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CE-MS for metabolomics: advancing performance and detection sensitivity

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Chapter 6

Assessing the suitability of capillary electrophoresis-mass spectrometry for biomarker discovery in plasma-based metabolomics

Based on

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Assessing the suitability of capillary electrophoresis-mass spectrometry for biomarker discovery in plasma-based metabolomics

Electrophoresis (2019)

* Authors contributed equally

Abstract

In metabolomics, CE-MS is a useful analytical technique for the profiling of polar and charged metabolites. However, the actual utility of this approach for biomarker discovery using metabolomics still needs to be assessed. Therefore, a simulated comparative metabolic profiling study for biomarker discovery by conventional CE-MS at low-pH separation conditions was performed, using pooled human plasma samples with spiked biomarkers. In this context, two studies have been carried out in this work. The focus of study I was on comparing two sets of plasma samples, in which one set (class I) was spiked with five isotope-labeled compounds, whereas another set (class II) was spiked with six different isotope-labeled compounds. In study II, the focus was also on comparing two sets of plasma samples, however, in this case the isotope-labeled compounds were spiked to both class I and class II samples but with concentrations which differ by a factor of two between both classes, and with the absence of one compound in each class. In both studies, blank pooled human plasma was used as a quality control sample. The main aim was to determine whether the CE-MS-based metabolomics approach could reveal the spiked biomarkers as the main classifiers, applying two different data analysis software tools (namely, MetaboAnalyst and Matlab). Unsupervised analysis of the recorded metabolic profiles revealed a clear distinction between class I and class II plasma samples in both studies. This classification was mainly attributed to the spiked isotope-labeled compounds, thereby emphasizing the utility of CE-MS in metabolomics for biomarker discovery.

Introduction

Metabolomics offers a new approach to explore changes in patterns for a large number of (endogenous) metabolites in biological media, such as blood, urine and cerebrospinal fluid ¹⁻⁶. Currently, a wide range of advanced analytical separation techniques are used for metabolic profiling of biological samples. The complex data sets generated by these analytical tools can be processed by software tools, for example XCMS ⁷, MZmine ⁸, MetAlign ⁹, or SpectConnect ¹⁰, and the main output is a peak table with the intensity of each chromatographic or electrophoretic peak, characterized by a specific retention or migration time, respectively, and one or more m/z values. Supervised and unsupervised chemometric approaches are often used to get visualization of the relations between the metabolic profiles and to define borders between groups of samples. Global profiling of (endogenous) metabolites in organisms has been vastly explored for its potential application in research areas, such as diagnosis of diseases ^{1, 3, 6}, guidance for personalized medicine ¹¹, and evaluation of therapeutic treatments ^{12, 13}. Despite the efforts dedicated to metabolomics for biomarker discovery, its impact on recent clinical practice is still rather limited due to various challenges encountered during the analytical process, including study design, sample handling, data acquisition and data analysis ¹⁴, which may potentially lead to contradictory results in reported biomarkers. For example, Slupsky *et al.* ¹⁵ indicated succinic acid to be among the down-regulated urinary metabolites in ovarian cancer patients, whereas Zhang *et al.* ¹⁶ obtained the opposite finding for this compound using a different analytical technique. Therefore, these studies clearly underscore the need for assessing the capability of a given analytical technique for delivering the right biomarkers in metabolomics using preferably multiple data analysis procedures. In principle, each data analysis procedure should provide the same chemical information/output when employing a single analytical technique for metabolic profiling. In this work, we have used MetaboAnalyst and Matlab as two data analysis software tools for analyzing metabolomics data obtained by CE-MS (**Fig. 1**).

CE is a separation technique that is well-suited for the highly efficient profiling of polar and charged metabolites, as compounds are separated according to their charge-to-size ratios. It provides complementary metabolic information compared to chromatography-based techniques. Until now, CE coupled to MS has been utilized for metabolic profiling of a wide range of biological samples in various application fields ¹⁷. However, in comparison to other analytical techniques the use of CE-MS in metabolomics is still underrepresented ¹⁸. CE-MS is often still considered by the scientific community as a rather complicated or not robust technique, in this case specifically the coupling of CE to MS, and often not fulfilling the criteria of repeatability and sensitivity for metabolomics studies.

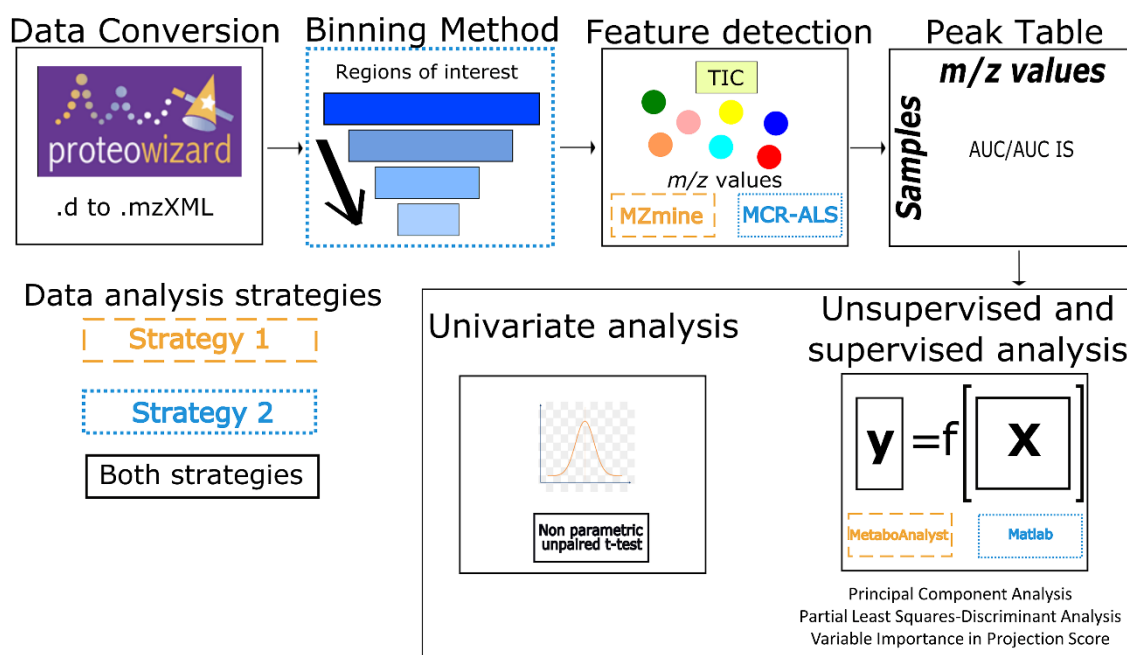


Figure 1. Overview of the data analysis tools used in this study. The tools of the first data analysis strategy are shown in orange (stripes) while in blue (dots) those for the second strategy are given. The workflow is similar starting with the data conversions to a readable file. Subsequently, data compression is needed for the MCR-ALS feature detection of the second strategy. After selecting the features a peak table is generated containing the corrected peak areas for each sample. The generated peak table can be further investigated using univariate, unsupervised and supervised analysis to discover potential biomarkers.

