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Matrix effect evaluation using multi-component post-column infusion in untargeted hydrophilic interaction liquid chromatography-mass spectrometry plasma metabolomics

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ABSTRACT

Metabolomics based on hydrophilic interaction liquid chromatography (HILIC) coupled with mass spectrometry (MS) is a powerful tool for polar metabolite identification and quantification to further contribute to biomarker discovery and disease mechanism elucidation. However, matrix effect (ME), which may lead to altered ionization efficiency due to co-eluting compounds, is a significant challenge during biological analysis. Therefore, ME evaluation plays a crucial role during method development. Two approaches to evaluate ME are using stable isotope labelled-internal standards (SIL-IS) and post-column infusion (PCI) of standards. In this study, we developed an untargeted HILIC-MS method by applying four PCI standards for ME evaluation. We found PCI is a compelling approach for ME assessment compared to SIL-IS method due to its advantage in untargeted analysis. Through the ME evaluation and chromatographic performance comparison of 18 SIL standards across three columns and three different mobile phase pH conditions, our findings revealed that the BEH-Z-HILIC column operated at pH 4 with 10 mM ammonium formate exhibited minimal ME and superior performance. The method showed exceptional linearity ($R^2 > 0.98$), reliable repeatability (RSD < 15 %), good inter-day precision (RSD < 30 %), and acceptable recovery (>75 %) for all SIL standards. Absolute matrix effect (AME) and relative matrix effect (RME) assessment in three plasma donors revealed a high consistency between PCI and SIL-IS approaches. Finally, this method coupled with the PCI approach was applied to 40 plasma samples. Fifty endogenous compounds were detected and their AME and RME were evaluated. Results showed that many compounds experienced severe ion suppression, though their ME variation between 40 samples is low. In conclusion, PCI method is a robust alternative for monitoring ME and evaluating ME of endogenous compounds during untargeted method optimization and biological analysis.

1. Introduction

Metabolomics has proven itself as an emerging field that seeks to identify and quantify metabolites for biomarker discovery and disease mechanism elucidation in a biological system [1–4]. Among the analytical techniques employed in metabolomics, liquid chromatography-mass spectrometry (LC-MS) has been the most widely used for metabolomics investigations due to its high sensitivity, enabling the detection and quantification of diverse metabolites with high precision [5–7]. LC-MS-based metabolomics can be applied in a targeted manner, which uses specific predefined precursor ion-product ion transitions (often referred to as tandem MS or MS/MS) [8,9] to achieve the highest sensitivity, and in an untargeted manner, which allows wide metabolome coverage for the discovery and screening of new metabolites [10–12].

Polar metabolites play a crucial role in the immune response and inflammatory process and are key biomarkers in clinical research [13, 14]. These metabolites, such as amino acids, organic acids and acylcarnitines, are for instance linked to rheumatoid arthritis [15] and oxidative stress in patients with COVID-19 [16]. Polar metabolites do

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not only have a diagnostic role but also a predictive capability for mortality [17]. Despite their biochemical importance, the analysis of polar metabolites by LC-MS is often challenged by poor retention on reversed-phase LC columns. Hydrophilic interaction liquid chromatography (HILIC) has emerged as a valuable chromatographic technique well-suited for polar metabolites, addressing this issue effectively. However, despite its potential advantages such as improved column retention and higher MS sensitivity because of increased ionization efficiency facilitated by a high proportion of organic mobile phase [18], HILIC has not been widely utilized by the scientific community. Common challenges associated with HILIC include complicated retention mechanisms, poor reproducibility, and low peak capacity [19]. The HILIC separation mechanism depends on the chemical properties of the stationary phase and mobile phase condition, including the type and concentration of the salt buffer, the pH, and the analyte structure [20]. Different HILIC columns and mobile phase conditions were systematically investigated in previous research by Contrepois et al. [11] and Hosseinkhani and coworkers [21]. Even though there is no consensus regarding the preferred HILIC stationary phase in the metabolomics field, the zwitterionic stationary phase seems to have better performance and wide coverage of polar metabolites in different biological matrices [11,21–24].

Despite LC-MS technology offering advanced features, developing a robust method that can be validated for routine application is challenging. A major challenge is the occurrence of matrix effect (ME) during biological analysis [25–28]. IUPAC defines ME as '*the combined effects of all components of the sample other than the analyte on the measurement of the quantity. If a specific component can be identified as causing an effect, then this is referred to as interference*' [29]. A prevalent interpretation of ME hypothesizes that the presence of co-eluting matrix interferences can impact the ionization efficiency of target analytes and influence the signal intensity due to the competition for available charges and access to the droplet surface during the electrospray process [27,30]. ME can be observed either as a decrease in signal (ion suppression) or as an increase in signal (ion enhancement). Apart from ionization, matrix interferences may also affect the extraction efficiency during sample preparation [31]. The occurrence of ME can significantly impact the precision, accuracy, linearity and limits of quantification and detection of the analytical method, leading to distorted or false results. The traditional way of minimizing ME involves the removal of all interfering co-eluting compounds during sample preparation prior to chromatography and ionization. This approach is only viable if the analytical method focuses on a narrow range of analytes with similar physicochemical properties. However, in metabolomics, analytical methods often target a plethora of compounds with varying physicochemical properties, particularly in untargeted analyses. Another possibility to reduce ME is to separate interferences from target analytes during chromatography by parameter optimization, including the chosen stationary phase, the particle size of the column and mobile phase conditions [32]. For instance, Chambers et al. [33] observed an obvious reduction in ME under different mobile phase pH conditions. Therefore, it is essential to assess and minimize ME during method development. The stable isotope labelled-internal standard (SIL-IS) method is a common and compelling approach to assess the ME. In this method, SIL standards are added to the samples prior to sample preparation and LC-MS analysis to evaluate the ME influence from sample preparation and ionization. Although effective, this becomes a costly approach if the analytical method, in the field of metabolomics, screens hundreds of metabolites and is hampered by the lack of commercial standards for some (unknown) metabolites. Alternative techniques for ME assessment include the post-extraction spike method [26], slope ratio analysis [34], and post-column infusion (PCI) of standards method [35]. The post-extraction spike method investigates the ME by comparing the response of the analyte in a standard solution to its response in a matrix which does not contain this analyte and spiked with the analyte at the same concentration after sample preparation. The Slope Ratio Analysis uses a calibration line added to the blank and matrix and compares the slopes of both calibration lines. Those two approaches are more focused on the quantitative evaluation of ME during validation, are retention time-dependent and require a blank matrix to be included. The PCI approach uses (a mixture of) SIL standard(s) or other physicochemically related standard(s) which is (are) continuously infused after the chromatographic separation prior to ionization, which only can evaluate the ME from ionization. Although it requires an additional pump, PCI is the most suitable approach for qualitative ME assessment to facilitate the optimal performance of untargeted method development, due to its retention time-independent character.

We hypothesize that ME evaluation by PCI method can facilitate untargeted HILIC method optimization and provide valuable information during biological analysis. To investigate this hypothesis, we first compared the ME evaluated by SIL-IS and PCI methods to explore the capacity of the PCI approach for ME assessment and the contribution of sample preparation to ME. Next, the PCI method was used for a HILIC column comparison between HILIC-Z, ZIC-cHILIC and BEH-Z-HILIC columns. In addition, the chromatographic performance of three columns was compared by using 18 SIL standards from different polar metabolite classes as our targets. Based on our results, the optimal column was selected and the salt concentration and pH of the mobile phases were optimized. Subsequently, linearity, repeatability, recovery, ME, and precision were characterized for this method. Finally, our HILIC-MS method with PCI was employed to investigate ME differences of polar metabolites in plasma samples from healthy and metabolically compromised older adults.

2. Materials and methods

2.1. Study cohort

The study consisted of two groups of 40 older adults from the Leiden Longevity Study (LLS) cohort [36] with high and low MetaboHealth scores. The MetaboHealth score was constructed from ^{1}H NMR-based metabolomics data in 44.000 individuals across all ages that predicts all-cause mortality and frailty among other endpoints [17]. This score is currently validated to indicate vulnerable older adults in the clinic, in population health and lifestyle intervention studies in the Vitality Oriented Innovations for the Lifecourse of the Ageing Society (VOILA) project. A high MetaboHealth score is associated with metabolically compromised older adults while a low score is associated with healthy older adults. In 2022, ethylenediaminetetraacetic acid (EDTA) plasma was isolated from the blood at the Leiden University Medical Center and stored at −80 °C for a couple of months until transport to the Metabolomics and Analytics Centre of Leiden University. There, all samples were stored at −80 °C until sub-aliquoting and metabolomics analysis.

2.2. Chemicals and materials

HPLC-grade acetonitrile (ACN), methanol (MeOH), isopropanol (IPA), chloroform and formic acid were purchased from Biosolve B.V. (Valkenswaard, Netherlands). Ammonium formate and ammonium hydroxide (28–30 wt% solution of ammonia in water) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was obtained from a Merck Milli-pore A10 purification system (Raleigh, USA). Chemical standards and SIL standards were purchased from CDN Isotopes (Quebec, Canada), Sigma-Aldrich, Cambridge Isotope Laboratories (Tewksbury, MA, USA) and Toronto Research Chemicals (Toronto, Canada). Table S1 provides detailed information of all standards used in this study. The Pooled EDTA plasma was obtained from Innovative Research (Peary Court Novi, MI, USA), pooled male and female ETDA plasma were purchased from Sanquin (Sanquin, Amsterdam, The Netherlands), and diverse ETDA plasma from individual donors was purchased from BioIVT (Westbury, NY, USA). The pH was measured by Five Easy Plus pH meter FP20 from Mettler Toledo (Port Melbourne,

Australia).

