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A comparison of hydrophilic interaction liquid chromatography and capillary electrophoresis for the metabolomics analysis of human serum

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ABSTRACT

Cationic, anionic, zwitterionic and, partially polar metabolites are very important constituents of blood serum. Several of these metabolites underpin the core metabolism of cells (e.g., Krebs cycle, urea cycle, proteins synthesis, etc.), while others might be considered ancillary but still important to grasp the status of any organism through blood serum analysis. Due to its wide chemical diversity, modern metabolomics analysis of serum is still struggling to provide a complete and comprehensive picture of the polar metabolome, due to the limitations of each specific analytical method. In this study, two metabolomics-based analytical methods using the most successful techniques for polar compounds separation in human serum samples, namely hydrophilic interaction liquid chromatography (HILIC) and capillary electrophoresis (CE), are evaluated, both coupled to a high-resolution time-of-flight mass spectrometer via electrospray ionization (ESI-Q-TOF-MS). The performance of the two methods have been compared using five terms of comparison, three of which are specific to metabolomics, such as (*1*) compounds' detectability (*2*) Pezzatti score (Pezzatti et al. 2018), (*3*) intra-day precision (repeatability), (*4*) ease of automatic analysis of the data (through a common deconvolution alignment and extrapolation software, MS-DIAL, and (*5*) time & cost analysis. From this study, HILIC-MS proved to be a better tool for polar metabolome analysis, while CE-MS helped identify some interesting variables that gave it interest in completing metabolome coverage in metabolomics studies. Finally, in this framework, MS-DIAL demonstrates for the first time its ability to process CE data for metabolomics, although it is not designed for it.

1. Introduction

The polar metabolome is constituted of cationic, anionic, zwitterionic and partially polar metabolites that differ in molecular structure and charge. Polar metabolites play important roles in various physiological processes and can serve as indicators of health or disease [1–5]. Therefore, the determination of polar metabolites has become a key area of research in the fields of biochemistry, clinical chemistry, and metabolomics. Mass spectrometry (MS) is the most widely applied detection technique to cover the polar metabolome, due to its high sensitivity, accuracy and unparalleled versatility [6]. In the analysis of the polar metabolome, MS is generally coupled with a separation technique such as Hydrophilic interaction liquid chromatography (HILIC), which separates compounds mostly on their hydrophilic properties. In recent years, capillary (zone) electrophoresis (CE) has been also deployed for the scope.

HILIC has demonstrated to be able to separate polar metabolites from all kind of matrices [7,8], like plants [9], urine [10], plasma [11], serum [12], etc. The strength of the HILIC technology separation is its repeatability in terms of retention time and peak shape, that provide very robust quantifications and identifications. In metabolomics mostly two kinds of HILIC columns are used: zwitterionic and amide [13]. Zwitterionic columns seems to be more versatile [14], while amide columns are sometimes preferred due to their smaller particle size allowing shorter runs and thinner peaks [13]. However, there is no perfect HILIC method for the comprehensive analysis of all metabolites in blood serum [15,16]. The range of polarities analyzable in HILIC are limited within a logP range of −5 to 0, thus the chemical complexity of the serum metabolome goes beyond the separation capabilities of HILIC stationary phases, resulting in lack of peaks separation (frequently

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linked to ion suppression), broad peak shape or double shaped peaks, and lack of compounds elution [17].

CE is an alternative technique for analyzing the polar metabolome. It is able to analyze a wide range of polarities while being a greener technique due to the lower amount of solvent necessary to perform the analysis. For many years, CE-MS has been considered a less suitable technique for metabolomics due to its lower sensitivity limited by the low sample injection volume, robustness, reproducibility and capillary longevity [18]. Some of these limitations have been overcome with the use of new instruments. Also, the inherent migration time variability issue has been solved by using effective electrophoretic mobility instead of migration time, which allows to use the migration of compounds as a robust identification parameter [19,20]. Low-pH background electrolytes (BGEs) are mostly used such as 1 M formic acid (pH 1.8), which allows the separation of cationic metabolites including amino acids, amines, and nucleosides. However, the separation of anionic metabolites seems a more complex task; there exist different approaches for the analysis of anionic compounds in CE but there is no consensus on the best one for metabolomics analysis.

In spite of the similar potential of HILIC and CE for the analysis of the polar metabolome, these two technologies seem to have a separate fate, running on distinct but parallel research environments. Direct comparisons between them are lacking and in general, most laboratories tend towards the use of LC-MS due to its greater versatility for the analysis of semi-polar and non-polar compounds. In this work, two broad analytical approaches have been developed and compared for the analysis of the polar metabolome of blood serum, using HILIC-MS and CE-MS, aiming to highlight the strength and the pitfalls of both methods, comparing them through several metabolomics-based parameters, like the scoring approach developed by Pezzatti et al. [21], but also intra-day precision, ease of automatic data deconvolution and analysis, and time & cost analysis. This comparison of two common and generic methods based on HILIC and CE technologies aims to pave the way for a coherent evaluation and choice in the analysis of the polar metabolome.