Over the past years, various studies have clearly indicated the long-term performance of CE-MS for metabolomics and peptide profiling studies¹⁹⁻²¹. For example, the group of Soga and co-workers, who introduced the first CE-MS methods for metabolomics in 2003²², has recently assessed the long-term performance of CE-MS for metabolic profiling of more than 8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52-month period²⁰. The study provided an absolute quantification of 94 polar metabolites in plasma with a similar or better reproducibility than other analytical platforms employed for large-scale metabolomics studies. The issue of migration-time repeatability for metabolic profiling studies can be tackled by converting migration times into electrophoretic mobilities, as recently demonstrated by Drouin *et al.*²³. This group has designed an experimental database for effective mobilities (μ_{eff}) measured for a large variety of charged metabolites, which was successfully implemented for the characterization of metabolites observed by CE-MS in tumor cell samples. Overall, we expect that this approach will be of great value for metabolomics studies, especially for the identification of metabolites when employing a library based on electrophoretic mobilities. Other improvements in CE-MS analyses, such as the use of novel interfaces^{24, 25} and multi-segment injection (MSI)²⁶, have clearly contributed to the potential of CE-MS of becoming a sensitive and high-throughput technique for metabolic profiling studies. Apart from increasing sample throughput, the MSI approach, developed by the group of Britz-McKibbin²⁶, could also be used to distinguish authentic

metabolite features from spurious signals in biological samples. The latter could readily be annotated based on their temporal signal pattern when using the MSI approach in combination with high-resolution tandem mass spectrometry.

Up till now, CE-MS has been used by various research groups for a wide range of metabolomics studies providing useful insights into questions/problems from different fields. Still, it is important to show the actual utility of CE-MS for comparative metabolic profiling studies, especially in order to convince the scientific community about the usefulness of this approach for biomarker discovery. An artificial metabolomics study was therefore designed to test the capability of CE-MS in finding the correct biomarkers in a comparative metabolic profiling study. For this, two studies have been carried out, in which the focus of study I was on comparing two sets of plasma samples, i.e. class I was spiked with five isotope-labeled compounds, whereas class II was spiked with six different isotope-labeled compounds. In study II, the focus was also on comparing two sets of plasma samples, however, in this case the isotope-labeled compounds were spiked to both class I and class II samples but with concentrations which differ by a factor of two between both classes, and with the absence of one compound in each class. Blank pooled human plasma (without spiking) was used as quality control (QC) sample to assess the performance of CE-MS over time. Overall, the strategy outlined in this paper could be considered as an approach to validate a (conventional) CE-MS method for metabolomics studies.

Materials and Methods

1. Chemicals and reagents

HPLC grade methanol and acetonitrile were obtained from Actua-All Chemicals (Oss, the Netherlands). HPLC grade chloroform was provided by Biosolve Chemicals (Valkenswaard, the Netherlands). Acetic acid (99-100%) and sodium hydroxide were purchased from VWR (Amsterdam, the Netherlands). Ammonium hydroxide (28-30%) was acquired from Acros Organics (Amsterdam, the Netherlands). Water in this work was produced by a Milli-Q® Advantage A10 Water Purification System from Millipore (Amsterdam-Zuidoost, the Netherlands). The standards of eleven ^{13}C , ^{15}N and/or D-isotope-labeled amino acids were purchased from Cambridge Isotope Laboratories (Apeldoorn, the Netherlands). In Study I, DL-phenyl-D5-alanine from CDN ISOTOPES (Nieuwegein, the Netherlands) was used as the internal standard (IS). In study II, an L-methionine sulfone-containing solution from Human Metabolome Technologies (Leiden, the Netherlands) was employed as IS. All compounds were dissolved in a mixture of water:acetonitrile (95:5, containing 0.5% v/v formic acid) and subsequently diluted to desired concentrations with water (see **Tables 1 and 2**). A solution of acetic acid (10% v/v in water, pH=2.2) was employed as BGE.

Table 1. An overview of the design of class I and class II plasma samples for study I (IS: DL-phenyl-D5-alanine). Sample 1 within class I is prepared by spiking Mix 1 to the blank plasma sample, and sample 2 within class I is prepared by spiking Mix 2 to the plasma sample, etc.

| Compound | <i>m/z</i> | Concentration (μM) | | | | | |
|---|------------|--|-------|-------|-------|--------|--------|
| | | Class I (<i>n</i> =6 samples per mix) | | | | | |
| | | Mix 1 | Mix 2 | Mix 3 | Mix 4 | Mix 5 | |
| L-Isoleucine (¹³ C; ¹⁵ N) | 134.099 | 40 | 36 | 50 | 40 | 36 | |
| L-Asparagine (¹³ C2; ¹⁵ N2) | 139.066 | 100 | 80 | 90 | 80 | 80 | |
| L-Glutamine (¹³ C2) | 149.081 | 20 | 15 | 30 | 30 | 30 | |
| L-Lysine (4,4,5,5-D4) | 151.135 | 10 | 10 | 15 | 12 | 15 | |
| L-Tryptophan (¹³ C11; ¹⁵ N2) | 218.124 | 40 | 48 | 50 | 36 | 50 | |
| Class II (<i>n</i> =5 samples per mix) | | | | | | | |
| | | Mix 6 | Mix 7 | Mix 8 | Mix 9 | Mix 10 | Mix 11 |
| Creatinine (N-methyl-D3) | 117.088 | 40 | 30 | 45 | 50 | 45 | 50 |
| L-Valine (D5) | 126.134 | 5 | 7.5 | 10 | 7.5 | 10 | 7.5 |
| L-Asparagine (2,3,3-D3) | 136.078 | 100 | 80 | 90 | 100 | 80 | 90 |
| L-Glutamine (2,3,3,4,4-D5) | 152.11 | 100 | 90 | 100 | 80 | 90 | 80 |
| L-Lysine (¹³ C6) | 153.129 | 40 | 35 | 50 | 45 | 35 | 40 |
| L-Glutamic acid (¹³ C5;D5; ¹⁵ N) | 159.103 | 40 | 45 | 30 | 50 | 30 | 40 |

2. Plasma sample preparation

Pooled human plasma, anti-coagulated with citrate, was obtained from Sanquin Blood Bank (Leiden, the Netherlands). For protein precipitation, methanol was added to pooled human plasma at a 5:1 ratio. The plasma/methanol mixture was vortexed for 1 min at room temperature before centrifugation at 16100 g at 4 °C for 10 min. Subsequently, 120 μL of the supernatant was transferred to an Eppendorf tube for liquid-liquid extraction, for which 300 μL methanol, 450 μL chloroform, 140 μL water, 50 μL internal standard solution (200 μmol/L for L-methionine and 60 μmol/L for DL-phenyl-D5-alanine), and 50 μL isotope-labeled compounds mix for classes I and II (50 μL water was used for the QC samples) were used to extract polar metabolites. **Tables 1 and 2** provide an overview of how the samples were prepared for each class of plasma samples within study I and II, respectively. The samples were vortexed for 2 min and then centrifuged at 16100 g at 4 °C for 10 min. 500 μL of the supernatant was centrifugally filtered using a 5 kDa cutoff filter (Millipore) at 12000 g at 4 °C for 1.5 h to further remove proteins. The filtered sample was evaporated in a CentriVap Concentrator (Labconco) and stored at -80 °C. The dried extract was reconstituted in 50 μL water prior to CE-MS analysis. Standards for calibration curves were generated by spiking the pooled human plasma with the mix of isotope labeled compounds at 10, 20, 40, 60, 80 and 100 μM, respectively.

