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ELECTROPHORESIS

RESEARCH ARTICLE

# Capillary electrophoresis–mass spectrometry for creatinine analysis in residual clinical plasma samples and comparison with gold standard assay

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## Abstract

When hospitalized, infants, particularly preterm, are often subjected to multiple painful needle procedures to collect sufficient blood for metabolic screening or diagnostic purposes using standard clinical tests. For example, at least 100 µL of whole blood is required to perform one creatinine plasma measurement with enzymatic colorimetric assays. As capillary electrophoresis–mass spectrometry (CE–MS) utilizing a sheathless porous tip interface only requires limited amounts of sample for in-depth metabolic profiling studies, the aim of this work was to assess the utility of this method for the determination of creatinine in low amounts of plasma using residual blood samples from adults and infants. By using a starting amount of 5 µL of plasma and an injection volume of only 6.7 nL, a detection limit (S/N = 3) of 30 nM could be obtained for creatinine, and intra- and interday precisions (for peak area ratios) were below 3.2%. To shorten the electrophoretic separation time, a multi-segment injection (MSI) strategy was employed to analyze up to seven samples in one electrophoretic run. The findings obtained by CE–MS for creatinine in pretreated plasma were compared with the values acquired by an enzymatic colorimetric assay typically used in clinical

**Abbreviations:** EMA, European Medicines Agency; ISTD, internal standard; MSI, multi-segment injection; QC, quality control.

Marlien van Mever and Bingshu He contributed equally to this study.

laboratories for this purpose. The comparison revealed that CE–MS could be used in a reliable way for the determination of creatinine in residual plasma samples from infants and adults. Nevertheless, to underscore the clinical efficacy of this method, a subsequent investigation employing an expanded pool of plasma samples is imperative. This will not only enhance the method's diagnostic utility but also contribute to minimizing both the amount and frequency of blood collection required for diagnostic purposes.

**KEYWORDS**
comparison enzymatic colorimetric assay, creatinine, metabolic profiling, multi-segment injection, residual plasma samples

## 1 | INTRODUCTION

In neonates, such as asphyxiated newborns, creatinine levels are frequently monitored multiple times per week upon admission to the hospital, requiring repeated blood sampling procedures involving heel lances or venipunctures [1, 2]. These procedures are not limited to sick infants; even healthy infants undergo approximately 12 needle procedures within their first year of life [3, 4]. Although necessary, these painful procedures can have immediate adverse effects, such as pain and enhanced anemia in preterm neonates, potentially necessitating blood transfusions [5, 6]. Furthermore, long-term effects were also observed regarding the changes in immune and cognition function, adaptations in brain development, such as cortical thickness and negative impacts on stress responsiveness and emotional health [7–9]. One of the solutions for reducing the required (starting) sample amount and subsequently easing the painful procedure for children is considering the use of microscale analytical methods that are well suited for analyzing small-volume biological samples.

Our group has developed capillary electrophoresis–mass spectrometry (CE–MS) methods employing a sheathless porous tip interface for the selective and sensitive profiling of polar ionogenic metabolites, including creatinine, in various volume-restricted biological samples over the past few years [10–12]. More recently, the Metabo-ring trial revealed that our CE–MS method can be used in a reproducible and robust way for compound annotation when using effective electrophoretic mobilities [13]. Moreover, a simulated metabolomics study using human plasma revealed that the right chemical information could be obtained when comparing two artificial sample sets based on their recorded metabolic profiles by CE–MS [14]. Another interesting feature of CE–MS, notably in the context of analyzing clinical samples, is the possibility of using a multi-segment approach, which allows the analysis of up to 10 samples within a single electrophoretic run, thereby improving sample throughput without compromising separation resolution [15–18]. Recently, CE–MS utilizing a multi-segment injection (MSI) approach has been used for the analysis of creatinine [19], polyamines [20], untargeted profiling of lipids [21], and for the monitoring of drug metabolism [17].