2. Materials & methods

2.1. Chemical standards

Ultrapure water has been obtained from a Milli-Q system (Merck, Millipore, Darmstad, Germany). LC-MS grade acetonitrile (MeCN), methanol (MeOH), isopropanol, chloroform, acetic acid, formic acid, water, ammonium formate and acetic acid have been purchased from Merck Lifesciences SLU (Madrid, Spain). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Panreac-Química (Madrid, Spain). The 72 chemical standards (STD) used in this study are reported in supplementary material (**Table S1**). The standards have been separated into five groups, amino-acids mix (AAmix), polar mix, non-polar mix, internal standards (IS) and capillary electrophoresis internal standards (CE-IS). All the standards have been purchased from Merck Lifesciences SLU, except stated otherwise.

2.2. Serum sample preparation

Serum samples have been obtained from anonymous healthy volunteers who donated samples to the Faculty of Pharmacy of the University of Granada (Spain). To increase the comparability of the two methods, the same extraction protocol has been used for both analytical methods, with the exception of an additional filtering step necessary for CE-MS analysis (Fig. 1). The samples were extracted following a Bligh&Dyer-like method [10] slightly modified. Briefly, 630 µL of cold methanol containing 2.5 ppm of the IS mix was added to 100 µL of blood serum, agitated and then 1290 µL of chloroform was added. The extracts were vortexed for 30 s and 400 µL of water was added and vortexed again. The extracts were centrifuged at 850 g for 5 min at 4 °C in a "Universal 320″ centrifuge (Hettich ZENtrifugen, Tuttlingen, Germany). Subsequently, 500 µL of the upper methanolic phase was placed in Eppendorf tubes and centrifuged again at 9000 g for 5 min at 4 °C. Finally, the supernatant was transferred in an Eppendorf tube and dried at 35 °C under nitrogen flow using a dryer EVA-EC System (VLM GmbH, Bielefeld, Germany).

The dry extracts followed different fates depending on the analytical platform used. For the HILIC-MS analysis, the samples were reconstituted in 50 µL of water, and then diluted with 100 µL of acetonitrile, pooled in quality control (QC) samples and transferred to amber vials prior to analysis. For the CE-MS analysis, the samples were reconstituted in 100 µL of 95/5 (v/v) water/acetonitrile containing 1.25 mg/L of the CE-IS mix, and filtered using the Amicon Ultra-0.5 Centrifugal Filter Unit (Merck Lifesciences SLU) at 11,500 g for 2 h at 4 °C. The filtered extracts were pooled in QC samples and transferred to amber vials prior to analysis.

The 72 chemical standards (STD) used in this work were separated into different groups according to their use (e.g., internal standards) and their chemical characteristics (**Table S1**). Three compounds, namely procaine, paracetamol and ethyl sulfate were pooled and used as CE internal standards (CE-IS) mix. Four deuterated compounds, namely 4-aminobutyric acid-2,2,3,3,4,4-d6, lauric-d23 acid, indole-3-acetic-2,2-d2 acid, L-tryptophan-(indole-d5), were pooled and used as internal standard (IS) mix. Furthermore, 25 were amino acids were pooled accordingly (AAmix). The remaining 40 compounds were separated according to their logP (i.e., 17 compounds had a logP ≤ 0 and they were pooled in the polar mix, while the remaining 23 compounds were pooled in the non-polar mix). QC samples obtained from the extraction of different serum samples were spiked either with the AA mix (QC-AA), the polar mix (QC-polar) or the non-polar mix (QC non-polar) for a final concentration of 2 mg/L for each compound, to obtain three different enriched QCs.