Table 2. Design of class I and class II plasma samples for study II (IS: L-methionine sulfone).

| Compound | <i>m/z</i> | Class I (<i>n</i> =30 samples) | Class II (<i>n</i> =30 samples) |
|---|------------|---------------------------------|----------------------------------|
| | | Concentration (μM) | Concentration (μM) |
| L-Lysine (4,4,5,5-D4) | 151.135 | 20 | 10 |
| L-Asparagine (¹³ C2; ¹⁵ N2) | 139.066 | 100 | 50 |
| L-Isoleucine (¹³ C; ¹⁵ N) | 134.099 | 20 | 40 |
| L-Tryptophan (¹³ C11; ¹⁵ N2) | 218.124 | 0 | 20 |

| | | | |
|--|---------|----|----|
| L-Glutamic acid ($^{13}\text{C}_5;\text{D}_5;^{15}\text{N}$) | 159.103 | 20 | 40 |
| L-Asparagine (2,3,3-D3) | 136.078 | 40 | 20 |
| L-Valine (D5) | 126.134 | 5 | 10 |
| L-Lysine ($^{13}\text{C}_6$) | 153.129 | 10 | 20 |
| L-Glutamine (2,3,3,4,4-D5) | 152.110 | 20 | 0 |
| L-Glutamine ($^{13}\text{C}_2$) | 149.081 | 10 | 20 |
| Creatinine (N-methyl-D3) | 117.088 | 10 | 20 |

3. CE-MS analysis

All fused-silica capillaries used were 70 cm in length with an internal diameter of 50 μm and obtained from BGB Analytik (Harderwijk, the Netherlands). Prior to first use a newly installed capillary was conditioned using the following rinsing steps: water for 2 min at 5 bar, 0.1 M sodium hydroxide for 10 min at 5 bar, water for 2 min at 5 bar, and BGE for 2 min at 5 bar. The samples were injected hydrodynamically at 50 mbar for 20 s, which corresponds to about 1.2% (~17 nL) of the total capillary volume.

The analyses were conducted on an Agilent 7100 CE instrument hyphenated to an Agilent 6230 Time of Flight (TOF) mass spectrometer (Agilent Technologies, Santa Clara, California), equipped with an ESI source via a co-axial sheath-liquid interface. The CE-MS approach used in this work was based on the work from Drouin *et al.*²³. The sheath-liquid, consisting of isopropanol/water (1:1, v/v) and acetic acid (200 μL added to a final volume of 100 mL sheath liquid), was delivered at a final flow-rate of 5 $\mu\text{L}/\text{min}$ by an Agilent 1260 Infinity II Isocratic Pump (Agilent Technologies) using a 1:100 splitter. A voltage of 30 kV was used for electrophoretic separation and detection was performed in positive MS mode. The MS parameters were as follows: drying gas was set at 100 $^\circ\text{C}$ with a flow-rate of 11 L/min, and the nebulizer gas at 0 psi. The capillary voltage was 5500 V, and the fragmentor, skimmer, and OCT1 RF voltages were set at 100, 50 and 150 V, respectively. The full scan MS acquisition covered the mass range from 50 to 1000 m/z at an acquisition rate of 1.5 spectra/s, which was controlled and monitored with MassHunter version B05.01 (Agilent Technologies). Between consecutive biological sample analyses, the capillary was flushed as follows: water for 30 s at 5 bar, methanol for 1 min at 5 bar, water for 30 s at 5 bar, 10% ammonium hydroxide for 1 min at 5 bar, water for 30 s at 5 bar and BGE for 2 min at 5 bar. The CE-MS data were stored as .d files.

The capillary cassette was thermostated at 22 $^\circ\text{C}$ and the sample tray maintained at 10 $^\circ\text{C}$ by means of a Julabo F12 circulator temperature controller (Boven-Leeuwen, the Netherlands). To assess the repeatability of CE-MS for metabolic profiling of plasma, the RSD for migration time and peak area were determined for 19 endogenous metabolites in a QC sample, which was analyzed in 16 consecutive runs. During the analysis of the individual plasma samples, every ten runs a QC sample was analyzed. In total 23 QC samples were analyzed in each study.

4. Data processing and chemometric analysis

An overview of the data analysis, by the software tools used in this study, is shown in **Figure 1**. Each data analysis strategy is described in detail below.

4.1. Strategy 1

The raw data were converted into mzXML format using ProteoWizard and imported into MZmine 2.32 for feature detection. The detailed detection process is listed in **supplementary file S1**. Considering that the peak area calculation function was not ideal in MZmine, the peak areas were calculated in the Data Acquisition module within MassHunter version B05.01 (Agilent Technologies). The peak areas were integrated based on a standard list generated by an untargeted analysis. Peak areas of the detected metabolites were corrected with the corresponding IS peak area (for study I with DL-phenyl-D5-alanine and for study II with L-methionine sulfone), and the peak area ratios were further used in the statistical analysis.

MetaboAnalyst (<http://www.metaboanalyst.ca>) was used for multivariate analysis, including principal component analysis (PCA) and partial least squares - discriminant analysis (PLS-DA) to identify the spiked markers as “biomarkers” to distinguish “class I” from “class II”. Auto-scaling was done prior to PCA to prevent highly responsive metabolites from dominating the model, and prior to PLS-DA to facilitate the discovery of the “spiked biomarkers”²⁷. The peak area ratios were also subjected to an unpaired non-parametric test (Wilcoxon rank-sum test, also known as Mann-Whitney U test) within MetaboAnalyst, and false discovery rates (FDR) were calculated to discover if those *m/z* values are significantly different between class I and II. The compounds responsible for distinguishing class I from class II samples were selected using the variable importance in projection (VIP) score employing the criteria of VIP>1 and false discovery rate (FDR)<0.05.

4.2. Strategy 2

As in strategy 1, data was generated in centroid mode at an Agilent CE-TOF-MS instrument and converted to mzXML files with the open-source file converter ProteoWizard. Compared to strategy 1, these files were imported and further analyzed in Matlab™ R2014a (The Mathworks, Natick, MA) instead of MetaboAnalyst. Due to storage requirements, a binning method was necessary to compress the data^{28, 29} (**Fig. 1**). The regions-of-interest (ROI) method was used to compress the generated Total Ion Current profile³⁰. Here, ROI values are searched among all measurement times in the recorded CE-MS profile. However, different input variables are needed to define an ROI, such as a signal threshold value, mass accuracy and the minimum time interval to be considered as a peak width^{30, 31}. In our study, these parameters were set at 1000 for the signal threshold, mass accuracy was set to 0.01 Da and the minimum time to elute a peak was set to 6 s. All parameter values were based on the protocol by Gorrochategui *et al.*³⁰. The following step was the feature detection step, which does not make use of MZmine, but is based on

Multivariate Curve Resolution - Alternating Least Squares (MCR-ALS) using the MCR-ALS toolbox³².

As in strategy 1, peak areas were further integrated in the Data Acquisition module within MassHunter version B05.01 (Agilent) and corrected with the corresponding IS peak area (for study I with DL-phenyl-D5-alanine and for study II with L-methionine sulfone). The peak area ratios were further utilized in Matlab™ R2014a (The Mathworks) to perform unsupervised PCA analysis, and supervised PLS-DA analysis. Autoscaling was also applied here as data pre-treatment method. The number of latent variables for the PLS-DA model was chosen based on a five-fold venetian-blind cross validation. Additionally, the PLS-DA model evaluation was based on the error rate, non-error rate and accuracy, based on the cross-validation and calibration results. Finally, compounds mainly responsible for distinguishing class I from class II samples were selected based on the variable importance in projection (VIP) score, with the aim to hopefully trace back the spiked markers and confirm the results of strategy 1. An additional confirmation was performed with the same non-parametric test as in strategy 1. All the m/z values resulting in a VIP value above 1 were analyzed with this univariate data analysis. Those resulting in a p -value below 0.05 are significantly different between both classes and are important for distinguishing class I from class II samples.

Results and discussion

1. CE-MS for cationic metabolic profiling

Up till now, most metabolomics studies using CE-MS employed a standard co-axial sheath-liquid interface and low-pH separation conditions to target cationic metabolites (i.e. basic compounds). In this study, this CE-MS approach was used in order to assess its capability of delivering proper chemical information in comparative metabolic profiling studies.

