn de verschillen tussen de profielen grotendeels te corrigeren, waardoor de profielen met elkaar vergeleken konden worden. Het verschil in retentietijden tussen de diverse metabolietprofielen verkregen met HILIC–MS was significant lager, waardoor het uitlijnen minder noodzakelijk was. PCA van de CE–MS en de HILIC–MS data resulteerde in een onderscheid tussen metabolietprofielen van met antibiotica behandelde ratten en controleratten. In totaal waren 17 en 29 componenten verantwoordelijk voor de verkregen verschillen met respectievelijk CE–MS en HILIC–MS, waarvan 10 verbindingen met beide technieken gevonden zijn. De relatieve af- of toename van deze componenten verkregen met CE–MS was gelijk aan die van HILIC–MS. Er konden 9 en 13 met respectievelijk CE–MS en HILIC–MS gevonden verbindingen geïdentificeerd worden op basis van accurate massa, waaronder hippuurzuur en indoxylsulfaat. Een in de literatuur beschreven onderzoek van dezelfde urinemonsters waarin gebruik gemaakt is van NMR resulteerde ook voor deze twee metabolieten in een significant verschil tussen de behandelde en onbehandelde ratten. Naast de gemeenschappelijk gevonden metabolieten zijn er met de verschillende analysemethoden ook unieke metabolieten gedetecteerd. Deze metabolieten konden niet met de twee andere toegepaste methoden gevonden worden. Dit toont de complementariteit en de toegevoegde waarde van CE–MS, HILIC–MS en NMR aan voor het profileren van metabolieten in biologische monsters.
In ***Hoofdstuk 7*** is de toepasbaarheid van CE–MS en HILIC–MS verder onderzocht. Urinemonsters van patiënten met de stofwisselingsziekten citrullinemie, midden-keten acyl-co-enzym A dehydrogenase (MCAD) deficiëntie en 3-methylcrotonyl-co-enzyme A carboxylase (MCC) deficiëntie zijn geanalyseerd en de metabolietprofielen zijn vergeleken met urineprofielen van controles. Voor de

metabole aandoening citrullinemie zijn 23 (CE–MS) en 25 (HILIC–MS) verbindingen gevonden, waarvan de concentratie significant hoger of lager was in de patiëntmonsters. Twee van deze onderscheidende componenten konden toegeschreven worden aan citrulline en orootzuur. Dit zijn reeds bekende biomarkers van citrullinemie. Met CE–MS werden zes en met HILIC–MS veertien componenten gevonden die significant verschilden tussen patiënten met MCAD deficiëntie en controles, waaronder hexanoylglycine en suberylglycine. De metabolieten 3-methylcrotonylglycine en 3-hydroxy-isovaleriaanzuur werden met HILIC–MS als bekende biomarkers voor MCC deficiëntie verhoogd in de urine van patiënten aangetroffen. Daarnaast zijn met CE–MS en HILIC–MS ook respectievelijk acht en vier onbekende metabolieten gevonden, waarvan de urineconcentraties significant verschilden tussen patiënten en controles.

Om onbekende verbindingen te kunnen identificeren zijn HILIC–MS/MS experimenten uitgevoerd, waarbij de monsters van de patiënten met MCAD deficiëntie als voorbeeld zijn genomen. Verschillende botsingsenergieën zijn toegepast om zoveel mogelijk metabolietfragmenten te verkrijgen. Met behulp van de verkregen fragmenten konden zes metabolieten geïdentificeerd worden, waaronder de biomarkers hexanoylglycine en suberylglycine. Op basis van de resultaten kan geconcludeerd worden dat CE–MS en HILIC–MS beide uitermate geschikt zijn voor het profileren en karakteriseren van anionische metabolieten in metabole aandoeningen. Daarnaast zijn MS/MS-experimenten belangrijk voor het identificeren van onbekende componenten.

Tot slot worden in ***Hoofdstuk 8*** algemene conclusies getrokken aangaande de mogelijkheden van CE–MS en HILIC–MS voor het profileren van anionische metabolieten. Daarbij wordt ook aandacht besteed aan de complementariteit van de twee systemen. Tevens worden enkele toekomstperspectieven bediscussieerd en worden aanbevelingen voor vervolgonderzoek gedaan.

Abbreviations

AB*x*-D*y*	samples taken at day *y* after the use of antibiotics for *x* days
BFS	bare fused-silica
BGE	background electrolyte
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
CGE	capillary gel electrophoresis
cIEF	capillary isoelectric focusing
DS	dextran sulfate
EI	electron ionization
EOF	electroosmotic flow
ESI	electrospray ionization
FAD	flavin adenine dinucleotide
FT-ICR	Fourier transform-ion cyclotron resonance
G6P	glucose-6-phosphate
GC	gas chromatography
HILIC	hydrophilic interaction chromatography
HPPA	hydroxyphenylpropionic acid
ITP	isotachophoresis
LC	liquid chromatography
LOD	limit of detection
MALDI	matrix-assisted laser desorption/ionization
MCAD	medium chain acyl-coenzyme A dehydrogenase
MCC	3-methylcrotonyl-coenzyme A carboxylase
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
PB	polybrene
PC	principal component
PCA	principal component analysis
PLS-DA	partial least squares discriminant analysis
Q	quadrupole
QC	quality control
RP	reversed-phase

R^2	coefficient of determination
RPW	reference peak warping
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
S/N	signal-to-noise
TBDMS-FA	N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide
TCA	tricarboxylic acid
TMCS	trimethylchlorosilane
TOF	time-of-flight
TSP	3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid
UP	ultra performance
UV	ultraviolet

List of publications

M.G.M. Kok, G.W. Somsen, G.J. de Jong, The role of capillary electrophoresis in metabolic profiling studies employing multiple analytical techniques: an overview, *submitted* (Chapter 2).

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