Based on the encouraging CE–MS studies reported until now for the profiling of charged metabolites in volume-restricted samples, the aim of this study was to assess the utility of our previously developed sheathless CE–MS method for the determination of creatinine in minute amounts of human plasma. Small aliquots of pooled human plasma were used for the initial part of the study to examine whether creatinine could be determined reliably when using the complete analytical workflow and isotope-labeled internal standards (ISTDs). An MSI strategy was also considered in order to speed up the electrophoretic analysis. Then, creatinine levels were determined in low amounts of residual plasma from adults and children, and the obtained findings have been compared with an enzymatic colorimetric analyzer (Architect c4000, Abbott Laboratories), which is typically used in clinical chemistry laboratories for creatinine measurements. Though used in a routine way, this approach requires at least 100 µL of blood for a single-creatinine measurement.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and materials

LC–MS-grade methanol, chloroform, and acetic acid were purchased from Biosolve B.V. Purified water was obtained from a Milli-Q PF Plus system (Merck Millipore). Amicon Ultra-0.5 Centrifugal Filter Unit, standard reagent creatinine (anhydrous, ≥98%), proline, valine, leucine, isoleucine, hypoxanthine, methionine, arginine,

**TABLE 1** Concentrations of creatinine, creatinine-$d_3$, and creatinine-$^{13}$C in calibrant solutions.

| Compound name | Concentration (μM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C0 | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 |
| Creatinine | 0 | 0.5 | 1 | 2.5 | 5 | 10 | 20 | 40 | 60 |
| Creatinine-$d_3$ | 0 | 0.5 | 1 | 2.5 | 5 | 10 | 20 | 40 | 60 |
| Creatinine-$^{13}$C | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

tryptophan, creatine, tyrosine, threonine, serine, alanine, asparagine, glycine, glutamic acid, histidine, lysine, glutamine, phenylalanine, and Deuterated Standard Creatinine-methyl-$^{13}$C were purchased from Sigma-Aldrich. Deuterated standard Creatinine-methyl-$d_3$ was purchased from Cayman Chemical.

## 2.2 | Preparation of standard solutions

All the amino acid standards mentioned above were dissolved in Milli-*Q* water to a concentration of 1 mg/mL as a single-standard solution. Subsequently, they were mixed into a mixture solution with 5 μM of each compound upon qualitative analysis.

Creatinine, creatinine-$^{13}$C, and creatinine-$d_3$ were weighed and dissolved in Milli-*Q* water to 1 mg/mL stock solutions. Creatinine stock solution (1 mg/mL) was then diluted in water, resulting in seven calibration concentration levels. Creatinine-$^{13}$C solution was spiked as ISTD for each calibration level to reach a final concentration of 5 μM. The concentration of ISTD was chosen to be in the middle of the dynamic range, that is, equivalent to C4 concentration. Final calibrant concentrations are shown in Table 1. When it comes to method validation, creatinine-$d_3$ was spiked in plasma in three different concentration levels [low-level (1 μM), medium-level (10 μM), and high-level (40 μM)] as a substitute for endogenous creatinine.

## 2.3 | Collection of clinical samples

Children were eligible for inclusion when their physician decided that a plasma creatinine measurement was necessary. All physicians working at the children's department of Spaarne Gasthuis were asked to alert one of the researchers when they requested a creatinine measurement for a child under 5 years of age. When alerted, one of the researchers would inform the parents for written consent. Children could only be included if written consent was obtained and if there was plasma left over after all measurements requested by the treating physician were finished. From the leftover material, at least 20 μL plasma was taken. A maximum of three samples (each from a different creatinine measurement) per patient was set to prevent confounding. The samples were stored and transferred at −20°C. The Advisory Committee on Local Feasibility of Spaarne Gasthuis tested and approved this study. Because only leftover material was used and the participants did not undergo any extra procedures, testing of the protocol by the Medical Ethical Review Committee was not needed.

For the development of the CE–MS method, adult leftover samples were used from the Atalmedial Medical Diagnostics Centre. Leftover samples in this clinical laboratory can be used anonymously for quality improvement and method development without the need for explicit written consent. Atalmedial routinely informs patients about this procedure, with the possibility to object against the use of their biomaterials for method development.

The sheathless CE–MS measurements were blinded to the outcome of the first creatinine measurement as well as to the participants' personal and health data.