For comparative metabolic profiling, the CE-MS method should provide consistent migration times and peak areas over time. Therefore, pretreated blank pooled human plasma was first analyzed for 16 consecutive runs (lasting around 8 hours in total). The RSD values for migration time, peak area, and peak area divided by IS, of 19 selected endogenous metabolites in this QC sample, were determined and are shown in Table 3. RSD values found are below 5.9%, 9.1%, and 4.5%, respectively. However, the lower RSD values are found for the corrected areas by the IS. For 16 of the 19 selected endogenous metabolites, the RSD values for migration time were below 3%. Therefore, we considered the overall findings acceptable to perform the proposed assessment study.

Table 3. Migration-time and peak-area repeatability (n=16) for selected endogenous metabolites in pooled human plasma obtained by CE-MS. Abbreviations: MT, migration time.

| Compound | m/z value | MT RSD(%) | Area RSD(%) | Area ratio 1 RSD(%)* | Area ratio 2 RSD(%)* |
|----------|-------------|-----------|-------------|----------------------|----------------------|
|----------|-------------|-----------|-------------|----------------------|----------------------|

| | | | | | |
|----------------------------|---------|-----|-----|-----|-----|
| Glycine | 76.039 | 1.6 | 8.9 | 3.4 | 3.3 |
| Serine | 106.05 | 2.1 | 8.4 | 3.1 | 2.8 |
| Proline | 116.071 | 2.4 | 6.7 | 2.9 | 2 |
| Valine | 118.086 | 2.1 | 6.6 | 2.4 | 1.6 |
| Threonine | 120.066 | 2.3 | 7.9 | 3.3 | 3.1 |
| Creatine | 132.077 | 1.7 | 7.1 | 2.9 | 2.5 |
| Asparagine | 133.061 | 2.3 | 7.1 | 2.5 | 2.1 |
| Ornithine | 133.097 | 1.2 | 7.7 | 2.8 | 2.5 |
| Glutamine | 147.076 | 2.3 | 7.6 | 2.6 | 2.2 |
| Glutamic acid | 148.06 | 2.4 | 6.9 | 2.9 | 2.4 |
| Phenyl-D5-alanine (IS2) | 171.123 | 2.4 | 6.1 | NA | NA |
| Arginine | 175.119 | 1.3 | 7.2 | 3.5 | 3 |
| L-Methionine Sulfone (IS1) | 182.048 | 2.7 | 6.6 | NA | NA |
| L-Alanine | 90.055 | 1.8 | 8.2 | 3.6 | 3 |
| L-Isoleucine | 132.102 | 2.1 | 4.4 | 4.2 | 3 |
| L-Leucine | 132.102 | 2.2 | 5.9 | 2.3 | 1.1 |
| L-Lysine | 147.113 | 1.2 | 7.4 | 2.6 | 2 |
| L-Methionine | 150.058 | 2.3 | 9.1 | 4.3 | 4.5 |
| L-Histidine | 156.077 | 1.4 | 6.5 | 2.4 | 2.4 |
| L-Phenylalanine | 166.086 | 2.4 | 6.1 | 2.8 | 1.5 |
| L-Tyrosine | 182.081 | 2.5 | 6.9 | 4 | 3.3 |

* Area ratio 1 is representing the corrected areas for the first internal standard, L-Methionine Sulfone. The second internal standard is Phenyl-D5-alanine and the correction for this internal standard resulted in the RSD values of area ratio 2.

2. Suitability of CE-MS for metabolic profiling of human plasma

To assess whether the CE-MS method for cationic metabolic profiling has the capability to deliver proper chemical information in biomarker discovery studies, a metabolomics study was simulated. For this purpose, isotope-labeled compounds were used as “spiked biomarkers”, while the capability of CE-MS to trace them back as biomarkers was examined. The selected isotope-labeled compounds included diverse chemical structures and were evenly spread over the analysis time. Another requirement was that the unlabeled form could be observed with a good detection sensitivity by CE-MS. Prior to performing the simulation study, some performance metrics of CE-MS for the analysis of the selected isotope-labeled compounds were determined. Special focus was on the accuracy of the method. The accuracy was determined comparing the spiked concentrations of the isotope-labeled compounds, with those experimentally estimated using calibration curves. The accuracy for all labeled compounds was found to be in the range of 85% to 115% (**Supplementary Table S1**).

Study I (**Table 1**) focused on analyzing three sets of plasma samples, i.e. class I is spiked with five isotope-labeled compounds, class II is spiked with six different isotope-labeled compounds, and set three consists of blank pooled human plasma (used as QC). In order to mimic a comparative metabolomics study, samples were constructed in a way as indicated in **Table 1**, in which the (introduced) concentration differences for the spiked compounds between the plasma samples can be found. In metabolomics, it is important to include QC samples to provide information about the robustness of the method ³³ and to mimic the sample composition, qualitatively and

quantitatively³⁴. Study II focused on more subtle differences by spiking the ‘markers’ in both groups with concentrations which differ by a factor 2 between both classes (**Table 2**), and with the absence of one compound in each class. For comparative metabolic profiling only compounds with RSD values for migration time and corrected peak area below 5 and 30%, respectively, as calculated for each class including QC samples ($n=23$), were considered for data analysis as those with higher values may be considered as spurious signals³⁵. **Supplementary Figure S1** shows extracted ion electropherograms obtained by CE-MS for the analysis of the spiked compounds in plasma samples of Group 2, Study II. **Supplementary Figure S2** shows extracted ion electropherograms obtained for the analysis of selected endogenous compounds in a QC sample by CE-MS (**Supplementary figure S2A**) including a mass spectrum for the same time window after noise subtraction (**Supplementary figure S2B**).

2.1. Data analysis for study I

The design of this first study introduced two groups of metabolites into individual classes, so it was merely the absence/presence of differences that needed to be distinguished. The whole corrected data matrix for the IS, including all the samples, which differs in composition of the mixtures mentioned in **Table 1**, are used for further data-analysis.

The first feature detection approach with MZmine (from strategy 1) resulted in more than 100 features. A feature is defined as a given mass-to-charge number with a defined migration time and intensity. Manual examination was then introduced to exclude falsely identified features, resulting in 70 features with peak heights above 1000. Except for the 11 compounds used for spiking, all the corrected peak areas detected in QC samples and class I and II samples showed variation far below 30% in RSD.

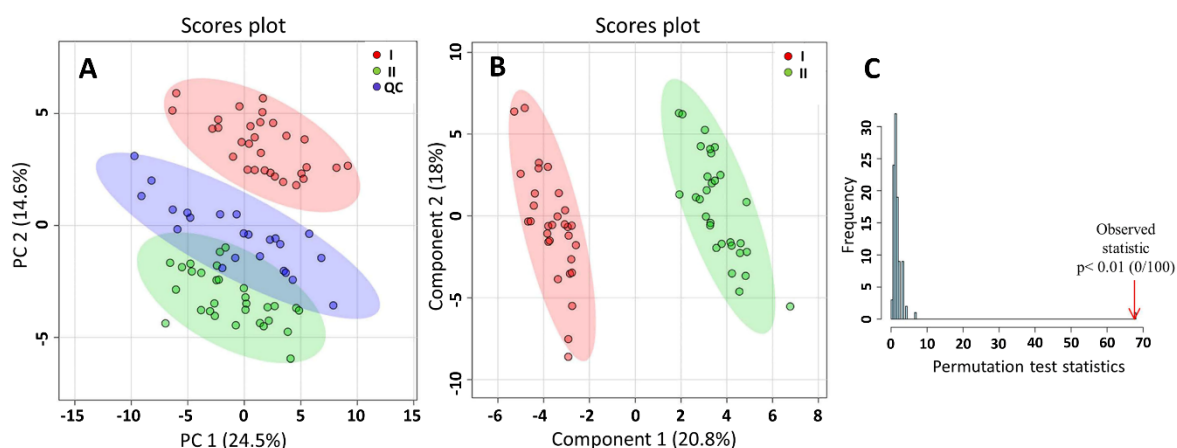


Figure 2. Multivariate results for study I obtained with MetaboAnalyst 4.0. (A) PC1-PC2 score plot for the area corrected by the IS. ●, ●, and ● symbols represent samples of class I, class II and QC group, respectively. The elliptic areas represent the 95% confidence regions; (B) PLS-DA scores plot. ● and ● symbols represent samples of class I and II, separately; (C) Permutation test results of the PLS-DA model (statistical test: separation distance (B/W)), number of permutations set at 100.