2.3. HILIC method

The LC system used in this work consisted of an Agilent 1290 Infinity II ultra-high pressure liquid chromatography (UHPLC) system capable to operate under pressure up to 1300 bar. The HILIC stationary phase was an ACQUITY Premier BEH Amide Column (2.1 mm × 150 mm,1.7 µm,) equipped with a dedicated ACQUITY Premier filter. The column was

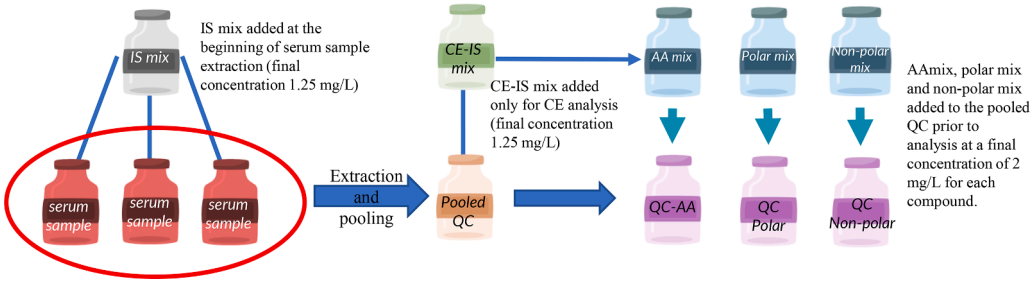

Fig. 1. Preparation of the three pooled QC samples spiked with different standard-mixes.

operated at a fixed temperature of 40 °C. Mobile phase A consisted of acetonitrile with 0.1% formic acid, while mobile phase B was 20 mM ammonium formate and 0.1% formic acid in water. The chromatographic gradient started from a 5% of B, rising to 10% of B at 0.3 min, 19% of B at 2 min, 34% of B at 5 min, 60% of B at 8 min, was hold at this level for 3.5 min, then returned to the initial 5% of B in half a minute and equilibrated until 16 min. The mobile phase flow rate was constant at 0.4 mL/min.

2.4. Capillary electrophoresis method

The experiments were carried out on a 7100-CE system from Agilent Technologies (Waldbronn, Germany). A 10% aqueous acetic acid solution was used as the BGE. The bare fused silica capillary used in this study was 80 cm in length with an internal diameter of 50 µm, and was purchased from Polymicro Tech. (Phoenix, AZ, USA). It was conditioned as follows before first use: 950 mbar, 5 min, MeOH; 950 mbar, 5 min, water; 950 mbar, 5 min, NaOH (1 M); 950 mbar, 5 min, water; 950 mbar, 25 min, HCl (1 M); 950 mbar, 5 min, water; 950 mbar, 5 min, HCl (0.1 M); 950 mbar, 5 min, water; and 950 mbar, 5 min, BGE. At the beginning of each day, the capillary was conditioned with the BGE for 5 min at 950 mbar.

Cationic and anionic profiling were performed separately. For both profiles, a 10% aqueous acetic acid solution was used as BGE, and the separation voltage was established at +30 kV and −30 kV (after initial 0.1 min CE voltage ramp up) for cationic and anionic profiling, respectively. In addition, a positive pressure of 50 mbar was applied at the inlet to generate a constant BGE flow towards the MS during both anion and cation separation and to prevent current drop, which as consequence reduces the analysis time. Samples were hydrodynamically injected at 50 mbar for 20 s. The capillary temperature was set at 25 °C.

2.5. Mass spectrometry and data acquisition

The MS detector used in this work was an Agilent 6550 iFunnel QToF LC/MS model, a quadrupole-time of flight-mass spectrometer equipped with a JetStream electrospray ion source (ESI-Q-TOF-MS). The instrument was operated in positive and negative ionization modes in both HILIC and CE analysis. The instrument was set to acquire in both MS and data dependent acquisition (DDA MS/MS) mode. High-energy (40 V) spectra were used to obtain fragment ions. Instrumental parameters were optimized for every combination of the separation technique and ionization polarity (Table 1).

The fragmentor and octopole RF voltages were set at 380 and 750 V, respectively. The instrument was calibrated and tuned according to the procedures recommended by the manufacturer. MS and MS/MS data were stored using the centroid mode. Accurate mass spectra were acquired in MS and MS/MS mode over a *m/z* range from 50 to 1000 and with an acquisition frequency of 3.33 Hz.

For CE-MS analysis, the ESI source included a coaxial sheath liquid interface with a sprayer and a platinum needle. The sheath liquid consisted of 5 mM acetic acid in a 50/50 (v/v) isopropanol/water including mass reference (0.1 µM purine and 0.015 µM HP-0921 from Agilent Technologies) for online reference mass calibration. Sheath liquid was delivered at a flow rate of 6 µL/min by an Agilent 1260-Infinity II isocratic pump.

The instrument was operated using the MassHunter WorkStation Data Acquisition software (version 10, Agilent).

Table 1
Optimized ionization conditions and acquisition parameters for each analytical method. * The nebulizer gas pressure was set to 0 psi during the injection.