2.3. Solution preparation

PCI solution. Leucine-enkephalin, fludrocortisone, glucose-d_7 and 3-fluoro-valine were selected as the PCI standards. All details of PCI standards are shown in Table S1. The PCI mixture solution was prepared with Milli-Q water/ACN (1:1, v/v). The PCI solution concentrations used in this study were 247.5 ng/mL leucine enkephalin, 650 ng/mL fludrocortisone, 4000 ng/mL glucose-d_7, and 3500 ng/mL 3-fluoro-valine.

SIL standard solution. 18 SIL standards from different compound classes were selected in the study including glutamic acid-d_5 and glutamine-d_5 (used during method development), glutamic acid-$^{13}C_2$ and glutamine-$^{13}C_2$ (used during method characterization and clinical sample measurement), leucine-d_3, glucose-$^{13}C_6$, creatinine-d_3, pyruvate-$^{13}C_3$, succinic acid-d_4, glycine-d_2, tryptophan-d_5, asparagine-$^{13}C_4$–$^{15}N_2$, aspartic acid-$^{13}C_4$-d_3–^{15}N, valine-d_8, alanine-d_3, lactate-$^{13}C_3$, indole-3-acetic acid-d_5, hypoxanthine-d_3, hippuric acid-$^{13}C_6$, and betaine-d_9. Most SIL standard stock solutions were prepared in Milli-Q water, except for hypoxanthine-d_3 and aspartic acid-$^{13}C_4$-d_3–^{15}N, which were prepared in 0.1 M sodium hydroxide. All SIL standards were stored at −80 °C until analysis. The working solution of SIL standards (Table S1) was prepared at the endogenous analogue concentration [37] by using the stock solution and diluting with Milli-Q water.

Calibration solutions. Stock solutions of 18 SIL standards were created at various concentrations using suitable solvents to facilitate method characterization (Table S1). A standard mixture was prepared by mixing 18 SIL standards and this mixture was serially diluted with Milli-Q water to obtain working calibration solutions at 7 concentration levels, as shown in Table S2. Stock solutions and standard mixture were stored at −80 °C until use, and calibration solutions were freshly prepared before each experiment.

2.4. Plasma sample preparation

Liquid-liquid extraction (LLE) was applied to remove the lipids and proteins from the plasma in this study. Polar metabolites were extracted from the plasma by using chloroform/MeOH/Milli-Q water adapted from the Bligh and Dyer approach [38]. Plasma aliquots (50 μL) were spiked with 10 μL of SIL standard working solution and quenched with 400 μL ice-cold methanol and 120 μL ice-cold Milli-Q water. The mixture was subsequently vortexed at speed 7 for 5 min, incubated at −20 °C for 20 min and then centrifuged for 10 min (18,000 × *g*, 4 °C) for protein precipitation. The supernatant (~ 520 μL) was transferred into a new Eppendorf tube and then mixed with 520 μL of chloroform and 350 μL of Milli-Q water. The mixture was vortexed at speed 7 for 5 min and incubated on ice for 10 min then centrifuged for 10 min (20,000 × *g*, 4 °C) for LLE. The upper layer (~ 720 μL) was collected and evaporated to dryness in a vacuum centrifuge coupled to a cold trap (Labconco, Kansas City, MO, USA). The residue was reconstituted with 50 μL of MeOH / Milli-Q water (6:4, v/v) vortexed at speed 7 for 5 min and centrifuged (20,000 × *g*, 4 °C, 10 min). The supernatant was transferred to a new Eppendorf tube to centrifuge again, after that, the supernatant (~30 μL) was transferred to an autosampler vial for further LC-MS analysis. To increase the quenching and minimize the residual enzymatic activity, all steps were performed on ice and all solvents for extraction were used ice-cold. For method characterization, calibration lines (n = 3) were created using pooled female EDTA plasma with 10 μL of spiked calibration working solutions. Repeatability and intra-day precision were evaluated at low (cal2), medium (cal4) and high (cal6) concentration levels from the calibration lines (Table S2). Inter-day precision was evaluated at low (cal2), medium (cal4) and high (cal6) concentration levels from 3 batches. Diverse EDTA plasma samples from three individual donors were used as three different plasma samples for recovery and ME evaluation. For recovery, plasma samples were prepared by spiking 10 μL of calibration working solutions at cal4 level before and after extraction and drying. Samples for the ME evaluation were prepared by spiking 10 μL of calibration working solution at cal4 level in plasma (matrix) and neat (matrix-free) samples after extraction and drying. Every plasma sample was prepared in three replicates. Quality control (QC) samples were prepared using pooled female EDTA plasma spiked with calibration working solution at the cal4 level. All QC samples were pooled together and used for the measurement of 3 batches.

During column comparison and mobile phases optimization, 100 μL pooled EDTA plasma samples (n = 3) and neat samples (n = 3) spiked with SIL standard working solution before sample preparation were prepared and reconstituted in 100 μL of MeOH / Milli-Q water (6:4, v/v).

2.5. LC-MS analysis

LC-MS analysis was performed using a Waters Acquity UPLC H—Class system coupled with a SCIEX TripleTOF 5600 mass spectrometer with an electrospray ionization source (ESI) that operated at negative mode. The ESI source parameters were set as follows: spray voltage - 4.5 kV, capillary temperature 400 °C, ion source gas 1: 20 psi, ion source gas 2: 50 psi, curtain gas 25 psi. Data were acquired in full scan mode at the *m/z* range of 50–900 Dalton (Da). Mobile phase A consisted of 90 % ACN and 10 % Milli-Q water with 10 mM ammonium formate at pH 4. Mobile phase B consisted of 10 % ACN and 90 % 10 mM ammonium formate in Milli-Q water at pH 4. The gradient started at 100 % A and was kept for 1 min, then B linearly increased to 15 % over 2 min and to 21 % from 2 to 5 min, then to 26 % from 2 to 7.5 min, to 40 % from 7.5 to 10 min whereat it was held for 1 min, then returned to 100 % A in 0.5 min and equilibrated the column for 6.5 min, resulting in an 18 min run time per analysis. A 90 % ACN solution was used as the weak needle wash, while 10 % ACN served as the strong needle wash. The wash program was set to 12 s prior to each injection. The autosampler temperature was set at 10 °C and the oven temperature was maintained at 30 °C. The injection volume was 3 μL.

During the column comparison, the buffer condition was 5 mM ammonium formate at pH 7 and the flow rate was 0.5 mL/min. Three HILIC columns were investigated in this study; the InfinityLab Poroshell 120 HILIC-Z column (2.7 μm, 2.1 mm × 100 mm, Agilent), the Atlantis Premier BEH Z-HILIC column (1.7 μm, 2.1 mm × 100 mm, Waters) and the SeQuant® ZIC®-cHILIC column (3 μm, 2.1 mm × 100 mm, Merck). Detailed information of the three columns is included in Table S3. The following buffer conditions: 5 mM, 10 mM and 20 mM ammonium formate and pH 4, pH 7 and pH 8 were investigated after selecting the optimal column at a 0.5 mL/min flow rate.

For method characterization and LLS cohort measurement, the buffer condition was 10 mM ammonium formate at pH 4 and the flow rate was 0.3 mL/min. In this study, the acidic and alkaline pH of the aqueous ammonium formate were adjusted using formic acid and ammonium hydroxide, respectively.

2.6. Continuous post-column infusion and data processing

The PCI was operated with an Agilent 1260 Infinity Isocratic Pump attached to an ALLTECH On-Line Degasser System 2000. PCI solution was infused at 20 μL/min and combined with the LC flow after chromatographic separation by using a T-piece prior to MS detection.

Data acquisition was conducted using Analyst (version 1.8.1). Peak integration was performed using Sciex OS (version 2.1.6). The *m/z* trace for each analyte and PCI standard was extracted within a mass tolerance window of ± 0.02 Da Histogram plots were made within Excel. The scatter plots were generated using GraphPad Prism (version 9.0). R Studio (version 4.3.1) was used to calculate the linearity by using different weighting factors and to assess the statistical ME significance between the groups using *t*-tests and plot graphs.

For ME visualization, the signal intensity of leucine-enkephalin,

glucose-d_7, fludrocortisone and 3-fluoro-valine were extracted at *m/z* 554.2620 *m/z* 232.1055, *m/z* 425.1981 and *m/z* 134.0623 by Sciex OS, respectively. The data points of the signal intensity of the infused PCI standards were smoothed by taking the mean value of five consecutive data points. The ME at each time point was calculated by using the following formula:

$$\mathrm{ME}\ (\%) = \frac{\textit{Response of PCI standard in plasma}}{\textit{Response of PCI standard in neat solution}} \times\ 100. \quad \text{(Formula 1)}$$

The ME profile was constructed by plotting the ME at each time point with the corresponding chromatographic run time in Excel (Microsoft). In the ME profile, values over 100 % reflect ion enhancement, whereas values below 100 % reflect ion suppression.

For a clear ME comparison between different columns and buffer conditions, a total of ion suppression percentage, ion enhancement percentage and ME was calculated. ME at each time point was calculated using Formula 1. Then, the ion suppression percentage was calculated by summing all ME values below 100 % (indicating ion suppression) and dividing by the sum of the ME in case of no ME (i.e. 100 % ME) at each time point during the targeted chromatographic run time. Similarly, the ion enhancement percentage was calculated by summing all ME values above 100 % (indicating ion enhancement) and dividing by the sum of the ME in case of no ME (i.e. 100 % ME) at each time point during the targeted chromatographic run time. All steps are processed in Excel (Microsoft).