Strategy 2 does not need alignment of the peaks and is therefore suitable for CE data where, especially the late-migrating analytes, may experience significant migration shifts between samples³¹. 67 features were investigated, resulting in the parameters for the best MCR-ALS model, with an explained variance of 99.1% and an LOF value of 9.3%. For 67 resolved compounds, which can be related to endogenous metabolites or spurious markers, the RSD values for corrected peak areas and migration times were maximally 29.0% and 3.8%, respectively.

PCA was first conducted to investigate relations between groups. Auto-scaling was adopted as data-pretreatment to strip away the dominance of highly responsive/abundant metabolites and to render all metabolites equally important. PCA plots thus generated from study I, using both data-analysis approaches, are displayed in **Figures 2A and 3A**. Good separation of the three groups was observed in both cases. However, **Figure 3A** will result in better separation of the groups, which may be the result of a different number of features in the **X**-matrix resolved by another feature selection method. It is worth noticing that samples in all groups in both PCA plots sprawled mainly along PC1, suggesting that most variation could be explained by the instrumental drift, while the difference between the groups was along PC2. However, no QC correction was performed because of the lack of spiked markers in the QC samples, which are pooled human plasma samples. The two spiked groups were well separated in **Figure 3B**. Then a supervised analysis is performed to build a classification model and to identify the features responsible for the classification.

PLS-DA is a commonly used classification method in metabolomics studies, because of its ability to identify biomarkers from the loadings of the model²⁹. In the first data analysis strategy with MetaboAnalyst, a five-component PLS-DA model was established based on the leave-one-out cross validation (LOOCV) results. The obtained PLS-DA plot is shown in **Figure 2B**. The LOOCV parameters, $R^2=0.994$ and $Q^2=0.979$, indicated an excellently fitting and predictive PLS-DA model. In order to prevent PLS-DA from overfitting the data, the established model was validated by performing a permutation test to determine whether differences observed between groups are significant^{36,37}. In each permutation, a PLS-DA model is established between the data (**X**) and the permuted class labels (**y**), utilizing the previously determined optimal number of components. Then the ratio of the between-group sum of the squares and the within-group sum of squares, indicated as B/W-ratio, is calculated for the class assignment predictions of each PLS-DA model built. These ratios can be plotted in a histogram known as “the distribution of random class assignments”³⁶. If the B/W ratio of the original class assignment is part of this distribution, the differences between the two class assignments cannot be deemed significant. In the permutation test in strategy 1, the class assignment was permuted 100 times (histogram shown in **Fig. 2C**). The bar pointed out by the arrow represents the original sample. A p-value below 0.01 in 100 permutations means that not even once ($<0.01 \times 100$) did the permuted data yield better performance (higher B/W) than the original label, suggesting the significant difference between these two classes.

The second data analysis approach resulted in a less complex PLS-DA model with only one latent variable, based on the values for the non-error rate and the not-assigned samples. The PLS-DA model was evaluated by five-fold venetian blind cross-validation instead of LOOCV. Good merits of the model were demonstrated with an excellent predictive ability of 100% accuracy and a zero-error rate. Comparing the two PLS-DA models shows that a simpler model was obtained with Strategy 2, which is the result of the better separation of the two classes observed in the unsupervised PCA plot in **Figure 3A**.

The validation of supervised models is often lacking in metabolomics studies ^{28, 29, 38, 39}. The validation of the established models in both strategies was performed with cross-validation, LOOCV or five-fold venetian blind cross-validation, respectively. These cross-validation approaches are often conducted when only a limited number of samples are involved, as in the present study, but it was also reported that this approach may have the risk of over-fitting, especially LOOCV ²⁹.

VIP scores are often applied to select variables that are important in the projection in PLS-DA models and for the differentiation of the groups. A variable with a VIP value above 1.0 may be considered important ⁴⁰. Data analysis strategy 1 revealed 17 features and strategy 2 revealed 16 features (**Supplementary Table S2**) with VIP scores above 1.0. In the results from both data analysis strategies, the 11 spiked “markers” were detected with VIP scores above 2.0. The false discovery rates (FDR) were obtained from the unpaired non-parametric test in order to assess the incidence of false positives. All 17 features had FDR values below 0.05 (data not shown), indicating that these features can indeed be regarded as potential “markers”.

The second data analysis strategy also took into consideration the results generated from the non-parametric test to confirm whether the results of the VIP score for the defined features were significantly different for comparing class I with class II, and it resulted in p-values below 0.0001 for all 16 *m/z* values. Furthermore, the PC1-PC2 loadings plot (**Fig. 3C**) showed similar findings as the statistical tests, i.e. five extra features (9, 13, 14, 20, 21), apart from the 11 spiked compounds are among the highest absolute loadings, indicating their contribution to the group classification. Among these detected features, *m/z* 158.101 showed a comparable VIP score to the rest of the spiked features in data analysis strategy 2. The individual standard solutions of the spiked compounds were injected and analyzed in an attempt to determine the source of feature *m/z* 158.101. **Figure 4** clearly shows that *m/z* 158.101 and *m/z* 159.103 are detected at the same migration time, thereby suggesting that *m/z* 158.101 could potentially be another labeled form of the same original compound (L-Glutamic acid). The reason why this feature was not detected in strategy 1 is that the peak height of *m/z* 158.101 did not always meet the peak height threshold of 1000, and got omitted from the feature list by the filtering function within MZmine.

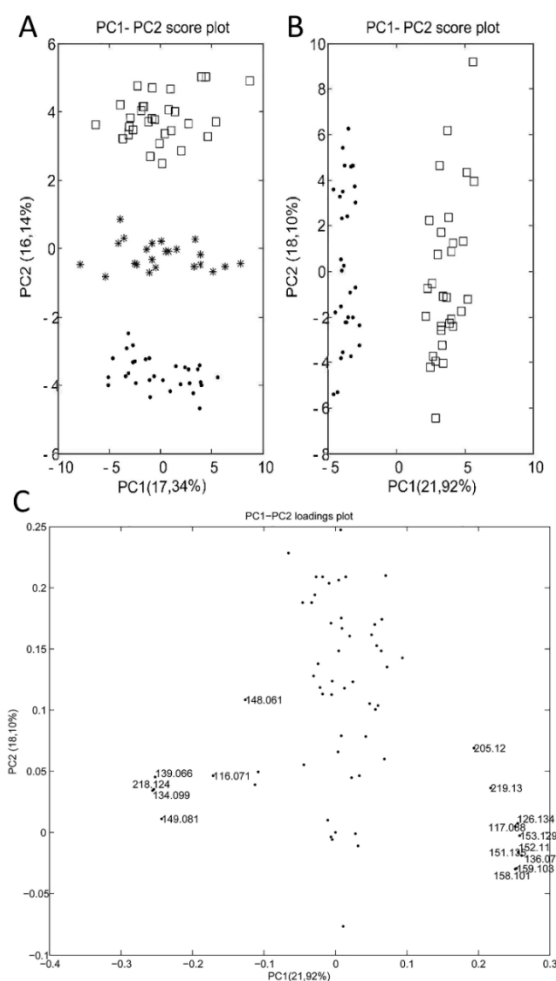


Figure 3. (A) PC1-PC2 score plot obtained for the **X** matrix of study I of the second data analysis strategy using internal standard correction and autoscaling; Quality Control samples are represented by stars; Class I by dots and Class II by squares; (B) PC1-PC2 score plot for the two groups using internal standard correction and autoscaling; (C) PC1-PC2 loadings plot (for numbers see supplementary materials **Table S2**).