## 2.4 | Sample preparation

The plasma samples were prepared through protein precipitation, Bligh and Dyer extraction, and an extra ultrafiltration step to gain clean samples for sheathless CE–MS analysis. For the purpose of method validation and adult sample quantification, 10 μL plasma was subsequently spiked with 10 μL water, 10 μL 50 μM creatinine-$^{13}$C, and 10 μL creatinine-$d_3$ solutions. Followed by 160 μL methanol for protein precipitation, samples were vortexed thoroughly for 5 min and centrifuged at 15,800 g for 10 min. Once the supernatant was collected, 90 μL water and 120 μL chloroform were added for the extraction of creatinine. After another round of vortex and centrifugation, a 200 μL sample was taken from the water/methanol layer. Finally, the ultra-filtered centrifugation step was applied with Amicon Ultra-0.5 Centrifugal Filter Unit at 13,000 g, 4°C for 60 min. The centrifugal filter was prerinsed with water prior to use. Considering the compatibility of the filter material, 200 μL methanol and 100 μL water were added in the filter tube together with the supernatant from the last step. Samples were evaporated to dryness and

reconstituted in 100 µL water for sheathless CE–MS analysis. For the quantification analysis in children samples, 5 µL plasma was used in the sample preparation, and the processed samples were reconstituted in 25 µL water. The volume of ISTD solution and other solvents were also adjusted in order to align with other samples.

## 2.5 | CE–MS instruments and conditions

Sheathless CE–MS analyses were performed on CESI 8000 Plus System (AB Sciex, Inc.) coupled to a Sciex TripleTOF 6600 MS via a Sciex Nanospray III ionization source. The MSI separation was carried out using an OptiMS fused-silica capillary cartridge (id is 30 µm across the whole capillary, 91 cm length in total; od is 150 µm), which was regulated at 25°C with recirculating liquid coolant. Overall, 10% acetic acid (1.75 M, pH = 2.2) was used as background electrolyte (BGE). Sheathless CE–MS separation was started with a rinsing procedure, including 1 min water rinse at 75 psi (517,106.8 Pa), 1 min 0.1 M NaOH rinse at 75 psi (517,106.8 Pa), and another 1 min water rinse at 85 psi (586,054.4 Pa), followed by BGE flushing for 2.5 min on separation capillary at 85 psi (586,054.4 Pa), and 1 min on conductive capillary at 80 psi (551,580.6 Pa). Seven samples and, in total, six BGE spacers were injected alternately using hydrodynamic injection [2 psi (13,789.5 Pa) for 20 s] and short rinsing procedure [20 psi (137,895.1 Pa) for 0.3 min], respectively. On completion of MSI, a voltage of 30 kV for electrophoresis separation was applied on the capillary for 25 min. BGE was refilled into the capillary at 85 psi (586,054.4 Pa) for 2.5 min after each measurement.

TOF–MS was operated in positive ionization mode with an ionspray voltage floating at 1530 V. Ion source gases 1 and 2 were set to 0 psi, and curtain gas at 5 psi (34,473.8 Pa). Data was recorded at a $m/z$ range from 65 to 500. Mass accuracy was calibrated daily using an ESI-positive calibration solution prior to analysis.

## 2.6 | Method validation

### 2.6.1 | Linearity of detector response and limit of detection

The linearity of detector response was evaluated by injecting academic calibration solutions ($n = 3$) on 3 consecutive days. The calibration range is shown in Table 1. The recorded calibration lines of creatinine were fitted to a $1/x^2$ weighted linear regression model. The limits of detection (LODs) and limits of quantification (LOQs) were calculated as $\text{LOD} = 3 \times \text{Sa}/b$, $\text{LOQ} = 10 \times \text{Sa}/b$, where Sa stands for the standard deviation of the $y$-intercept, $b$ is the slope of the calibration curve.

### 2.6.2 | Precision and accuracy

The intra- and interday precisions and accuracy were evaluated by spiking three different concentrations of creatinine-$d_3$ [low-level (1 µM), medium-level (10 µM), and high-level (40 µM)] into pooled plasma samples over 3 different days ($n = 5$ per day). Precision was expressed as the relative standard deviations (RSD) of the peak area ratio of creatinine-$d_3$ and creatinine-$^{13}$C. An RSD of less than 15% was within the tolerance limits of the European Medicines Agency (EMA) guidelines. Accuracy was evaluated by back calculation of creatinine-$d_3$ concentrations based on the linear regression equation, and criteria of 15% relative error compared with their nominal concentrations were employed according to the EMA guideline.