2.7. ME evaluation method

For the SIL-IS method, SIL standards were spiked into both plasma and neat solutions prior to sample preparation. This was done to assess ME during ionization, as well as the potential ME that could occur during sample preparation, such as differences in analyte solubility between plasma and neat solution, and variations in extraction efficiency between plasma and neat solution. The ME for each SIL standard was calculated using the following formula:

$$\mathrm{ME}\,(\%) = \frac{\textit{Peak Area of SIL standard in the plasma spiked before}}{\textit{Peak Area of SIL standard in the neat solution spiked before}} \times 100. \quad \text{(Formula 2)}$$

For ME evaluation by the PCI method during characterization and biological plasma measurement, the RT window of each compound was selected based on the widest peak width of this compound over all samples, including the plasma samples and neat samples. According to the widest RT window for each compound, ME was calculated as the area of the ME profile (calculated using Formula 1) in this RT window.

2.8. Method characterization

Linearity. The linearity of selected SIL standards in plasma was evaluated by building calibration lines ($n = 3$). The calibration lines of the SIL standards applied in plasma were designed based on using the concentration levels of their endogenous analogues as cal4. The calibration range for each spiked SIL standard is presented in Table S2.

Precision, Repeatability and Recovery. The intra-day precision and repeatability were expressed as the relative standard deviation (RSD) of the peak area at low (cal2), medium (cal4) and high (cal6) concentration levels in triplicate. The inter-day precision was evaluated as the RSD of the peak areas in triplicates on 3 different days at low (cal2), medium (cal4) and high (cal6) concentration levels ($n = 9$). The same QC samples were injected every 10 injections across all batches. The intra-day and inter-day precision of QC samples were assessed by the RSD of 6 injections within a batch and 14 injections over 3 batches. The recovery was calculated by dividing the average peak areas added before extraction by the average peak areas added after extraction of each SIL standard from three plasma donors at the cal4 concentration level in three replicates.

Carry-over. Carry-over was evaluated by analyzing cal0 plasma samples following the highest plasma calibration standard (cal7 in this study). Since SIL standards were used as analytes for method characterization, cal0 plasma samples were selected as blank samples to avoid retention time variations caused by differences between neat solutions and plasma. Carry-over was calculated as the mean ratio of the analyte response in the cal0 samples to its response at the lower limit of quantification (LLOQ), which in this study refers to cal1 or cal2 (for glutamic acid-$^{13}C_2$), across the three replicates.

ME assessment. Absolute matrix effect (AME) was defined by Matuszewski et al. [26] as the comparison between the signal response of a standard in a matrix sample to its response in a neat solution, relative matrix effect (RME) was the variation between diverse plasma samples. It is more important to evaluate the RME during clinical sample analysis. Therefore, AME and RME were both evaluated during method characterization, they were assessed by adding SIL standards at the cal4 concentration level in three different plasma donors. The AME was evaluated by comparing the peak area of each SIL standard obtained in the plasma (post-extraction) with the neat sample. The RME was expressed as the RSD of the AME in three plasma donors.

3. Results and discussion

In this study, ME evaluation was investigated during the HILIC method optimization. We first evaluated the ME by comparing the peak area of SILs spiked in plasma and neat solution. This was compared with ME evaluation using four standards that were post-column infused during injection of plasma and neat solution. The PCI method was applied to further method optimization, characterization and biological application.

3.1. Matrix effect evaluation

ME evaluation by the SIL-IS method. The ME of 18 SIL standards was assessed when using a BEH Z-HILIC, HILIC-Z and a ZIC-cHILIC column. The applied HILIC-MS method was adapted from Hosseinkhani et al. [21]. In this experiment, ME for each SIL standard was calculated using Formula 2. Kollipara et al. [39] introduced 80 %–120 % as the acceptable range to quantify matrix effects for bioanalytical analysis. In the absence of established official criteria for ME, this study adopts the 80 %–120 % range as the acceptance threshold for ME evaluation. Details of ME values and Retention Times (RT) of each compound on each column are shown in Table S4. Overall, 11 out of 54 compound measurements meet the ME acceptance criteria (80–120 %; highlighted in black), while the rest suffer from ion suppression (ME $<$ 80 %; 40 measurements, highlighted in blue) or ion enhancement (ME $>$ 120 %; 3 measurements, highlighted in red). The ME of each SIL standard on each column for three replicates are visualized as a scatter plot in Fig. S1. The ME of each SIL standard was presented by blue circles, red squares and green triangles for ZIC-cHILIC, HILIC-Z and BEH Z-HILIC columns, respectively, with the vertical error bars representing the standard deviations (SD) from the triplicate analyses. Among the 18 SIL standards, 16 standards exhibited minimal ion suppression in the BEH Z-HILIC column, while glucose-$^{13}C_6$ and indole-d_5–3-acetic acid showed slightly higher suppression than ZIC-cHILIC column.

ME evaluation comparison: PCI vs SIL-IS. To investigate the overall ME during the whole LC run time, a multi-component PCI approach was developed by infusing four standards to monitor the ME. The four standards were selected considering their physical properties, ionization behaviour, availability and cost [40]. Specifically, glucose-d_7 and 3-fluoro-valine were chosen to represent the targeted compound classes, including sugars and amino acids. Leucine-enkephalin was selected due to its low cost, wide availability, and physicochemical properties, which are similar to those of amino acids. Given the

structural similarity, we hypothesize that organic acids could be effectively evaluated using 3-fluoro-valine or leucine-enkephalin. Fludrocortisone was selected due to its affordability, ease of procurement, and its distinct profile compared to the other compounds, thereby adding variability for potential broader applications. An appropriate concentration of the PCI standards is important for ME evaluation, the signal of targets will be suppressed if the concentration is too high, while PCI will not assess ME properly due to the background noise if the concentration is too low. Therefore, the PCI solution concentration was optimized based on a previous study [41], the concentration details are provided in **Method 2.3**. Initially, the efficacy of this approach was explored by comparing the ME assessed using SIL-IS and PCI methods. ME profiles were generated for the three columns by applying the same LC-MS method to both neat and plasma samples. A comparison of the ME profiles of four PCI standards and ME values derived from 18 SIL standards was made for the BEH Z-HILIC column (Fig. 1), HILIC-Z column (Fig. S2) and ZIC-cHILIC column (Fig. S3). Fig. 1 illustrates a strong correlation between both approaches, ME values from 17 SIL standards (except for Indole-d_5–3-acetic acid) coincide with at least one ME profile of a PCI standard, demonstrating its effectiveness. As mentioned in **Method 2.4**, SIL standards were spiked before the sample preparation in plasma and neat solution. Therefore, the ME evaluated by SIL-IS includes ME from differences between plasma and neat solution during sample preparation and ionization, while the PCI approach can only monitor the ME from ionization. Fig. 1 shows that the ME of most compounds evaluated by SIL-IS method aligns with at least one of the ME profiles. This result indicates that this PCI method can be used as an alternative to the SIL-IS method. Additionally, it suggests the difference between plasma and neat solution caused by sample preparation is minimal and ME is mainly caused by ionization. Indole-d_5–3-acetic acid might be an exception as the ME evaluated by the SIL-IS and PCI methods differed on each of the three columns.

In all three columns (Figs. 1, S2, S3), there are some co-eluting compounds, resulting in an enhanced effect on the ME profiles of leucine enkephalin and 3-fluoro-valine. Most interference was observed for 3-fluoro-valine, creating a high peak in its ME profile, which eluted at 2.0 min, 1.1 min and 1.5 min in BEH Z-HILIC column (Fig. 1), HILIC-Z column (Fig. S2) and ZIC-cHILIC column (Fig. S3), respectively. Moreover, the ME profile of fludrocortisone exhibited some ion enhancement on the BEH Z-HILIC (Fig. 1) and HILIC-Z column (Fig. S2). Figs. S2 and S3 indicate that not all SIL standards are accurately monitored by the current four PCI standards, suggesting the need of more chemically representative (additional) PCI standards. According to the results, the PCI approach demonstrates excellent ability in quantitative ME assessment.

3.2. HILIC column comparison

In a previous study, a HILIC column evaluation by Hosseinkhani et al. [21], the results showed ZIC-cHILIC column operated at pH 7 with 5 mM ammonium formate exhibited superior performance than the BEH-amide column and other mobile phases. However, a new HILIC column has emerged recently [42]. In this study, three zwitterionic stationary phase columns (BEH Z-HILIC, HILIC-Z and ZIC-cHILIC) with different functional groups and column dimensions were selected and the performance of ME and chromatography were investigated by using 18 SIL standards as our target analytes with PCI of four standards.