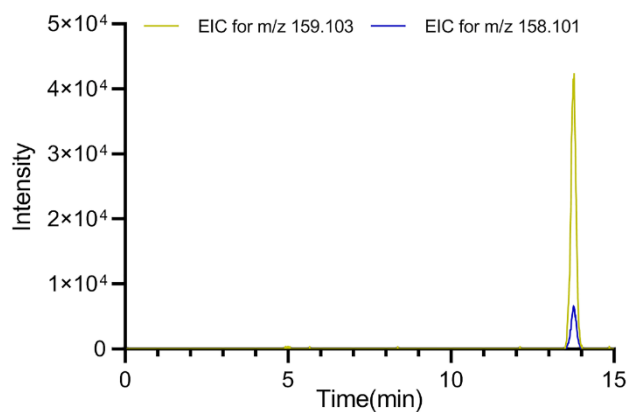


Figure 4. Extracted ion electropherogram obtained by injecting the standard solution of compound m/z 159.103, resolving the contaminant m/z 158.101.

Apart from the features discussed above, there are still some unaccounted features with a VIP score above 1.0. However, the reason why these variables ended up being “markers” is not clear at this stage. Strategy 2 resulted in 5 unaccounted markers (9, 13, 14, 20, 21), which could be related to an impurity. Strategy 1 resulted in 6 spurious markers (13, 15-19). Strategy 2 resulted in better results for all steps performed in study I. The separation of the different groups was clearer, and the PLS-DA model was much simpler for a better performance and less unknown markers are indicated. In the future, it would be interesting to investigate the importance of the unaccounted markers in more detail.

2.2. Data analysis for study II

Study I showed that spiked “markers” were detected by both data analysis strategies, but it is important to stress that in real-life metabolomics studies, changes in the abundance of metabolites tend to be more subtle than those introduced in study I, where spiked metabolites were present in one group and not in the other. In the second study more subtle differences (**Table 2**) were introduced between the two classes, which anyway still might be larger than the very small metabolic differences that may actually occur between healthy and diseased individuals.

The data from the second study were subjected to the same analysis processes as study I. The application of MZmine resulted in 73 features, among which only 3 features had RSD values above 30%. Those features were deleted prior to further data analysis. The MCR-ALS model in strategy 2 resulted in 90 features with 99.2% explained variance and 9.2% LOF. After removing features with RSD values of peak area ratios over 30%, 84 remained in the data set.

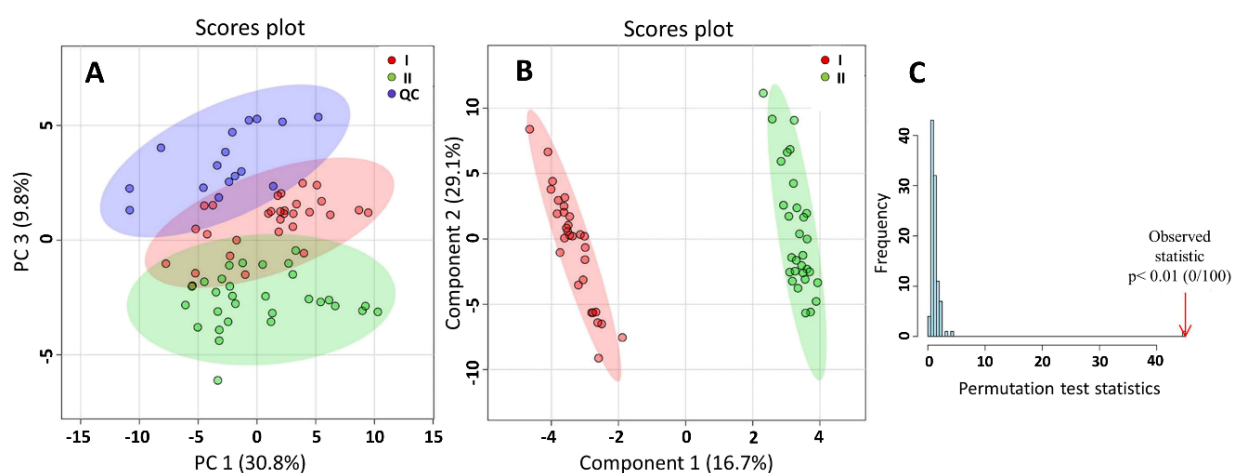


Figure 5. Multivariate results for study II obtained with MetaboAnalyst 4.0. A) PC1-PC3 score plot for the area corrected by the IS. ●, ● and ● symbols represent samples of class I, class II and QC group, respectively. The elliptic areas represent the 95% confidence regions; B) PLS-DA scores plot. ● and ● symbols represent samples of class I and II, respectively; C) Permutation test results of the PLS-DA model (statistical test: separation distance (B/W)), number of permutations set at 100.

PCA score plots were generated after auto-scaling the peak area ratios in both strategies, as shown in **Figures 5A and 6A**. As in study I, the QC samples were distributed along PC1, indicating that the largest variation in the first PC was not related to the group information. The auto-scaled data were well separated along PC3. The PC1-PC2 score plot for only the two spiked groups (**Fig. 6B**) shows that these groups tend to be separated, despite the subtle differences between the profiles.

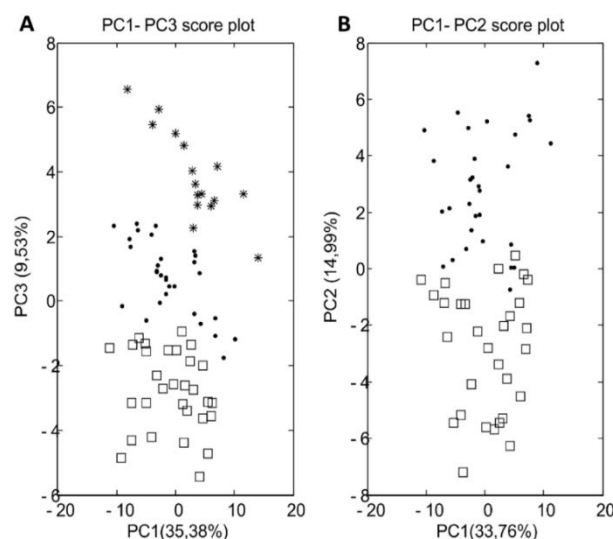


Figure 6. A) PC1-PC3 Score plot of study II obtained with the second data analysis strategy using internal standard correction and autoscaling. Quality Control samples are represented by stars, class I by dots and class II by squares; B) PC1-PC2 Score plot for the two groups using internal standard correction and autoscaling.

A PLS-DA model with five components was established in the first data analysis strategy, using MetaboAnalyst, with parameters $R^2=0.998$ and $Q^2=0.995$ acquired by LOOCV. A 100-permutations test using separation distance (B/W) as test parameter, was performed with the observed statistic having a $p<0.01$ (**Fig. 5C**). The second data analysis strategy resulted in a PLS-DA model with one latent variable, with good predictive ability (100% accuracy and non-error rate) and the model was validated as mentioned above by the same cross-validation method, resulting in good parameters (100% accuracy and non-error rate). Based on the criteria $VIP>1.0$, and FDR or $p<0.05$ in the non-parametric tests in both data analysis strategies, the 11 spiked compounds were identified as “biomarkers” in study II (**Supplementary Table S3**). However, again in Strategy 2 the peak with m/z value 158.101 was indicated as a biomarker.