### 2.6.3 | Recovery and matrix effects

Recovery and matrix effects were evaluated by spiking creatinine-$^{13}$C and creatinine-$d_3$ solutions to pooled plasma samples ($n = 5$) or water ($n = 5$). Recovery was calculated using the peak area ratio of creatinine-$d_3$ and creatinine-$^{13}$C measured before and after extraction. Similarly, the matrix effect was calculated by the peak area ratio of creatinine-$d_3$ and creatinine-$^{13}$C spiked within pooled plasma and water, both spiked before extraction.

## 2.7 | Data preprocessing

SCIEX MultiQuant version 3.0.3 (SCIEX) was used for peak integration and identification. The precursor ions selected for peak picking were $m/z$ 114.0662, 115.0662, and 117.0850 for creatinine, creatinine-$^{13}$C, and creatinine-$d_3$, respectively. Mass tolerance was set to 5 ppm. Absolute quantitation was calculated using the equation of the calibration curve and peak area ratios between creatinine and creatinine-$^{13}$C. For method performance comparison, creatinine concentrations from CE–MS were compared to the Architect c4000 results using Bland–Altman plots. To compute their disparities, statistical analysis was performed on GraphPad Prism 10. The following formulas were utilized:

Difference = Conc. Architect − Conc. CE–MS (see Figure 2A,D later)

Difference% = 100% × (Conc. Architect − Conc. CE–MS)/Conc. Architect (see Figure 2B,E later).

## 3 | RESULTS AND DISCUSSION

As indicated in Section 1 and considered a logical next step given our previous studies, the aim of this work was to assess the utility of sheathless CE–MS for diagnostic purposes in a clinical setting by performing the determination of creatinine in low amounts of plasma, using residual samples from the clinic, as a test case. To achieve this goal, method development was focused on describing performance metrics for creatinine analysis, including sample preparation and adjustment on MSI. A key aspect was the comparison of the creatinine concentration values obtained by CE–MS in plasma with an assay typically employed in clinical chemistry labs for this purpose in order to assess whether the same findings can be obtained by CE–MS but with significantly less blood material.

### 3.1 | CE–MS method for creatinine determination

Huang et al. proposed a CE–MS method using MSI for the quantification of urinary creatinine using a simple dilute-and-shoot sample preparation [19]. However, in this study, the aim was to determine creatinine in residual human plasma samples. For the latter matrix, rigorous sample preparation is needed prior to CE–MS analysis, in particular when using porous tip capillaries that have an inner diameter of only 30 µm. Therefore, sample preparation comprised Bligh and Dyer extraction and ultrafiltration, including 5–50 times dilution of the residual plasma samples from children and adults to avoid detector saturation. In the case of using MSI, two pooled plasma segments were injected at the beginning and the end as quality control (QC) samples. As for the academic calibrant line, C0 samples were injected. Creatinine-$^{13}$C and creatinine-$d_3$ were spiked in the QC samples as ISTDs to correct potential migration time shifts of creatinine. In order to obtain good peak shapes for creatinine when using MSI, the hydrodynamic injection time was optimized. As a result, samples were injected under 2.0 psi for 20 s, which corresponds to about 6.7 nL, followed by a BGE plug of 20 psi for 0.3 min (corresponding to 9.4% of the total capillary volume) among sample segments. The BGE plug length was also optimized for baseline separation of peaks when using the injection of seven discrete sample plugs in a single electrophoretic run.

The modified sheathless CE–MS method was validated according to the EMA guidelines for the validation of analytical methods, including linearity, precision, accuracy, recovery, and quantification. Typical electropherograms obtained for creatinine in academic calibrant solutions with multi-segment CE–MS are shown in Figure S1. The eight calibrant points were able to run within two measurements. Within a linear range from 0.5 to 40 µM, the coefficient of determination ($R^2$) value reached 0.998, and all the back calculation concentrations of the calibration standards were within ±10% of the nominal value, indicating an acceptable response function satisfactory for creatinine within the calibration range. The LOD and LOQ of creatinine were 0.03 and 0.09 µM, respectively (Table 2), when using an injection volume of 6.7 nL, which enabled the reliable determination of endogenous creatinine plasma levels. Although no peak tailing was observed for 60 µM creatinine, the accuracy of back calculation concentration was above 15%, presumably attributed to detector saturation, and as such, this calibrant solution was excluded from the calibration range.