ME profiles comparison between columns. PCI was applied to evaluate the overall ME disparities across the three HILIC columns. Fig. S4 depicts the ME profiles up to 14 min as this is the range for untargeted metabolomics analysis in this method. Notably, the four PCI standards showed different ME performances between different columns. Leucine enkephalin predominantly observed ion suppression across three columns. Glucose-d_7 exhibited ion suppression on BEH Z-HILIC and HILIC-Z columns while showing ion enhancement from 9 to 12 min on the ZIC-cHILIC column. Fludrocortisone indicated significant

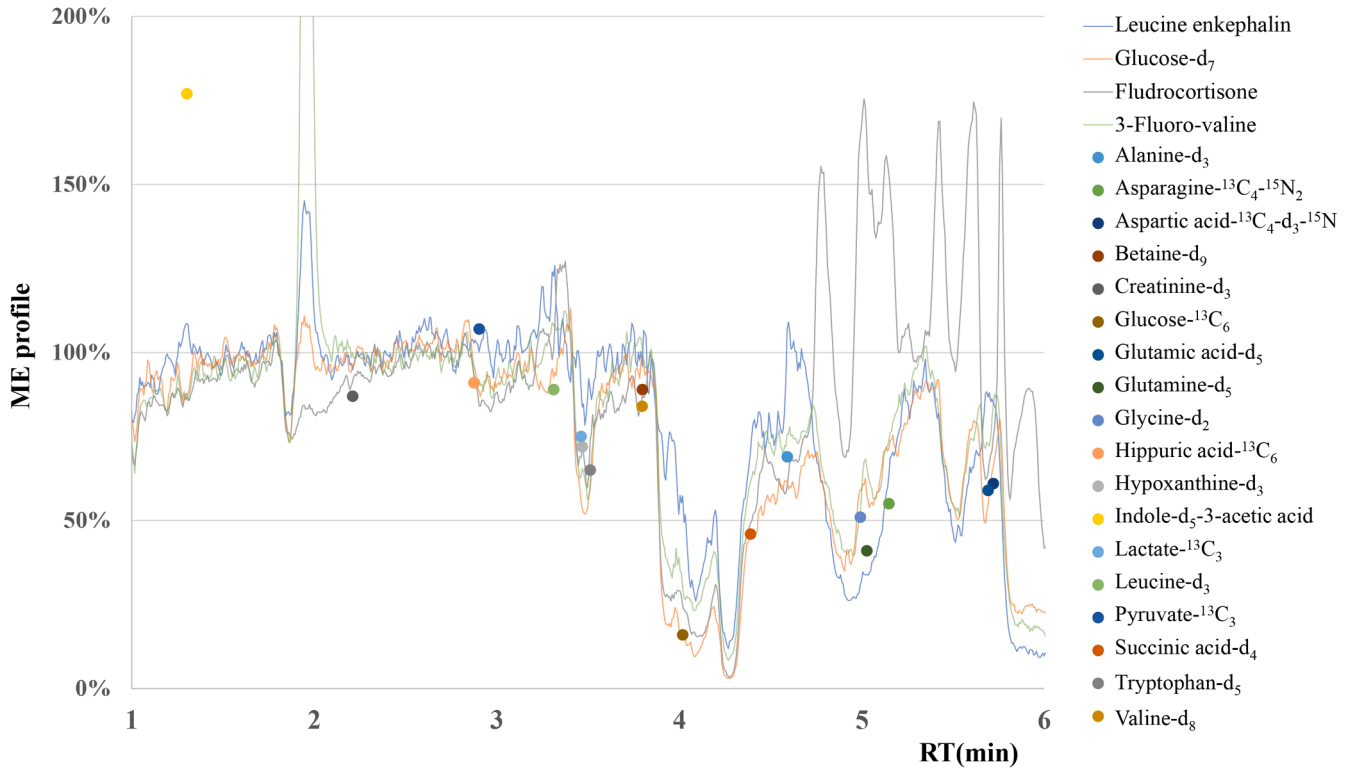


Fig. 1. A comparison of four ME profiles of four PCI standards and ME values of 18 SIL standards in plasma on the BEH Z-HILIC column. Four ME profiles are shown in 4 curves and ME values of 18 SIL standards are presented in 18 colored dots. ME over 100 % means ion enhancement and below 100 % means ion suppression. 3-Fluoro-valine shows ion enhancement above 200 % around 2 min, this figure only shows ME below 200 %.

ion suppression around 3 min on ZIC-cHILIC and HILIC-Z columns and at 4 min on the BEH Z-HILIC column. It also exhibited ion enhancement across all columns, with extremely high enhancement at 10 and 12 min on the ZIC-cHILIC column. 3-fluoro-valine mostly showed ion suppression on all columns, except enhanced ME at 2 min on the BEH Z-HILIC column.

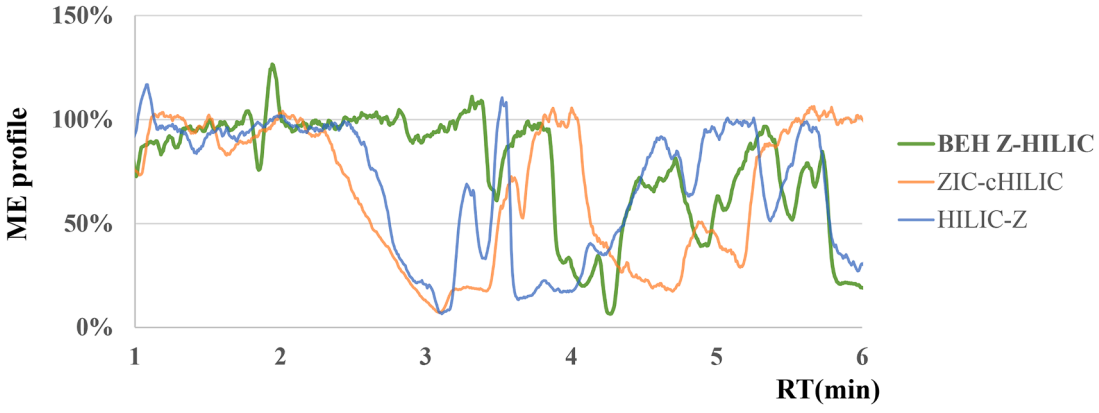
Given the varying ME performances of each PCI standard, the median of four PCI standards was used to generate ME profiles for each column to ensure equitable ME evaluation between columns. First, the ME profile was employed to provide a qualitative assessment of the ME in plasma. Seven interferences were detected by inspecting the mass spectra differences in suppressed RT regions (Fig. S5). Subsequently, the extracted ion chromatogram of each interferent was generated, with the response presented as 100 % of the maximum intensity across three columns. For instance, lactic acid showed the highest response on the BEH Z-HILIC column, therefore the peak of lactic acid reached 100 %, which means it has a higher signal compared to other columns. The ME profile and response of the interferences are presented in Fig. S6. The extent of ion suppression observed overlapped with the signal of interferences. For instance, EDTA and citric acid exhibited a higher signal on the ZIC-cHILIC column, resulting in more severe corresponding ME compared to the other two columns. Overall, EDTA and citrate, as widely used anticoagulants in plasma, were identified as the external sources causing significant ion suppression, consistent with previous studies [43,44]. As shown in Fig. S6, citric acid exhibited a broad and pronounced peak across three columns, potentially caused by both endogenous citric acid and external anticoagulant. Additionally, sodium formate and potassium formate clusters emerged as another primary source from plasma and mobile phase, leading to pronounced ion suppression across all columns. Further methods are required for more accurate identification of co-eluting compounds, but this was not included in this study.

For a quantitative ME assessment, the chromatographic region of the 18 SIL standards (1–6 min) was compared for the three columns. To facilitate a column comparison, a total of ion suppression and ion enhancement percentages was calculated. Fig. 2A shows ME profiles of plasma on three columns, with the green curve indicating the BEH Z-HILIC column, showing less ME than other columns. The summarized ME percentages are shown in Fig. 2B, revealing 22.81 %, 34.67 % and 31.87 % ion suppression and 0.94 %, 0.68 % and 0.44 % ion enhancement for the BEH Z-HILIC, ZIC-cHILIC and HILIC-Z columns, respectively. Overall, the BEH Z-HILIC column exhibits the least ion suppression, attributable to its superior separation between SIL standards and interferences.

The separation of compounds is determined by the interaction between the stationary phase and mobile phases [45]. As shown in Table S3, variations in the characteristics of each column could lead to different separation mechanisms and efficiency. This could explain the different retention of the same interferences shown in Fig. S6, as well as the varied ME of analytes and ME profiles seen in Figs. 1, S2 and S3 between the three columns. Additionally, the Van Deemter equation provides insight into column efficiency, indicating that smaller particle and pore size can reduce the eddy diffusion and longitudinal diffusion contributes to a lower theoretical plate height, which leads to a better column performance [46]. Overall, BEH Z-HILIC column uses zwitterionic sulfobetaine as the stationary phase based on ethylene-bridged hybrid (BEH) particles with a size of 1.7 μm. This likely contributed to its enhanced separation efficiency for analytes and interferences, thereby resulting in less ME compared to the other two columns.

Chromatographic performance comparison. A standard mixture of 18 SIL standards from diverse compound classes was analyzed to investigate the chromatographic performance in all three columns. A scoring approach was applied to compare the column performance of those standards based on the retention time, peak sensitivity (peak area), peak shape (width and symmetry) and ME [11,21]. The performance of each SIL standard was assessed as excellent, good, acceptable or poor according to the calculated total score (shown in Table S5). A metabolite with an excellent score retains on the column to avoid ion suppression in the void zone, has a narrow peak with minimal tailing and high sensitivity, and experiences ME with 80 % to 120 %, which can

(A)



(B)

ME (%)	BEH Z-HILIC	ZIC-cHILIC	HILIC-Z
Ion suppression	22.81	34.67	31.87
Ion enhancement	0.94	0.68	0.44
Total	23.74	35.35	32.31

Fig. 2. (A): The ME profiles generated by the median of four PCI standards on three HILIC columns: BEH Z-HILIC (green), ZIC-cHILIC (orange), HILIC-Z (blue). (B): Total ion suppression and ion enhancement on each column from 1 to 6 min.

be accurately identified and quantified. For metabolites evaluated as good, their good peak shape together with MS signal and slight ME provide reliable quantification results. Metabolites categorized as acceptable can be used for qualitative purposes but might have inaccurate quantitative results. The remaining metabolites were classified as poor, usually because of broad peaks, low MS intensity or high ion suppression. Fig. S7 shows the performance score per column of each metabolite. On the HILIC-Z column, 39 % of the compounds had an excellent or good performance score, while on the ZIC-cHILIC column, 33 % of the compounds achieved the same performance. Overall, the BEH Z-HILIC column was superior in chromatographic performance, as most (72 %) of tested standards achieved an excellent or good performance score. This good performance observed on the BEH Z-HILIC column might be explained by its smaller particle and pore size and the use of sulfobetaine as a stationary phase with the BEH function group [42]. The BEH Z-HILIC column was chosen as the most suitable column for further analysis.