Again features with peak heights over 1000 were extracted for further data analysis, because smaller peaks are difficult to measure precisely and might increase the chance of false biomarker identification^{41, 42}. For a reliable detection of low abundant metabolites with the current CE-MS set-up, the use of an in-capillary preconcentration technique is needed^{43, 44}.

In summary, both data processing and analysis strategies resulted in similar findings, despite the small differences observed with the VIP scores. An interesting phenomenon is that the three groups were better separated in the PCA score plots using the second strategy. Additionally, the

better separation may be the result of simpler PLS-DA models in the second strategy compared to the 5 component PLS-DA model in the first strategy. This might be the consequence of the different numbers of m/z values included in the **X**-matrix. However, the better separation results between the groups with data analysis strategy 2 makes this method more suitable. However, strategy 2 is more time-consuming and more difficult for an analyst less skilled with data analysis approaches. In real-life cases, targeted metabolomics studies are usually required after untargeted analysis, quantifying the earlier indicated potential “biomarkers”, in order to validate and quantify the extent of changes in “biomarkers”. The essential message here is that all spiked features could be distinguished by univariate and multivariate analyses of the recorded sheath-liquid CE-MS data. This clearly emphasizes the utility of sheath-liquid CE-MS in metabolomics studies of human plasma samples.

Conclusions and perspectives

In metabolomics, CE-MS has become a useful analytical technique for the profiling of highly polar and charged compounds. In the context of biomarker discovery, it is important to assess whether a given analytical technique provides the proper chemical information and does not result in false positive or negative decisions. In this study, the utility of CE-MS for this purpose was evaluated. Different chemometric analysis procedures were used in order to confirm each other’s results and to show that both data analysis strategies give similar information. As shown, the second strategy reveals fewer spurious markers in study I and shows a better separation between the groups in study II. However, the latter approach is more difficult to perform than the use of the MetaboAnalyst software.

Additionally, in this work the two data analysis strategies resulted in very similar outcomes, as expected, and showed that CE-MS in combination with data analysis tools may help to uncover the spiked “biomarkers”. Overall, this work emphasized the capability of CE-MS in metabolic profiling studies of human plasma. The usefulness of CE-MS for comparative metabolic profiling may also be evaluated using a comparison or cross-validation with another analytical technique, such as, for example HILIC-MS or NMR spectroscopy. In this case it would be important to focus in such a study on the compounds that can be covered by each analytical technique. For a follow-up study, it would also be interesting to use very small differences in concentration levels for the spiked compounds between sample groups in order to better simulate the actual biological situation in which metabolic differences may be very subtle or to make use of real-life samples.

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Supplementary Materials

Supplementary file S1: MZMine 2.32 procedure and settings

Raw data import

Peak detection

- Mass detection:
 - Mass detector: Centroid
 - Noise level: 10^3
 - MS level: 1
- Chromatogram builder:
 - Min time span (min): 0.02
 - Min height: 10^3
 - M/Z tolerance: 0.01 m/z or 12 ppm
 - Retention time: 5-20 min
- Chromatogram deconvolution:
 - Algorithm: Local minimum search
 - Chromatographic threshold: 65%
 - Search minimum in RT range (min): 0.05
 - Minimum relative height: 5%
 - Minimum absolute height: 1000
 - Min ratio of peak top/edge: 2
 - Peak duration range(min): 0.05-0.8

Alignment

- Retention time normalizer:
 - MZ tolerance: 0.01 m/z or 12 ppm
 - Retention time tolerance: 15% Relative
 - Minimum standard intensity: 1000
- Alignment: RANSAC Aligner
 - MZ tolerance: 0.01 m/z or 12 ppm
 - RT tolerance: 10% Relative
 - RT tolerance after correction: 5% Relative
 - RANSAC iterations 10000
 - Minimum number of points 20%
 - Threshold value: 0.05
 - Linear model ☒
- Filtering: peak list rows filter
 - Minimum peaks in a row: 14
 - Minimum peaks in an isotope pattern: 0
 - m/z : auto range
 - Retention time: 5-20 min
 - Peak duration range: 0-0.8
- Gap filling: Same RT and m/z gap filler
 - MZ tolerance: 0.005 m/z or 12 ppm
- Filtering: duplicate peak filter
 - MZ tolerance: 0.005 m/z or 12 ppm
 - RT tolerance: 5%

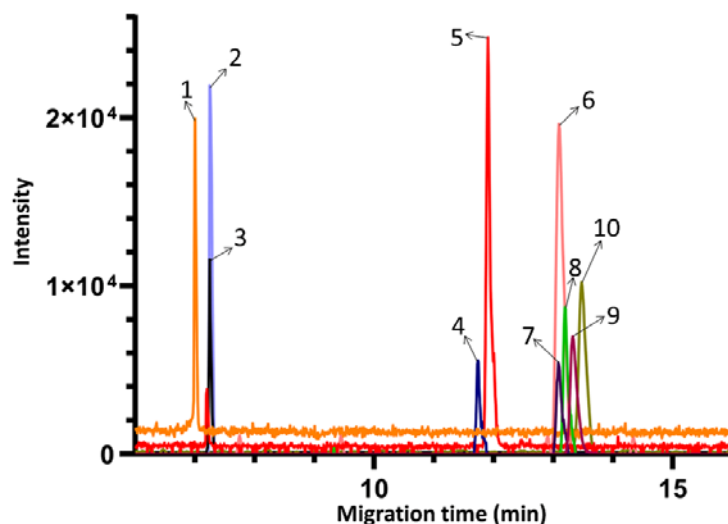


Figure S1. Extracted ion electropherograms obtained by CE-MS for the analysis of spiked compounds in plasma samples from Group 2, Study II. The highlighted compounds are: 1. Creatinine (N-methyl-D3); 2. L-Lysine ($^{13}\text{C}_6$); 3. L-Lysine (4,4,5,5-D4); 4. L-Valine (D5); 5. L-Isoleucine (^{13}C ; ^{15}N); 6. L-Asparagine ($^{13}\text{C}_2$; $^{15}\text{N}_2$); 7. L-Asparagine (2,3,3-D3); 8. L-Tryptophan ($^{13}\text{C}_{11}$; $^{15}\text{N}_2$); 9. L-Glutamine ($^{13}\text{C}_2$); 10. L-Glutamic acid ($^{13}\text{C}_5$; D5; ^{15}N).