The intra- and interday precisions were assessed using three different concentrations (low-level [1 µM], medium-level [10 µM], and high-level [40 µM], $n = 5$ for each concentration) of ISTDs spiked in pooled plasma. Triplicate samples were prepared for each level, and samples were measured on 3 consecutive days. Intra- and interday precisions were below 3.16%, indicating that the repeatability was within the tolerance limits (Table 3). In addition, during method development and sample measurement, no significant signal decrease was observed, which demonstrated stable performance over time.

Recovery and matrix effects were determined using creatinine-$d_3$ and were above 87% for the three concentration levels. Matrix effects were around 50% (Table 3), and deuterated creatinine was therefore employed to compensate for matrix effects and ensure quantification accuracy.

### 3.2 | Comparison with enzymatic colorimetric assay

To determine whether our CE–MS method yielded the right creatinine values in the plasma samples obtained from our clinical collaborators, a comparison with the gold standard assay for creatinine measurements is required, in this case, an enzymatic colorimetric assay using the Architect c4000. For the reliable determination of creatinine, both creatinine-$^{13}$C and creatinine-$d_3$ were spiked into human plasma samples, and QC samples were injected as the first and last sample plugs. As shown in Figure 1, the isotope-labeled ISTDs showed a similar variation trend to endogenous creatinine during the electrophoretic run, indicating that analyte response fluctuations during CE–MS analysis can be corrected by using peak area ratio for quantification.

**TABLE 2** Overview of validation parameters determined for creatinine and creatinine-$d_3$ by capillary electrophoresis–mass spectrometry (CE–MS).

| Compound | Calibration ranges (µM) | *m/z* | Slope | $R^2$ | LOD (µM) | LOQ (µM) |
|---|---|---|---|---|---|---|
| Creatinine | 1–40 | 114.0662 | 0.16 | 0.998 | 0.03 | 0.09 |
| Creatinine-$d_3$ | 1–40 | 115.0662 | 0.15 | 0.997 | 0.05 | 0.15 |

*Note*: $R^2$, coefficient of determination.
Abbreviations: LODs, limits of detection; LOQs, limits of quantification.