3.3. Mobile phases optimization based on BEH Z-HILIC column

The concentration and pH of the buffer significantly influence the retention and chromatographic performance in HILIC [47]. Ammonium formate was chosen because it stabilizes better in more acidic conditions, due to the pK_a value of formate being 3.8, and it can provide narrower peak widths compared to ammonium acetate, as demonstrated in the work of Lioupi et al. [48]. Initially, three concentrations of ammonium formate (5, 10, 20 mM) were tested on the BEH Z-HILIC column. The results indicated that 20 mM suppressed the MS signal of some analytes, while most analytes showed higher signals at 10 mM (Data are not shown). Consequently, based on 10 mM ammonium formate, three different pH levels: acidic (4), neutral (7) and alkaline (8) were investigated for the BEH Z-HILIC column. The objective was to minimize the ME and maximize the number of detected metabolites with excellent or good performance scores.

In pursuit of an untargeted method, the PCI approach was employed to evaluate ME in plasma at different mobile phase conditions throughout the whole chromatographic runtime. Fig. S8A indicates ME profiles generated by the median of the four PCI standards at three different pH conditions from 0 to 12 min, while Fig. S8B summarizes the total ion suppression and enhancement observed at each condition. It clearly shows that 10 mM ammonium formate at pH 4 exhibited the least ion suppression (14.68 %) compared to pH 7 and pH 8, which indicates ME reduction through adjustment of mobile phase pH. Individual ME profiles of each PCI standard at each condition are shown in Fig. S9. Furthermore, ME calculated using 18 individual SIL standards matched the results of PCI profiles, with reduced ME observed at pH 4. Detailed information can be found in Table S6.

Besides the ME variation comparison, the chromatographic performance of 18 SIL standards at each condition was evaluated using the same scoring approach as described in **Result 3.1**. Fig. 3 displays the chromatographic performance of each metabolite at each pH condition, with 56 % of compounds demonstrating excellent performance at pH 4 with 10 mM ammonium formate. Among three tested pH conditions, the BEH Z-HILIC column operated the best at pH 4 with the highest number of metabolites with excellent scores. Altogether, the BEH Z-HILIC column operated at the acidic condition yielded optimal results with minimal ME and better peak performance of hydrophilic metabolites profiled in plasma. This could be explained by increased ionization efficiencies and retention time changes of some analytes under the acidic condition [49], consequently achieving higher signal and better separation.

3.4. Method characterization

Characterization parameters. Linearity, repeatability, intra-day precision and inter-day precision from calibration samples and QC samples, recovery and carry-over are summarized in Table 1. Excellent linearity ($R^2 > 0.98$) was obtained for all 18 SIL standards with a wide linear range. The selection of an appropriate weighting factor is crucial for ensuring the accuracy and reliability of the calibration curve [50,51]. In this study, three weighting factors (1, 1/X and 1/X^2) were tested to generate the best-fit calibration curves, with the most suitable weighting factor selected for each SIL according to the highest R^2 and maximum calibration points. Calibration points were accepted if the residual error was below 20 % compared to the nominal concentration, and a minimum of six concentration levels were used to construct each calibration curve. Except for succinic acid-d_4, excellent repeatability and intra-day precision (RSD < 15 %) for all SIL standards were achieved at three concentration levels (Low, Medium and High). Good inter-day precision (RSD < 30 %) was obtained at all concentration levels for all SIL standards. Excellent intra-day precision (RSD < 17 %) and inter-day precision (RSD < 20 %) from QC samples indicated the stability of this method during the whole analysis. Good recoveries were obtained for 14 SIL standards (within 80–120 %), and slightly lower recoveries (> 67 %) were noted for alanine-d_3, indole-d_5–3-acetic acid, succinic acid-d_4, and tryptophan-d_5. The carry-over for 14 SIL standards was lower than 5 % LLOQ, except for glutamic acid-$^{13}C_2$, glutamine-$^{13}C_2$, glycine-d_2, and tryptophan-d_5. Due to the 2 Da mass difference with the non-labelled analyte, the high carry-over for glutamic acid-$^{13}C_2$, glutamine-$^{13}C_2$, and glycine-d_2 was attributed to contributions from naturally occurring isotopes of the endogenous compounds. For tryptophan-d_5, a low-intensity interference with *m/z* 206.1033 was observed in the

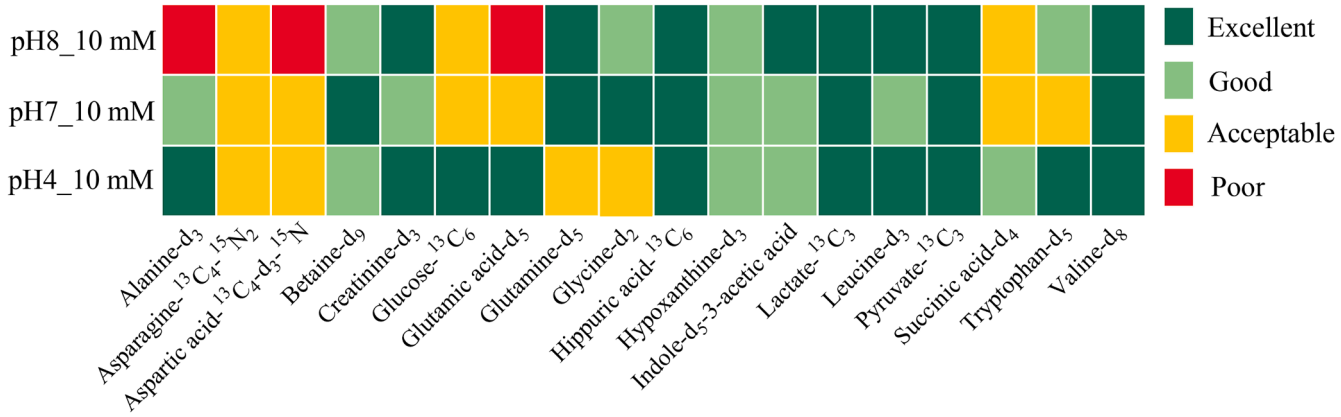


Fig. 3. Individual chromatographic performance score of 18 polar metabolites from different compound classes at three pH conditions. 10 mM refers to 10 mM ammonium formate. Dark green means excellent (Score ≥ 6), light green means good (4 ≤ Score < 6), yellow means acceptable (0 < Score < 4), and red means poor (Score ≤ 0).

Table 1
Method characterization parameters.

Component Name	Weighting factor	Linear range (μM)	R^2	Repeatability(%)			Inter Precision(%) $n = 9$			QC, n = 6	QC, $n = 14$	Recovery (%) $n = 9$	Carry-over(%) n = 3
				Intra Precision $n = 3$						Intra Precision (%)	Inter Precision (%)		
				Cal2	Cal4	Cal6	Cal2	Cal4	Cal6	Cal4	Cal4	Cal4	
Alanine-d_3	1/X	50–3200	1	5	10	3	12	8	11	12	10	75	1.7
Asparagine-$^{13}C_4$–$^{15}N_2$	1/X^2	9.375–600	0.99	4	13	8	11	14	18	6	7	84	1.3
Aspartic acid-$^{13}C_4$-d_3–^{15}N	1/X^2	5–320	0.98	10	10	7	19	19	21	16	13	87	4.2
Betaine-d_9	1/X^2	9.375–600	0.98	7	11	8	15	12	12	14	12	87	0.8
Creatinine-d_3	1/X^2	12.5–800	0.98	7	8	6	19	20	20	14	14	86	0.1
Glucose-$^{13}C_6$	1/X^2	75–4800	0.99	6	10	11	19	19	19	17	15	88	0.4
Glutamic acid-$^{13}C_2$	1/X	25–800	0.99	1	10	7	17	21	23	13	11	93	57.0
Glutamine-$^{13}C_2$	1/X^2	62.5–4000	0.99	9	7	7	14	11	16	11	11	85	11.9
Glycine-d_2	1/X^2	37.5–2400	0.98	11	4	9	24	24	27	4	18	86	39.3
Hippuric acid-$^{13}C_6$	1/X^2	1.375–88	0.98	7	8	5	16	16	16	13	11	87	0.0
Hypoxanthine-d_3	1/X^2	6.25–400	0.98	9	7	7	11	12	12	14	10	87	0.3
Indole-d_5–3-acetic acid	1/X^2	1.5625–100	0.99	7	5	6	12	18	20	8	14	75	0.9
Lactate-$^{13}C_3$	1/X^2	93.75–6000	0.99	6	8	5	20	21	20	14	15	87	0.6
Leucine-d_3	1/X^2	6.25–400	0.98	10	10	8	16	17	16	12	13	88	0.2
Pyruvate-$^{13}C_3$	1/X^2	12.5–800	0.98	10	6	4	21	19	21	12	14	83	0.6
Succinic acid-d_4	1/X	6.25–400	0.99	3	7	22	22	22	19	16	20	67	0.1
Tryptophan-d_5	1/X^2	6.25–400	0.99	2	4	4	22	18	20	6	15	79	7.4
Valine-d_8	1/X^2	25–1600	0.98	7	10	7	16	16	16	15	13	87	0.1

plasma samples, resulting in a false high carry-over.