Parameters	unit	HILIC-MS ESI+	HILIC-MS ESI-	CE-MS ESI+	CE-MS ESI-
D rying gas temperature	°C	160	150	125	225
Drying gas flow	L/min	15	15	11	11
Nebulizer gas pressure	psi	35	35	10*	10*
S heath gas temperature	°C	260	250	100	100
Sheath gas flow	L/min	10	10	3.5	3.5
Vcap	V	3500	3000	2000	2500
Nozzle voltage	V	600	600	2000	2000
Acquisition range	*m/z*	50–1000	50–1000	50–1000	50–1000
Scan rate	spectra/s	10	10	10	10

2.6. Data processing and analysis

Manual detection and identification of the peaks resulting from the injection of the STD mix and the QC samples spiked with STD mix were performed using Agilent MassHunter Qualitative Analysis 10.0 software, while quantification of the peaks was performed using Agilent MassHunter Quantitative Analysis software. Each chromatographic/electrophoretic peak was manually curated. The tolerance limit was set at 10 ppm for accurate mass and 0.4 min for retention time (RT) in HILIC, and at 10 ppm for accurate mass and 3 min for migration time (MT) in CE.

For automatic deconvolution and analysis of the data, the chromatograms (or electropherograms) were converted to the open-source .mzML format using the MSConvert software from ProteoWizard [22], using the following parameters: peak picking (vendor algorithm) MS levels 1–2, subset polarity according to the polarity of the chromatograms (or electropherograms). The .mzML files were deconvoluted and analyzed using MS-DIAL software [23]. This software has been chosen because it has been recently ranked first in a direct comparison with further popular peak picking software [24].

Comparative evaluation of the two separation methods was performed using five terms of comparison. The first was compound detectability, quantified as the number of compounds that could show a clear peak in the chromatograms/electropherograms. The second, developed by Pezzatti et al., was a scoring approach expressly developed for the comparison of metabolomics methods [21]. This approach evaluates the retention/migration time, signal-to-noise ratio (S/N), relative intensity, and peak shape of each peak. The sum of all the scores from each peak gives a cumulative score for each method using different analytical platform (i.e., the higher the score, the better the overall performance of the platform). The third term of comparison was the intra-day precision. Analytical signals from 10 consecutive injections of the spiked QC were integrated for all the spiked compounds, and the unit-variance was scaled to attain all the compounds at the same scale. Thus, the coefficient of variation (CV) was calculated for all compounds. The fourth term of comparison was the performance of automatic data deconvolution and analysis. The capability of automatic data analysis of the datasets obtained from the two platforms was evaluated in terms of accuracy, reproducibility, and prognostic capabilities. The last term of comparison was the analysis time and cost.

3. Results & discussion

3.1. HILIC-MS and CE-MS method improvement

The HILIC method derived from Arapitsas et al. [25] was selected for this work with few precautions. Mobile phase A was deprived of any salt due to apparent precipitation problems when added at concentrations above 5 mg/L, as also reported by Schellinger and Carr [26]. The maximum water concentration was limited to 60% as recommended by the column manufacturer to achieve maximum separation reproducibility. The equilibration time was set to a total of 5 min (i.e., 4 min run + 1 min during the injection time) as suggested by McCalley [27], for a total analytical time of 16 min. The positive CE-MS method derived from an inter-laboratory study of metabolite determination, involving CE

16-MS different platforms [28]. CE conditions are described in Section 2.4 and were selected based on the most common or the mean values of the CE conditions used in the mentioned study. It is remarkable that most metabolomics studies are performed by CE-MS in positive mode, since most compounds are detected in this mode. However, in this study, a CE-ESI(-)-MS method was additionally tested, and three different aqueous BGE [i.e. 10% acetic acid (pH 2.2) [29], 50 mM ammonium bicarbonate (pH 8.5) [30] and 50 mM ammonium acetate (pH 8.5)] were compared. Finally, 10% aqueous acetic acid solution was selected as BGE because it allowed the detection of at least 30% more metabolites in serum than with the other two BGEs tested [29]. Therefore, the same 80 cm capillary and BGE were used in positive and negative ionization modes.

The MS settings of both HILIC-MS and CE-MS were optimized to increase the specific sensitivity of the analytical methods. In the HILIC-MS methods, the critical parameters to optimize of the Agilent Jetstream ESI source were the gas temperature, the sheath gas temperature, the capillary voltage, and the nozzle voltage. Also, dry gas temperature was tested. Lower sheath gas temperature and higher capillary voltage led to higher signal intensities for the studied metabolites. Furthermore, lowering the nozzle voltage to 600 V decreased the noise level across the whole chromatogram. In the CE-MS methods, the sheath liquid flow rate, drying gas flow and temperature, sheath gas flow and temperature, capillary voltage, nozzle voltage and nebulizer gas pressure were optimized. The sheath liquid flow rate and the capillary voltage were the parameters that most influenced the signal intensities. A sheath liquid flow of 6 μL/min, and a capillary voltage of 2000 V for positive ionization mode and 2500 V for negative ionization mode led to higher signal intensities These values offered higher S/N values for most of the compounds without ion suppression. All the ranges of the parameters tested in this study are reported in the supplementary material (**Tables S2 and S3**).