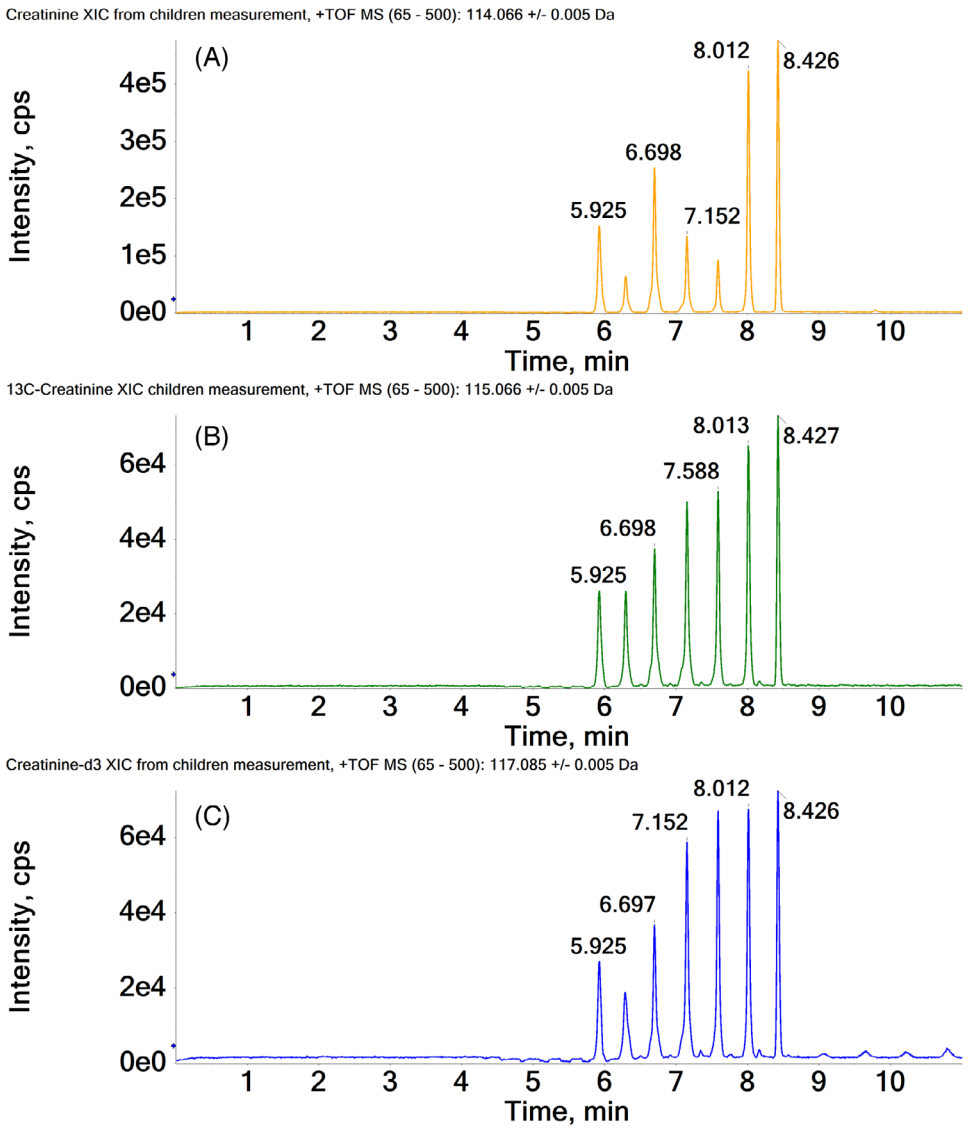


**FIGURE 1** Extracted ion electropherogram of (A) creatinine, (B) creatinine-$^{13}$C, and (C) creatinine-$d_3$ obtained with sheathless capillary electrophoresis–mass spectrometry (CE–MS) in children's plasma samples.

For a comprehensive comparison between sheathless CE–MS and Architect c4000, Bland–Altman plots and regression lines were constructed to illustrate concentration disparities relative to the standard concentrations determined with Architect c4000.

In adult samples, the mean creatinine concentration difference is 7.12 µM (0.73%), which is indicated by the red line in Figure 2A,B. The difference values were distributed close to zero and mean difference line, particularly in cases of lower creatinine concentrations. For the higher