Furthermore, ME of three diverse plasma donors was investigated and the results of the AME ($n = 3$) and RME ($n = 3$) are presented in Table S7. For 61 % of the SIL standards, the AME fell within the acceptable range (80 %−120 %). However, significant AME was observed for 6 SIL standards with alanine-d_3, asparagine-$^{13}C_4$–$^{15}N_2$ and glycine-d_2 values below 30 %, with glucose-$^{13}C_6$ and tryptophan-d_5 values between 50 % and 80 %, and glutamic acid-$^{13}C_2$ values above 120 %. RME reflects the variation between diverse plasma samples, which is important for large-scale clinical studies. In our results, all targets exhibited RME lower than 10 %, indicating minimal ME variation among the three plasma donors.

AME and RME evaluation. To select the most suitable PCI standard for ME assessment of each target, the AME was calculated for each SIL standard and assessed by four PCI standards, which were plotted with the error bars representing the standard deviation calculated from three plasma donors (Fig. 4A). The optimal PCI standard was chosen based on minimal difference between the AME calculated by SIL standards and the AME evaluated by PCI standards, along with minimal RME between three plasma donors (Table S8). With the exception of aspartic acid-$^{13}C_4$-d_3–^{15}N, glutamic acid-$^{13}C_2$ and tryptophan-d_5, accurate ME evaluation was observed by one or more PCI standards for each SIL standard. After selecting the optimal PCI standard for each SIL standard, the correlation between the AME calculated by the SIL-IS and the optimal PCI standard was evaluated. Fig. 4B illustrates a good correlation between SIL-IS and the optimal PCI standard, with an R^2 of 0.90, indicating that the optimal PCI standard could serve as a viable alternative to SIL-IS method for ME evaluation.

Furthermore, considering the untargeted aim of this study, the median of four PCI standards was used to assess AME and compared to the AME assessment by SIL-IS (Fig. S10 and Table S9). Table S9 presents the AME evaluation of SIL-IS and the median of four PCI standards, with most SIL standards showing similar ME evaluation, with AME difference below 20 %. Slightly higher AME differences (below 35 %) were noted

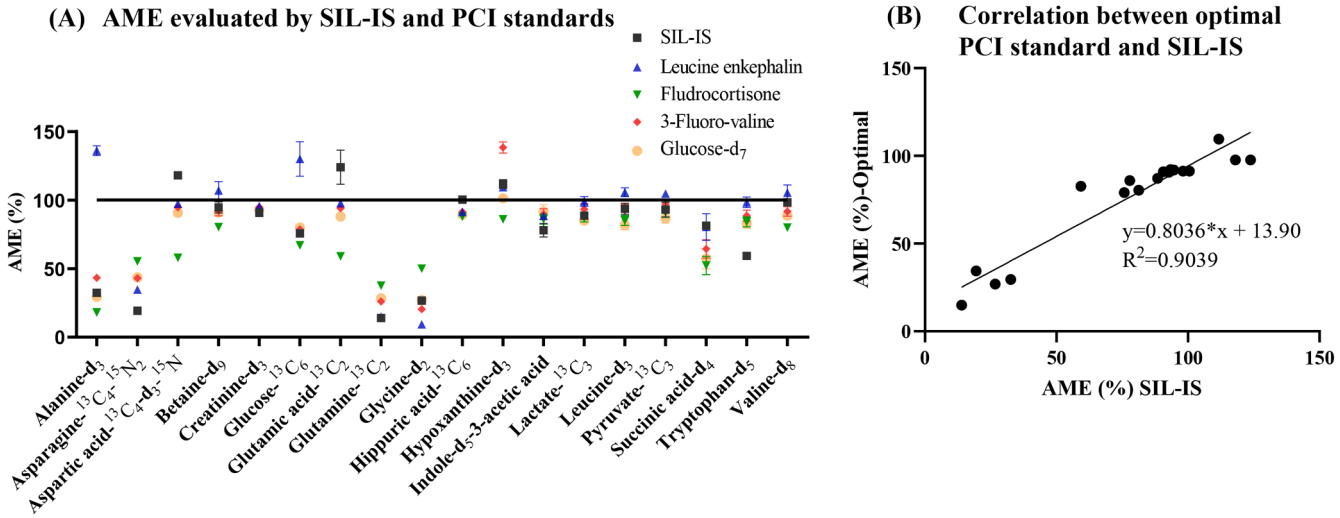


Fig. 4. (A): The selection of the most suitable PCI standard for 18 SIL standards showing the AME evaluated by SIL-IS method and four PCI standards in three different plasma donors with their standard deviations (vertical error bars). The black line indicates no ME. (B): Correlation between ME evaluated by optimal PCI standard and SIL-IS method.

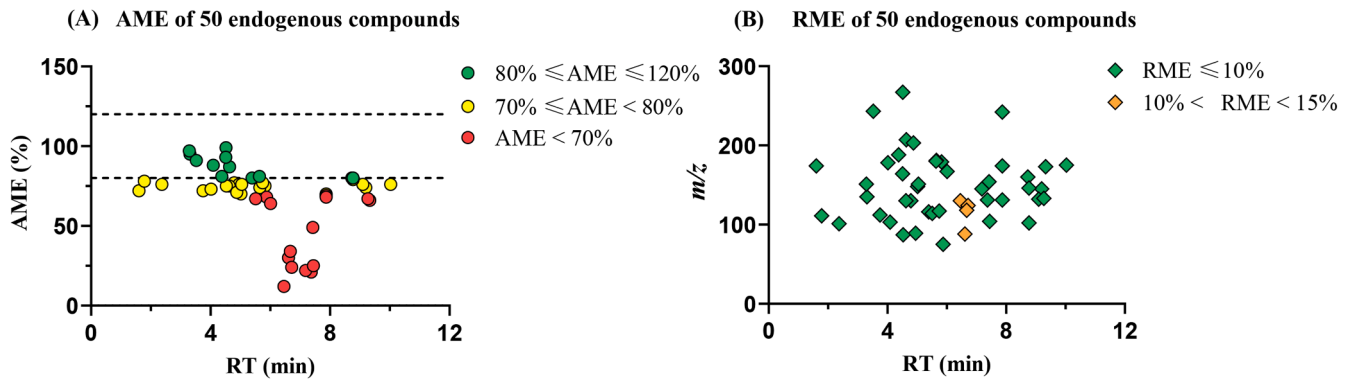


Fig. 5. (A): AME of 50 endogenous compounds. Green circles mean those compounds have an acceptable AME; yellow circles mean those compounds experienced between 20 % and 30 % ion suppression in those plasma samples; Red circles mean those compounds exhibited above 30 % ion suppression. The two lines represent AME values of 80 % and 120 % .(B): RME of 50 endogenous compounds. Green rhombi mean their RME is equal to or below 10 %, and orange rhombi mean their RME is between 10 % and 15 %.

for asparagine-$^{13}C_4$–$^{15}N_2$, aspartic acid-$^{13}C_4$-d_3–^{15}N, glutamic acid-$^{13}C_2$ and tryptophan-d_5. Fig. S10A indicates the AME calculated by each SIL standard and evaluated by the median of PCI standards during three plasma donors. Fig. S10B shows the correlation between SIL-IS and the median of four PCI standards, the R^2 is 0.81, demonstrating a good ability of PCI for ME assessment compared to SIL-IS method. Therefore, this PCI approach shows promising prospects for ME assessment in untargeted metabolomics analysis.

3.5. AME and RME evaluation in LLS cohort by PCI

To assess the applicability of this untargeted HILIC-MS method with PCI, it was applied to analyze 40 plasma samples for polar metabolites. These plasma samples were collected from patients with high MetaboHealth score ($n = 20$) or low MetaboHealth score ($n = 20$). One sample was excluded because of the low signal, leaving 39 samples for further analysis. Based on commercial authentic standards, 50 endogenous polar metabolites were detected and identified in these samples, with the RT and *m/z* information of measured compounds summarized in Table S10. The objective was to evaluate the AME and RME of those 50 compounds across 39 plasma samples using the PCI approach. The AME of endogenous compounds cannot be directly calculated due to absent signal in the neat solution samples. Hence, the median AME assessed by four PCI standards was used to represent the AME of each endogenous compound, and the average AME across all samples was calculated for each compound (Table S10 and Fig. 5). Twelve compounds, presented as green circles, exhibited AME between 80 % to 120 %, indicating acceptable ME across 39 plasma samples in Fig. 5A. Twenty compounds displayed slightly higher ion suppression, represented by yellow circles. The remaining compounds, shown as red circles, experienced significant ion suppression. The RME of 50 compounds was illustrated in Fig. 5B, plotting the *m/z* and RT of compounds. Four compounds (4-hydroxyproline, alanine, taurine and threonine) showed a slightly increased variation (RME > 10 %) over 39 plasma samples, also experiencing high ion suppression. The majority of compounds demonstrate RME below 10 %. Overall, those 39 plasma samples showed a comparable ME between samples, but they still exhibited relatively high ME.

This PCI approach offers the possibility to evaluate ME of endogenous compounds throughout untargeted analysis, providing valuable insights into sample-to-sample ME variability in clinical studies. In addition, low RME variation is important for accurate quantification for which the LLS results confirm potential PCI applicability.