3.2. HILIC-MS vs CE-MS

3.2.1. Compounds separation and detectability

The separation of the compounds and their detectability have been evaluated in matrix (serum), spiked with pure standards all at the same concentration. This has been necessary to avoid the fact that some compounds could result undetected for confounding factors, like storage time of the samples, different sample-sets, or their concentration in the specific sample-set is lower than their detection limit. This improves the replicability of the experiments between different methods, at different times and also in different laboratories. The sensitivity of the analytical methods for the different compounds is evaluated in Section 3.2.2 (peak intensity and S/N).

After the analysis, the chromatographic peaks of the QC-AA, QC-polar and QC-non-polar were manually integrated using the MassHunter Qualitative Analysis 10.0 software (Agilent) to extract the peak data of each compound. Table 2 reports all the most intense ions observed (i.e., protonated molecules, deprotonated molecules, sodium adducts, etc.) and the retention/migration time of all the compounds analyzed in this study in spiked samples and also injected as pure standards. Table 2 also indicates the compounds that could not be detected by the HILIC-MS and/or the CE-MS method, either in positive or negative ionization modes. All the peaks were identified at level 1 using the retention/migration time of pure standards injected in the same conditions as well their MS and MS/MS spectra, as reported in the supplementary material (**Table S4**).

The total number of detectable compounds in the spiked samples in HILIC-MS (positive and negative ionization modes) was 52 because the analyzable compounds were often the same in both modes. In contrast, the overlap of compounds detectable by CE-MS in the two ionization modes was limited to 3 compounds (i.e. kynurenic acid, glutathione oxidized, and inosine) and the total number of analyzable compounds was higher (i.e. 55 compounds). However, taking the ionization modes singularly, 33 and 49 compounds were detected by HILIC-MS in matrix applying positive and negative ionization modes, respectively, whereas 38 and 20 compounds were detected by CE-MS (in matrix) applying each ionization mode, respectively. The effect of the matrix was limited for HILIC-MS because just the signal from two compounds disappeared in comparison with the injection of pure standards (kynurenic acid and acetylcysteine), both in positive mode. In CE-MS the presence of the matrix determined the disappearance of the signal of five compounds: chenodeoxycholic and deoxycholic acids, dopamine, and ADP in negative and the internal standard indole-3-acetic-2,2-d2 acid in positive ionization mode. In the case of cysteine and acetylcysteine, the dimers were detected by CE-MS because their formation is a common process that happens when diluted in aqueous solvents and in presence of oxygen that can favor the dimerization process (formation of covalent bonds between the thiol groups (-SH), disulfide bond (-S-S-) [12]. Both methods showed a higher sensitivity for amino acids, likely due to the mild acidic conditions used in both instruments during the analysis, which favors the charge of zwitterionic compounds, helping their separation (especially in CE-MS) and their ionization.

The HILIC-MS method was unable to detect compounds exhibiting high logP (above its retention range), such as short chain fatty acids (with the sole exception of butyric acid), cholesterol, and lauric-d23 acid. Moreover, 3 polar compounds (i.e. xanthosine, UDP and ADP) did not show any detectability, likely due to their very low logP (~ -5), which is close to HILIC capabilities [31] and require different analytical conditions [32]. Surprisingly, serotonin and L-homocysteine could not be detected neither as pure standard, despite their logP is within the range of the HILIC column. Acetylcysteine and acetyl-glutamic acid could not be detected in the spiked QC due to matrix effect. Finally, 2-picolinic acid perfectly co-elute with its isomer (i.e., nicotinic acid).

At these conditions, the CE-MS method showed some disadvantages with respect to the HILIC-MS method since it did not allow the separation of isomers, such as isoleucine and leucine, isovaleric from valeric acid, and fructose from glucose. Among the polar compounds, phospho-enol-pyruvic acid and pyruvic acid were not detected probably due to the lack of sensibility for these two compounds. Pyruvic acid and phospho-enol-pyruvic acid have previously been separated using basic buffers [33], as well as ADP [34]. Among the less polar compounds, deoxycholic acid, chenodeoxycholic acid and dopamine, despite detectable when injected in solvent, were not detected in serum due to the matrix effect.