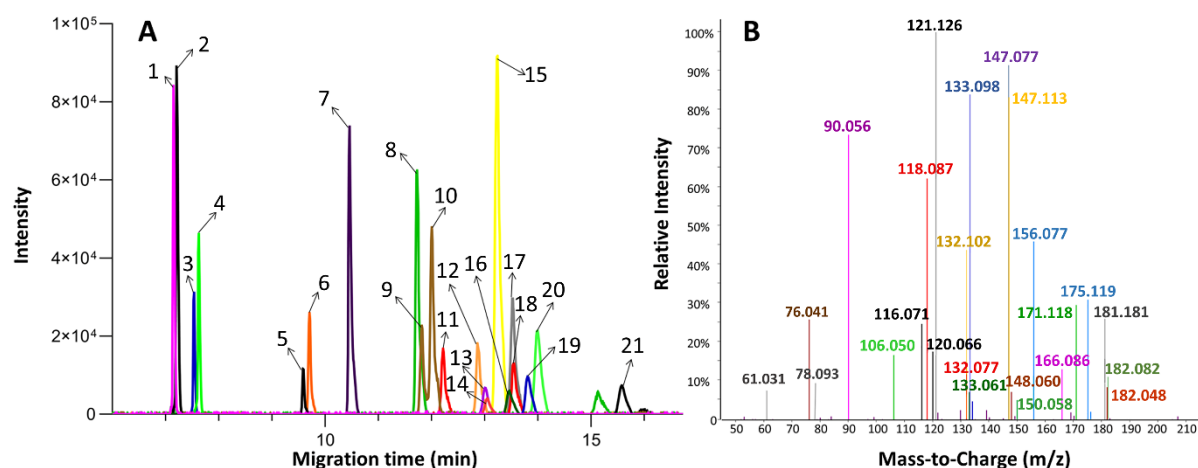


Figure S2. Extracted ion electropherograms. (A) obtained for selected endogenous compounds in a QC sample from Study II by CE-MS, and mass spectrum (B) extracted from the same time window after noise subtraction. The highlighted compounds are as follows: 1. Ornithine (m/z 133.098); 2. L-Lysine (m/z 147.113); 3. Arginine (m/z 175.119); 4. L-Histidine (m/z 156.077); 5. Creatine (m/z 132.077); 6. Glycine (m/z 76.041); 7. L-Alanine (m/z 90.056); 8. Valine (m/z 118.087); 9. L-Isoleucine (m/z 132.102); 10. L-Leucine (m/z 132.102); 11. Serine (m/z 106.050); 12. Threonine (m/z 120.066); 13. Asparagine (m/z 133.061); 14. L-Methionine (m/z 150.058); 15. Glutamine (m/z 147.077); 16. Glutamic acid (m/z 148.060); 17. Phenyl-D5-alanine (IS2) (m/z 171.118); 18. L-Phenylalanine (m/z 166.086); 19. L-Tyrosine (m/z 182.082); 20. Proline (m/z 116.071); 21. L-Methionine sulfone (IS1) (m/z 182.048).

Table S1. CE-MS performance metrics for the analysis of isotope-labeled compounds in plasma.

Precision and accuracy of peak areas were based on the analysis of three plasma samples spiked with 40 μM of the labeled compounds.

| Metrics | Determination coefficient (R ²)* | Precision (%RSD) (n=3) | Accuracy (Mean \pm SD) (n=3) |
|--|--|------------------------|--------------------------------|
| Creatinine(N-methyl-D3) | 0.9992 | 8.10% | 100.27 \pm 5.45 |
| L-Asparagine (¹³ C ₂ ; ¹⁵ N ₂) | 0.9909 | 3.00% | 89.37 \pm 1.35 |
| L-Asparagine (2,3,3-D3) | 0.9973 | 7.50% | 95.60 \pm 3.30 |
| L-Glutamic acid (¹³ C ₅ ; D ₅ ; ¹⁵ N) | 0.9995 | 4.20% | 102.40 \pm 2.95 |
| L-Glutamine (¹³ C ₂) | 0.9987 | 5.30% | 94.10 \pm 1.56 |
| L-Glutamine (2,3,3,4,4-D5) | 0.998 | 5.10% | 98.40 \pm 1.14 |
| L-Isoleucine (¹³ C; ¹⁵ N) | 0.9992 | 7.70% | 104.27 \pm 6.26 |
| L-Lysine (¹³ C ₆) | 0.9991 | 9.50% | 92.93 \pm 5.39 |
| L-Lysine (4,4,5,5-D4) | 0.9991 | 12.80% | 91.43 \pm 8.04 |
| L-Tryptophan (¹³ C ₁₁ ; ¹⁵ N ₂) | 0.9988 | 4.80% | 102.80 \pm 3.38 |
| L-Valine (D5) | 0.9991 | 3.70% | 101.47 \pm 1.91 |

*Peak areas were corrected with IS2. Linearity determined in the range from 10 to 100 μM .

Table S2. Variable Importance in Projection (VIP) scores obtained for study I using data analysis strategies 1 and 2. The reported *m/z* values have VIP values above 1.0.

| Number | <i>m/z</i> value | Compound | VIP score strategy 1 | VIP score strategy 2 |
|--------|------------------|--|----------------------|----------------------|
| 1 | 152.110* | L-Glutamine (2,3,3,4,4-D5) | 2.27 | 2.17 |
| 2 | 151.135* | L-Lysine (4,4,5,5-D4) | 2.16 | 2.17 |
| 3 | 136.078* | L-Asparagine (2,3,3-D3) | 2.26 | 2.17 |
| 4 | 218.124* | L-Tryptophan (¹³ C ₁₁ ; ¹⁵ N ₂) | 2.25 | 2.15 |
| 5 | 134.099* | L-Isoleucine (¹³ C; ¹⁵ N) | 2.24 | 2.14 |
| 6 | 153.129* | L-Lysine (¹³ C ₆) | 2.23 | 2.14 |
| 7 | 139.066* | L-Asparagine (¹³ C ₂ ; ¹⁵ N ₂) | 2.14 | 2.12 |
| 8 | 159.103* | L-Glutamic Acid (¹³ C ₅ ; D ₅ ; ¹⁵ N) | 2.20 | 2.11 |
| 9 | 158.101 | Unknown | NA | 2.09 |
| 10 | 126.134* | L-Valine (D5) | 2.14 | 2.09 |
| 11 | 117.088* | Creatinine (N-methyl-D3) | 2.18 | 2.08 |
| 12 | 149.081* | L-Glutamine (¹³ C ₂) | 2.13 | 2.05 |
| 13 | 116.071 | Proline | 1.33 | 1.27 |
| 14 | 148.061 | Glutamic acid | NA | 1.16 |
| 15 | 158.154 | Unknown | 1.49 | NA |
| 16 | 148.079 | Unknown | 1.30 | NA |
| 17 | 246.205 | Unknown | 1.06 | NA |
| 18 | 184.170 | Unknown | 1.24 | NA |
| 19 | 104.111 | Unknown | 1.30 | NA |
| 20 | 219.130 | Unknown | NA | 1.58 |
| 21 | 205.120 | Unknown | NA | 1.37 |

* These *m/z* values are related to the 11 spiked compounds. NA, not applicable

Table S3. Variable Importance in Projection (VIP) scores obtained for study II using data analysis strategies 1 and 2. All m/z values have VIP values above 1.0.

| m/z value | Compound | VIP score strategy 1 | VIP score strategy 2 |
|-------------|--|----------------------|----------------------|
| 218.124* | L-Tryptophan ($^{13}\text{C}11,^{15}\text{N}2$) | 2.50 | 2.63 |
| 136.078* | L-Asparagine (2,3,3-D3) | 2.47 | 2.60 |
| 159.103* | L-Glutamic acid ($^{13}\text{C}5;\text{D}5;^{15}\text{N}$) | 2.38 | 2.57 |
| 126.134* | L-Valine (D5) | 2.45 | 2.57 |
| 149.081* | L-Glutamine ($^{13}\text{C}2$) | 2.45 | 2.57 |
| 158.101 | Unknown | NA | 2.57 |
| 134.099* | L-Isoleucine ($^{13}\text{C};^{15}\text{N}$) | 2.46 | 2.55 |
| 139.066* | L-Asparagine ($^{13}\text{C}2;^{15}\text{N}2$) | 2.46 | 2.55 |
| 151.135* | L-Lysine (4,4,5,5-D4) | 2.41 | 2.53 |
| 117.088* | Creatinine (N-methyl-D3) | 2.35 | 2.50 |
| 153.129* | L-Lysine ($^{13}\text{C}6$) | 2.38 | 2.47 |
| 152.110* | L-Glutamine (2,3,3,4,4-D5) | 2.49 | 2.44 |

* These m/z values are related to the 11 spiked compounds. NA, not applicable.