4. Conclusions

In this study, an untargeted HILIC-MS method was developed for polar metabolite analysis by applying a multi-component PCI approach for ME evaluation. This strategy facilitated column selection and optimization of mobile phase conditions and demonstrated that ME can be minimized by selecting the most suitable column and buffer condition, emphasizing the significance of ME evaluation in method development. The strong correlation observed between ME assessment using PCI and SIL-IS approaches provides compelling evidence for the efficacy of PCI in ME evaluation for untargeted analysis. Furthermore, a link to clinical metabolomics analysis was made through the LLS cohort which was used to evaluate the AME and RME of endogenous compounds and demonstrates an impressive capability to monitor ME during untargeted metabolomics analysis.

In conclusion, ME assessment provides valuable information during method development and application. Optimization of chromatographic parameters has shown significant improvements when guided by ME evaluation. The integration of the PCI approach with HILIC-MS provides an auspicious ability to evaluate ME during untargeted metabolomics analysis, paving the way for more accurate and reliable metabolite quantification in complex biological systems. Future analysis may benefit from the use of more chemically representative PCI standards to cover a broader range of targets. Moreover, this PCI approach could be used as a powerful tool to correct ME for accurate quantification of polar metabolites in clinical studies. Moving forward, its use could further advance clinical metabolomics, contributing to more precise biomarker discovery and a deeper understanding of metabolic pathways in health and disease.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT (OpenAI) in order to improve the readability of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Mengle Zhu: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lieke Lamont:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Pascal Maas:** Writing – review & editing, Visualization, Formal analysis. **Amy C. Harms:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Marian Beekman:** Writing – review & editing, Resources. **P. Eline Slagboom:** Writing – review & editing, Resources. **Anne-Charlotte Dubbelman:** Writing – review & editing, Visualization, Supervision,

Methodology, Investigation, Conceptualization. **Thomas Hankemeier:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Thomas Hankemeier, Anne Charlotte Dubbelman, Amy C Harms has patent #NL2022019B1, EP3881068A1, US20220003726A1, CN113196052A, WO2020101499A1 pending to Universiteit Leiden. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465580.

Data availability

Data will be made available on request.

References

[1] C.H. Johnson, J. Ivanisevic, G. Siuzdak, Metabolomics: beyond biomarkers and towards mechanisms, Nat. Rev. Mol. Cell Biol. 17 (7) (2016) 451–459, https://doi.org/10.1038/nrm.2016.25.

[2] D.S. Wishart, Emerging applications of metabolomics in drug discovery and precision medicine, Nat. Rev. Drug Discov. 15 (7) (2016) 473–484, https://doi.org/10.1038/nrd.2016.32.

[3] H. Pang, W. Jia, Z. Hu, Emerging applications of metabolomics in clinical pharmacology, Clin. Pharmacol. Ther. 106 (3) (2019) 544–556, https://doi.org/10.1002/cpt.1538.

[4] M.M. Rinschen, J. Ivanisevic, M. Giera, G. Siuzdak, Identification of bioactive metabolites using activity metabolomics, Nat. Rev. Mol. Cell Biol. 20 (6) (2019) 353–367, https://doi.org/10.1038/s41580-019-0108-4.

[5] B. Zhou, J.F. Xiao, L. Tuli, H.W. Ressom, LC-MS-based metabolomics, Mol. Biosyst. 8 (2) (2012) 470–481, https://doi.org/10.1039/c1mb05350g.

[6] E. Gorrochategui, J. Jaumot, S. Lacorte, R. Tauler, Data analysis strategies for targeted and untargeted LC-MS metabolomic studies: overview and workflow, TrAC Trends Anal. Chem. 82 (2016) 425–442, https://doi.org/10.1016/j.trac.2016.07.004.

[7] P. Miggiels, B. Wouters, G.J.P. van Westen, A.C. Dubbelman, T. Hankemeier, Novel technologies for metabolomics: more for less, TrAC Trends Anal. Chem. 120 (2019), https://doi.org/10.1016/j.trac.2018.11.021.

[8] A. Mardinoglu, E. Bjornson, C. Zhang, M. Klevstig, S. Soderlund, M. Stahlman, M. Adiels, A. Hakkarainen, N. Lundbom, M. Kilicarslan, B.M. Hallstrom, J. Lundbom, B. Verges, P.H. Barrett, G.F. Watts, M.J. Serlie, J. Nielsen, M. Uhlen, U. Smith, H.U. Marschall, M.R. Taskinen, J. Boren, Personal model-assisted identification of NAD(+) and glutathione metabolism as intervention target in NAFLD, Mol. Syst. Biol. 13 (3) (2017) 916, https://doi.org/10.15252/msb.20167422.

[9] V.R. Varma, A.M. Oommen, S. Varma, R. Casanova, Y. An, R.M. Andrews, R. O'Brien, O. Pletnikova, J.C. Troncoso, J. Toledo, R. Baillie, M. Arnold, G. Kastenmueller, K. Nho, P.M. Doraiswamy, A.J. Saykin, R. Kaddurah-Daouk, C. Legido-Quigley, M. Thambisetty, Brain and blood metabolite signatures of pathology and progression in Alzheimer disease: a targeted metabolomics study, PLoS Med. 15 (1) (2018) e1002482, https://doi.org/10.1371/journal.pmed.1002482.

[10] P.A. Vorkas, G. Isaac, M.A. Anwar, A.H. Davies, E.J. Want, J.K. Nicholson, E. Holmes, Untargeted UPLC-MS profiling pipeline to expand tissue metabolome coverage: application to cardiovascular disease, Anal. Chem. 87 (8) (2015) 4184–4193, https://doi.org/10.1021/ac503775m.

[11] K. Contrepois, L. Jiang, M. Snyder, Optimized analytical procedures for the untargeted metabolomic profiling of human urine and plasma by combining hydrophilic interaction (HILIC) and reverse-phase liquid chromatography (RPLC)-mass spectrometry, Mol. Cell Proteom. 14 (6) (2015) 1684–1695, https://doi.org/10.1074/mcp.M114.046508.

[12] K. Spagou, H. Tsoukali, N. Raikos, H. Gika, I.D. Wilson, G. Theodoridis, Hydrophilic interaction chromatography coupled to MS for metabonomic/metabolomic studies, J. Sep. Sci. 33 (6–7) (2010) 716–727, https://doi.org/10.1002/jssc.200900803.

[13] R. Coras, J.D. Murillo-Saich, M. Guma, Circulating pro- and anti-inflammatory metabolites and its potential role in rheumatoid arthritis pathogenesis, Cells 9 (4) (2020), https://doi.org/10.3390/cells9040827.

[14] A. Alonso, A. Julia, M. Vinaixa, E. Domenech, A. Fernandez-Nebro, J.D. Canete, C. Ferrandiz, J. Tornero, J.P. Gisbert, P. Nos, A.G. Casbas, L. Puig, I. Gonzalez-Alvaro, J.A. Pinto-Tasende, R. Blanco, M.A. Rodriguez, A. Beltran, X. Correig, S. Marsal, I. Consortium, Urine metabolome profiling of immune-mediated inflammatory diseases, BMC Med. 14 (1) (2016) 133, https://doi.org/10.1186/s12916-016-0681-8.

[15] S.K. Srimadh Bhagavatham, S.K. Pulukool, S.S. Pradhan, S. R, A. Ashok Naik, M. D. V, V. Sivaramakrishnan, Systems biology approach delineates critical pathways associated with disease progression in rheumatoid arthritis, J. Biomol. Struct. Dyn. 41 (14) (2023) 6969–6990, https://doi.org/10.1080/07391102.2022.2115555.

[16] N. Karu, A. Kindt, A.J. van Gammeren, A.A.M. Ermens, A.C. Harms, L. Portengen, R.C.H. Vermeulen, W.A. Dik, A.W. Langerak, V.H.J. van der Velden, T. Hankemeier, Severe COVID-19 Is characterised by perturbations in plasma amines correlated with immune response markers, and linked to inflammation and oxidative stress, Metabolites 12 (7) (2022), https://doi.org/10.3390/metabo12070618.

[17] J. Deelen, J. Kettunen, K. Fischer, A. van der Spek, S. Trompet, G. Kastenmuller, A. Boyd, J. Zierer, E.B. van den Akker, M. Ala-Korpela, N. Amin, A. Demirkan, M. Ghanbari, D. van Heemst, M.A. Ikram, J.B. van Klinken, S.P. Mooijaart, A. Peters, V. Salomaa, N. Sattar, T.D. Spector, H. Tiemeier, A. Verhoeven, M. Waldenberger, P. Wurtz, G.Davey Smith, A. Metspalu, M. Perola, C. Menni, J. M. Geleijnse, F. Drenos, M. Beekman, J.W. Jukema, C.M. van Duijn, P.E. Slagboom, A metabolic profile of all-cause mortality risk identified in an observational study of 44,168 individuals, Nat. Commun. 10 (1) (2019) 3346, https://doi.org/10.1038/s41467-019-11311-9.

[18] P. Hemstrom, K. Irgum, Hydrophilic interaction chromatography, J. Sep. Sci. 29 (12) (2006) 1784–1821, https://doi.org/10.1002/jssc.200600199.

[19] Y. Guo, Recent progress in the fundamental understanding of hydrophilic interaction chromatography (HILIC), Analyst 140 (19) (2015) 6452–6466, https://doi.org/10.1039/c5an00670h.

[20] G. Schuster, W. Lindner, Comparative characterization of hydrophilic interaction liquid chromatography columns by linear solvation energy relationships, J. Chromatogr. A 1273 (2013) 73–94, https://doi.org/10.1016/j.chroma.2012.11.075.