3.2.2. Pezzatti's score

A score to evaluate LC–MS peaks elaborated from a LC–MS based metabolomics perspective from Pezzatti et al. [21] was used in this study to evaluate the performance of HILIC–MS and CE–MS methods. Peak detectability was assessed using the four well-established criteria (i.e., apparent retention factor (k_{app}), S/N, peak intensity, and peak shape). The sum of the score of each compound allows to compare the two analytical methods as already reported by Narduzzi et al. [10]. Table 3 shows a comparison of the scores obtained for each criterion for the compounds analyzed by both methods. The k_{app} score for HILIC conditions was calculated using the difference between the RT of each compound and the RT of the front peak, to exclude the effect of the system dead volume, as suggested by Pezzatti et al. [21]. In CE the system, dead volume does not really exist. In fact, the MT of the neutral reference IS (paracetamol) can be considered as the limit of separation ability of the CE-MS. Consequently, the k_{app} score for CE conditions was calculated using the difference between the MT of each compound and the MT of paracetamol.

After each individual score (i.e., k_{app}, S/N, I and S) was determined, the total score, which expresses overall performance, was calculated using the following equation (from [21]):

A similar total score (~ 28) was obtained for HILIC and CE methods. In general, CE-MS performed better than HILIC-MS for the peak intensity and peak shape scores and similarly for the S/N score. This outcome

Table 2
Compounds detected in the QC samples by the HILIC-MS and CE-MS methods. *ND = non-detected. **picolinic acid co-eluted with nicotinic acid. ***leucine co-migrated with isoleucine. **** glucose co-migrated with fructose. ***** valeric acid co-migrated with isovaleric acid.

		HILIC-MS					CE-MS				
QC	Standards name	*m/z*	Adduct	RT (min)	ND* in QC	ND* as pure standard	*m/z*	Adduct	MT (min)	ND* in QC	ND* as pure standard
AA mix	glycine	74.0248	$[M-H]^-$	6.28			76.0393	$[M+H]^+$	7.32		
AA mix	alanine	88.0405	$[M-H]^-$	7.03			90.055	$[M+H]^+$	7.64		
AA mix	serine	104.0354 106.0498	$[M-H]^-$ $[M+H]^+$	6.67			106.0498	$[M+H]^+$	8.25		
AA mix	proline	114.0561 116.0705	$[M-H]^-$ $[M+H]^+$	5.49			116.0705	$[M+H]^+$	6.15		
AA mix	valine	116.0718	$[M-H]^-$	5.41			118.0863	$[M+H]^+$	8.12		
AA mix	threonine	118.051 120.0654	$[M-H]^-$ $[M+H]^+$	6.28			120.0654	$[M+H]^+$	8.47		
AA mix	cysteine	120.0125 122.0269	$[M-H]^-$ $[M+H]^+$	5.72			241.0311	$[(2M-2H)+H]^+$	8.96		
AA mix	asparagine	131.0463 133.0607	$[M-H]^-$ $[M+H]^+$	6.74			133.0608	$[M+H]^+$	8.5		
AA mix	ornithine	131.0827 133.0971	$[M-H]^-$ $[M+H]^+$	7.57			133.0971	$[M+H]^+$	6.15		
AA mix	aspartic acid	132.0303 134.0447	[M-H]- $[M+H]^+$	7.03			134.0448	$[M+H]^+$	9.09		
AA mix	hydroxyproline	130.051 132.0654	[M-H]- $[M+H]^+$	6.11			132.0654	$[M+H]^+$	9.36		
AA mix	isoleucine	130.0874 132.1018	[M-H]- $[M+H]^+$	4.84			132.1018	$[M+H]^+$	8.19		
AA mix	leucine	130.0874 132.1018	[M-H]- $[M+H]^+$	5.01			132.1018	$[M+H]^+$		X***	X***
AA mix	L-homocysteine				X	X	136.0432	$[M+H]^+$	8.15		
AA mix	glutamine	145.0619 147.0763	[M-H]- $[M+H]^+$	6.6			147.0763	$[M+H]^+$	8.59		
AA mix	glutamic acid	146.046 148.0604	[M-H]- $[M+H]^+$	6.58			148.0604	$[M+H]^+$	8.64		
AA mix	lysine	145.0983 147.1127	[M-H]- $[M+H]^+$	7.69			147.1127	$[M+H]^+$	6.19		
AA mix	methionine	148.0438 150.0582	[M-H]- $[M+H]^+$	1.2			150.0582	$[M+H]^+$	8.53		
AA mix	histidine	154.0623 156.0767	[M-H]- $[M+H]^+$	7.52			156.0767	$[M+H]^+$	6.39		
AA mix	acetylcysteine	162.0231	$[M-H]^-$	3.62	X		325.0523	$[(2M-2H)+H]^+$	11.47		
AA mix	phenylalanine	164.0718 166.0862	[M-H]- $[M+H]^+$	4.78			166.0862	$[M+H]^+$	8.7		
AA mix	arginine	173.1045 175.1189	[M-H]- $[M+H]^+$	7.57			175.119	$[M+H]^+$	6.35		
AA mix	tyrosine	180.0667 182.0811	[M-H]- $[M+H]^+$	5.46			182.0811	$[M+H]^+$	8.77		
AA mix	citric acid	191.0198	$[M-H]^-$	6.58			191.0198	$[M-H]^-$	13.19		
AA mix	tryptophan	203.0827 205.0971	[M-H]- $[M+H]^+$	4.81			205.0971	$[M+H]^+$	8.56		
polar mix	pyruvic acid	87.0082	$[M-H]^-$	3.26							
polar mix	fumaric acid	115.0032	$[M-H]^-$	3.12			115.0032	$[M-H]^-$	11.69		
polar mix	succinic acid	117.0188 119.0344	[M-H]- $[M+H]^+$	1.9			117.0188	$[M-H]^-$	12.86		
polar mix	2-picolinic acid	122.0248	$[M-H]^-$		X**	X**	124.0393	$[M+H]^+$	9.3		
polar mix	nicotinic acid	122.0242 124.0398	[M-H]- $[M+H]^+$	2.12			124.0398	$[M+H]^+$	7.46		
polar mix	xanthine	151.0256 153.0412	[M-H]- $[M+H]^+$	3.95			151.0256	$[M-H]^-$	13.38		
polar mix	2,3-pyridine-dicarboxylic acid	166.0141	$[M-H]^-$	5.68			166.0141	$[M-H]^-$	10.44		
polar mix	phospho-enol-pyruvic acid	166.9751	$[M-H]^-$	7.67							
polar mix	uric acid	167.0211	$[M-H]^-$	5.33			167.0211	[M-H]-	13.03		
polar mix	fructose	179.0556	$[M-H]^-$	5.54			203.0532	$[M+Na]^+$	9.57		
polar mix	glucose	179.0556	$[M-H]^-$	5.66			203.0532	[M+Na]+		X****	X****
polar mix	glucose-6-phosphate	259.0219	$[M-H]^-$	3.11			259.0219	$[M-H]^-$	8.54		