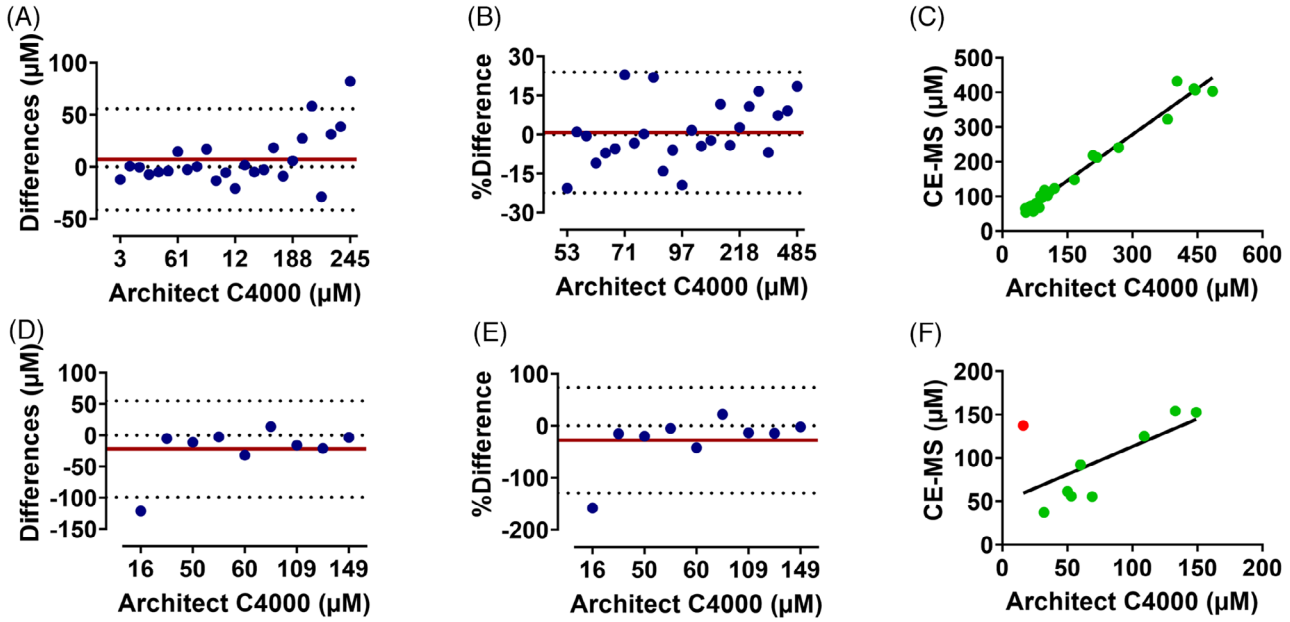


**FIGURE 2** Correlation between creatinine concentrations measured with sheathless capillary electrophoresis–mass spectrometry (CE–MS) and Architect C4000 for 25 adult (A–C) and 9 children samples (D–F). Bland–Altman plots where concentration differences are presented as micromolar (A and D) and (B and E) as a percentage; (C and F) regression lines between the two methods.

**TABLE 3** Intraday ($n = 5$) and interday ($n = 15$) precisions (relative standard deviations [RSD]%), accuracy (relative error [RE]%), matrix effect, and recovery of creatinine-$d_3$ calculated by using creatinine-$^{13}C$ as internal standard in plasma by capillary electrophoresis–mass spectrometry (CE–MS).

| Concentration levels | 1 µM | 10 µM | 40 µM |
|---|---|---|---|
| Intraday precision (%) | 3.0 | 2.7 | 1.8 |
| Interday precision (%) | 3.2 | 2.8 | 2.5 |
| Accuracy (%) | 0.6 | 8.5 | 15.1 |
| Matrix effect (%) | 51.1 | 33.3 | 43.7 |
| Recovery (%) | 94.4 | 94.4 | 87.6 |

concentration levels, larger differences were observed, most presumably attributed to MS detector saturation. The determination coefficient ($R^2$) for adult sample concentrations was 0.9789, whereas the correlation of concentrations for children samples appears to be suboptimal due to the presence of an outlier (Figure 2C,F). However, if we exclude the outlier, concentration differences of children samples are clustered around the mean difference line as well (Figure 2D,E). The important notion is that only 5 µL of plasma as starting material is needed for CE–MS, whereas 100 µL is required for the enzymatic colorimetric assay. Most of the data points are closely aligned with the mean difference line and remain within the limits of agreement (±2SD), indicating a good agreement between the two methodologies.

### 3.3 | CE–MS for metabolic profiling of residual plasma samples

Along with the ability for creatinine quantification, the utilization of high-resolution TOF–MS in full scan mode and low-pH BGE condition in CE enabled a wide range of polar ionogenic metabolites to be detected in the plasma sample. Some amino acids were identified based on their accurate mass and migration time paired with authentic standards. Extracted ion electropherograms of these amino acids and some compounds that still need to be identified are shown in Figure 3. Data were acquired from QC plasma samples. Despite seven sample plugs being injected in the same electrophoretic run, the peaks from the same compound were baseline separated except for a few isomers, such as leucine and isoleucine. However, as these two compounds were partially overlayed in the electropherogram (Figure 3C), better separation could be potentially gained by adjusting the composition of the BGE (i.e., the viscosity and thus the electrophoretic mobility). Using a longer capillary is not an option with our sheathless CE–MS, as the capillaries from the vendor have a defined capillary length. The main purpose of this part of the study was to solely demonstrate that, next to creatinine, many more metabolites could be detected by CE–MS in plasma, offering the possibility of further exploring the role of the proposed CE–MS method for biomarker discovery studies using metabolomics in a neonatology/pediatric context.