[21] F. Hosseinkhani, L. Huang, A.C. Dubbelman, F. Guled, A.C. Harms, T. Hankemeier, Systematic evaluation of HILIC stationary phases for global metabolomics of human plasma, Metabolites 12 (2) (2022), https://doi.org/10.3390/metabo12020165.

[22] R. Zhang, D.G. Watson, L. Wang, G.D. Westrop, G.H. Coombs, T. Zhang, Evaluation of mobile phase characteristics on three zwitterionic columns in hydrophilic interaction liquid chromatography mode for liquid chromatography-high resolution mass spectrometry based untargeted metabolite profiling of Leishmania parasites, J. Chromatogr. A 1362 (2014) 168–179, https://doi.org/10.1016/j.chroma.2014.08.039.

[23] R.A. Sonnenberg, S. Naz, L. Cougnaud, D. Vuckovic, Comparison of underivatized silica and zwitterionic sulfobetaine hydrophilic interaction liquid chromatography stationary phases for global metabolomics of human plasma, J. Chromatogr. A 1608 (2019) 460419, https://doi.org/10.1016/j.chroma.2019.460419.

[24] T. Zhang, D.J. Creek, M.P. Barrett, G. Blackburn, D.G. Watson, Evaluation of coupling reversed phase, aqueous normal phase, and hydrophilic interaction liquid chromatography with Orbitrap mass spectrometry for metabolomic studies of human urine, Anal. Chem. 84 (4) (2012) 1994–2001, https://doi.org/10.1021/ac2030738.

[25] M. Cortese, M.R. Gigliobianco, F. Magnoni, R. Censi, P.D. Di Martino, Compensate for or minimize matrix effects? Strategies for overcoming matrix effects in liquid chromatography-mass spectrometry technique: a tutorial review, Molecules 25 (13) (2020), https://doi.org/10.3390/molecules25133047.

[26] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS, Anal. Chem. 75 (13) (2003) 3019–3030, https://doi.org/10.1021/ac020361s.

[27] H. Trufelli, P. Palma, G. Famiglini, A. Cappiello, An overview of matrix effects in liquid chromatography-mass spectrometry, Mass Spectrom. Rev. 30 (3) (2011) 491–509, https://doi.org/10.1002/mas.20298.

[28] W.M. Niessen, P. Manini, R. Andreoli, Matrix effects in quantitative pesticide analysis using liquid chromatography-mass spectrometry, Mass Spectrom. Rev. 25 (6) (2006) 881–899, https://doi.org/10.1002/mas.20097.

[29] Matrix effect. International Union of Pure and Applied Chemistry (IUPAC), 2019. https://doi.org/10.1351/goldbook.M03759.

[30] N.B. Cech, C.G. Enke, Practical implications of some recent studies in electrospray ionization fundamentals, Mass Spectrom. Rev. 20 (6) (2001) 362–387, https://doi.org/10.1002/mas.10008.

[31] I. Marchi, V. Viette, F. Badoud, M. Fathi, M. Saugy, S. Rudaz, J.L. Veuthey, Characterization and classification of matrix effects in biological samples analyses, J. Chromatogr. A 1217 (25) (2010) 4071–4078, https://doi.org/10.1016/j.chroma.2009.08.061.

[32] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects, J. Chromatogr. B

Anal. Technol. Biomed. Life Sci. 877 (23) (2009) 2198–2207, https://doi.org/10.1016/j.jchromb.2009.01.003.
[33] E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, J.R. Mazzeo, Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 852 (1–2) (2007) 22–34, https://doi.org/10.1016/j.jchromb.2006.12.030.
[34] M. Sulyok, R. Krska, R. Schuhmacher, A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples, Anal. Bioanal. Chem. 389 (5) (2007) 1505–1523, https://doi.org/10.1007/s00216-007-1542-2.
[35] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom. 13 (12) (1999) 1175–1185, https://doi.org/10.1002/(sici)1097-0231(19990630)13:12<1175::Aid-rcm639>3.0.Co;2-0.
[36] R.G. Westendorp, D. van Heemst, M.P. Rozing, M. Frolich, S.P. Mooijaart, G. J. Blauw, M. Beekman, B.T. Heijmans, A.J. de Craen, P.E. Slagboom, G. Leiden, Longevity study, nonagenarian siblings and their offspring display lower risk of mortality and morbidity than sporadic nonagenarians: the Leiden Longevity Study, J. Am. Geriatr. Soc. 57 (9) (2009) 1634–1637, https://doi.org/10.1111/j.1532-5415.2009.02381.x.
[37] D.S. Wishart, A. Guo, E. Oler, F. Wang, A. Anjum, H. Peters, R. Dizon, Z. Sayeeda, S. Tian, B.L. Lee, M. Berjanskii, R. Mah, M. Yamamoto, J. Jovel, C. Torres-Calzada, M. Hiebert-Giesbrecht, V.W. Lui, D. Varshavi, D. Varshavi, D. Allen, D. Arndt, N. Khetarpal, A. Sivakumaran, K. Harford, S. Sanford, K. Yee, X. Cao, Z. Budinski, J. Liigand, L. Zhang, J. Zheng, R. Mandal, N. Karu, M. Dambrova, H.B. Schiöth, R. Greiner, V. Gautam, HMDB 5.0: the human metabolome database for 2022, Nucleic Acids Res. 50 (D1) (2022) D622–d631, https://doi.org/10.1093/nar/gkab1062.
[38] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (8) (1959) 911–917, https://doi.org/10.1139/o59-099.
[39] S. Kollipara, G. Bende, N. Agarwal, B. Varshney, J. Paliwal, International guidelines for bioanalytical method validation: a comparison and discussion on current scenario, Chromatographia 73 (3–4) (2011) 201–217, https://doi.org/10.1007/s10337-010-1869-2.
[40] A.C. Dubbelman, B. van Wieringen, L.Roman Arias, M. van Vliet, R. Vermeulen, A. C. Harms, T. Hankemeier, Strategies for using postcolumn infusion of standards to correct for matrix effect in LC-MS-based quantitative metabolomics, J. Am. Soc. Mass Spectrom. (2024), https://doi.org/10.1021/jasms.4c00408.
[41] P. Zhu, A.C. Dubbelman, C. Hunter, M. Genangeli, N. Karu, A. Harms, T. Hankemeier, Development of an untargeted LC-MS metabolomics method with postcolumn infusion for matrix effect monitoring in plasma and feces, J. Am. Soc. Mass Spectrom. (2024), https://doi.org/10.1021/jasms.3c00418.
[42] T.H. Walter, B.A. Alden, K. Berthelette, J.A. Field, N.L. Lawrence, J. McLaughlin, A. V. Patel, Characterization of a highly stable zwitterionic hydrophilic interaction chromatography stationary phase based on hybrid organic-inorganic particles, J. Sep. Sci. 45 (8) (2022) 1389–1399, https://doi.org/10.1002/jssc.202100859.
[43] C. Chin, Z.P. Zhang, H.T. Karnes, A study of matrix effects on an LC/MS/MS assay for olanzapine and desmethyl olanzapine, J. Pharm. Biomed. Anal. 35 (5) (2004) 1149–1167, https://doi.org/10.1016/j.jpba.2004.01.005.
[44] C. Ghosh, C.P. Shinde, B.S. Chakraborty, Influence of ionization source design on matrix effects during LC-ESI-MS/MS analysis, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 893-894 (2012) 193–200, https://doi.org/10.1016/j.jchromb.2012.03.012.
[45] P. Jandera, Stationary phases for hydrophilic interaction chromatography, their characterization and implementation into multidimensional chromatography concepts, J. Sep. Sci. 31 (9) (2008) 1421–1437, https://doi.org/10.1002/jssc.200800051.
[46] J. Grinias, J. Godinho, Liquid chromatography column design and dimensional analysis of the van deemter equation, LCGC North Am. 40 (8) (2022) 367–370, https://doi.org/10.56530/lcgc.na.kh7671g4.
[47] L. Nováková, L. Havlíková, H. Vlčková, Hydrophilic interaction chromatography of polar and ionizable compounds by UHPLC, TrAC Trends Anal. Chem. 63 (2014) 55–64, https://doi.org/10.1016/j.trac.2014.08.004.
[48] A. Lioupi, C. Virgiliou, T.H. Walter, K.M. Smith, P. Rainville, I.D. Wilson, G. Theodoridis, H.G. Gika, Application of a hybrid zwitterionic hydrophilic interaction liquid chromatography column in metabolic profiling studies, J. Chromatogr. A 1672 (2022) 463013, https://doi.org/10.1016/j.chroma.2022.463013.
[49] J. Liigand, A. Laaniste, A. Kruve, pH effects on electrospray ionization efficiency, J. Am. Soc. Mass Spectrom. 28 (3) (2017) 461–469, https://doi.org/10.1007/s13361-016-1563-1.
[50] A.M. Almeida, M.M. Castel-Branco, A.C. Falcão, Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods, J. Chromatogr. B 774 (2) (2002) 215–222, https://doi.org/10.1016/S1570-0232(02)00244-1.
[51] H. Gu, G. Liu, J. Wang, A.F. Aubry, M.E. Arnold, Selecting the correct weighting factors for linear and quadratic calibration curves with least-squares regression algorithm in bioanalytical LC-MS/MS assays and impacts of using incorrect weighting factors on curve stability, data quality, and assay performance, Anal. Chem. 86 (18) (2014) 8959–8966, https://doi.org/10.1021/ac5018265.