(*continued on next page*)

Table 2 (*continued*)

		HILIC-MS					CE-MS				
QC	Standards name	*m/z*	Adduct	RT (min)	ND* in QC	ND* as pure standard	*m/z*	Adduct	MT (min)	ND* in QC	ND* as pure standard
polar mix	inosine	267.073 269.0886	[M-H]- $[M+H]^+$	4.31			267.073 269.089	$[M-H]^-$ $[M+H]^+$	13.41		
polar mix	xanthosine				X	X	285.0835	$[M+H]^+$	11.36		
polar mix	inosine-5′-monophosphate (I5M)	347.0393 349.0549	[M-H]- $[M+H]^+$	7.39			347.0393	$[M-H]^-$	9.1		
polar mix	uridine-5′-diphosphate (UDP)				X	X	402.9949	$[M-H]^-$	12.99		
polar mix	adenosine-5′-diphosphate (ADP)				X	X				X	
non-polar mix	butyric acid	89.0602	$[M+H]^+$	1.69			87.0452	$[M-H]^-$	13.26		
non-polar mix	valeric acid				X	X	101.0603	$[M-H]^-$		X*****	X*****
non-polar mix	isovaleric acid				X	X	101.0603	$[M-H]^-$	13.28		
non-polar mix	hexanoic acid				X	X	115.0765	$[M-H]^-$	13.29		
non-polar mix	octanoic acid				X	X	143.1072	$[M-H]^-$	13.3		
non-polar mix	dopamine	152.0712 154.0868	[M-H]- $[M+H]^+$	4.42						X	X
non-polar mix	allantoin	157.0362 181.0338	[M-H]- $[M+Na]^+$	3.98			157.0362	$[M-H]^-$	13.37		
non-polar mix	decanoic acid	171.1385 195.1361	$[M+H]^-$ $[M+Na]^+$	1.09			171.1385	$[M-H]^-$	13.32		
non-polar mix	L-citrulline	174.0879 176.1035	[M-H]- $[M+H]^+$	6.84			176.103	$[M+H]^+$	7.87		
non-polar mix	serotonin				X	X	177.1028	$[M+H]^+$	6.88		
non-polar mix	N-acetyl-glutamic acid	190.0715	$[M+H]^+$	5.62	X		243.0619	$[M—HCOO]^-$	13.37		
non-polar mix	kynurenic acid	188.0348 190.0504	[M-H]- $[M+H]^+$	4.24			188.0348 190.0504	$[M-H]^-$ $[M+H]^+$	11.09		
non-polar mix	3,4-dihydroxy-L-phenylalanine	196.061 198.0766	[M-H]- $[M+H]^+$	5.95			198.0766	$[M+H]^+$	7.87		
non-polar mix	dodecanoic acid	199.1698	$[M-H]^-$	1.07						X	X
non-polar mix	L-kynurenine	207.077 209.0926	[M-H]- $[M+H]^+$	4.84			209.0926	$[M+H]^+$	7.61		
non-polar mix	melatonin	231.1134 233.129	[M-H]- $[M+H]^+$	1.19			233.129	$[M+H]^+$	10.11		
non-polar mix	arachidonic acid	305.248	$[M+H]^+$	1.095						X	X
non-polar mix	glutathione reduced	306.076 308.0916	[M-H]- $[M+H]^+$	6.88			308.0916	$[M+H]^+$	8.4		
non-polar mix	cholesterol				X	X				X	X
non-polar mix	chenodeoxycholic acid	391.2849 415.2825	$[M-H]^-$ $[M+Na]^+$	1.35						X	
non-polar mix	deoxycholic acid	391.2849 431.2774	$[M-H]^-$ $[M+K]^+$	1.42						X	