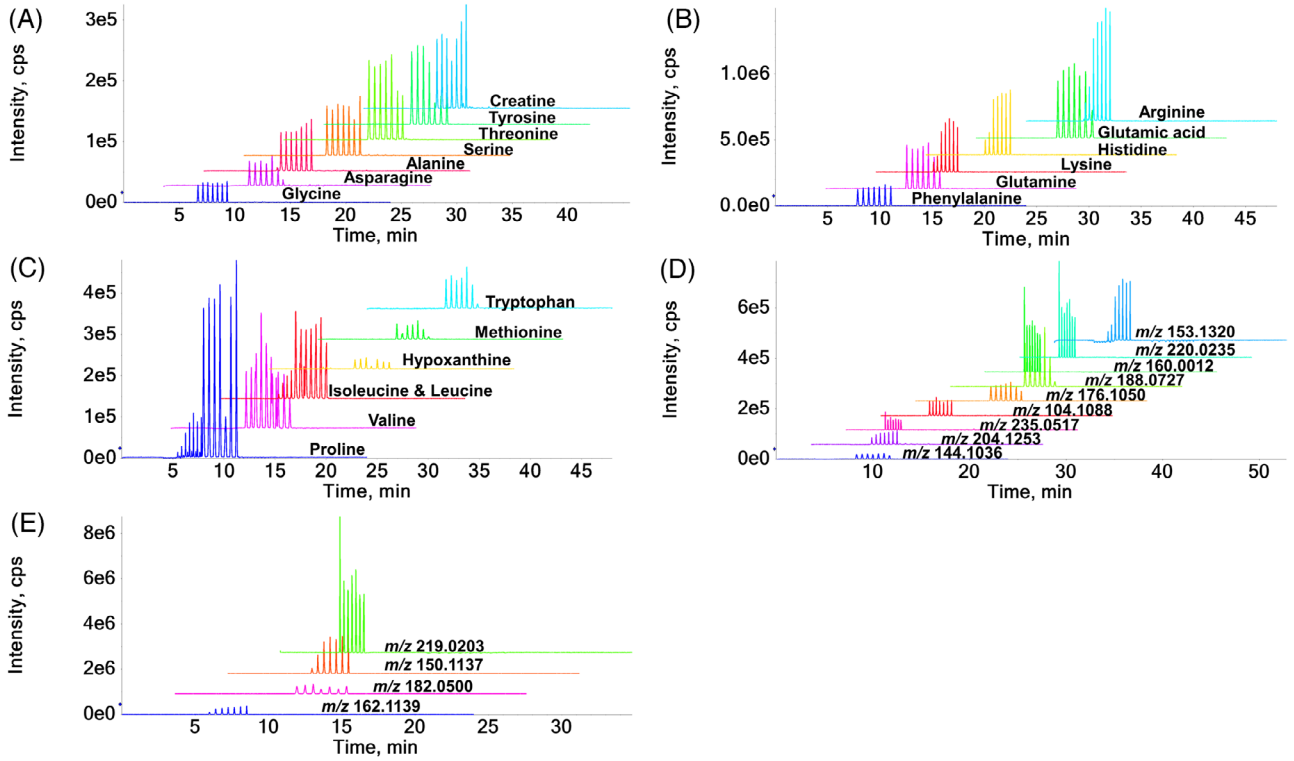


**FIGURE 3** Multiple extracted ion electropherograms for a selected number of analytes detected in human plasma next to creatinine (using a starting amount of 5 μL) by sheathless capillary electrophoresis–mass spectrometry (CE–MS) in positive ion mode: (A–C) amino acids identified by accurate mass and migration time; (D and E) identities of compounds are unknown at this stage. Separation conditions: background electrolyte (BGE), 10% acetic acid (1.75 M, pH 2.2); separation voltage, 30 kV.

## 4 | CONCLUDING REMARKS

In this study, sheathless CE–MS was used for the determination of creatinine in volume-limited plasma samples with the aim of assessing its utility for clinical studies, in particular for samples originating from a neonatology/pediatric context. A comparison with a gold standard assay has been made and revealed that sheathless CE–MS yielded comparable creatinine concentration values but with the use of significantly less plasma sample, that is, 5 versus 100 μL required for the clinical assay. Still, a follow-up study with many more plasma samples is needed for the comparison with the clinical assay in order to determine whether CE–MS is ready for handling samples from pediatrics, in particular for biomarker discovery studies, as the current study clearly revealed that, apart from creatinine, many more polar and charged metabolites could be observed by CE–MS in the volume-limited plasma sample.

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## CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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