(*continued on next page*)

Table 2 (*continued*)

QC	Standards name	HILIC-MS m/z	Adduct	RT (min)	ND* in QC	ND* as pure standard	CE-MS m/z	Adduct	MT (min)	ND* in QC	ND* as pure standard
non-polar mix	cholic acid	407.2798	$[M-H]^-$	2.34			407.2798	$[M-H]^-$	13.33		
non-polar mix	glutathione oxidized	611.1442 613.1598	[M-H]- $[M+H]^+$	8.64			611.1442 613.16	$[M-H]^-$ $[M+H]^+$	18.8		
IS mix	4-aminobutyric acid-2,2,3,3,4,4-d6				X	X	110.1083	$[M+H]^+$	5.93		
IS mix	indole-3-acetic-2,2-d2 acid				X	X				X	
IS mix	L-tryptophan-(indole-d5)	208.114 210.1285	[M-H]- $[M+H]^+$	4.81			210.1285	$[M+H]^+$	7.96		
IS mix	lauric-d23 acid									X	X
CE-IS mix	ethyl sulfate						124.9914	$[M-H]^-$	6.25		
CE-IS mix	paracetamol						150.0561	$[M-H]^-$	13.59		
CE-IS mix	paracetamol						152.0706	$[M+H]^+$	10.39		
CE-IS mix	procaine						237.1598	$[M+H]^+$	6.33		
Total peaks detected					52				55		

Table 3
The Pezzatti's score [21] obtained for the two methods in each parameter and their total score.

Method	Peak intensity (I) score	k_{app} score	S/N score	Peak shape (S) score	Total, score
HILIC	36.0	34.7	11.7	41.5	**27.9**
CE	43.8	23.5	12.6	46.0	**27.8**

indirectly emphasizes the similar sensitivity obtained for CE-MS in comparison with HILIC-MS without the need of applying any pre-concentration approach or using sheathless CE-MS [35].This result is not so obvious, considering that 5 µl were injected in HILIC-MS while the injection volume is only of a few nanoliters in CE-MS. On the other hand, the apparent migration of CE-MS resulted worse than HILIC-MS, mostly due to the limited separation between paracetamol and most of the compounds in negative mode. Indeed, the low pH conditions allow a better detectability (as reported above) at the cost of lower separation.

3.2.3. *Intra-day precision (repeatability)*

Intra-day (including intra-batch) precision or repeatability is a key parameter in metabolomics analysis to assure the comparability among samples and to avoid biased statistical analysis. For this reason, the repeatability of both methods was evaluated. After analyzing the QC-AA, QC-polar and QC-non-polar mixtures in both platforms for 10 times consecutively, the cumulative coefficient of variation (CV,%) was calculated averaging the CV across injection of all the compounds for each method. Peak area correction using IS (4-aminobutyric acid-d6 or paracetamol) was tested. As shown in the supplementary material (**Fig. S1**), there were no differences in peak area variability between the standards with and without IS correction, and in some cases, variability was even higher when IS correction was applied. So, no signal corrections were applied. The areas of the peaks have been z-scaled and averaged to build the trend plots (Fig. 2) that help to visualize their trend injection after injection. As shown in Fig. 2, both methods have a

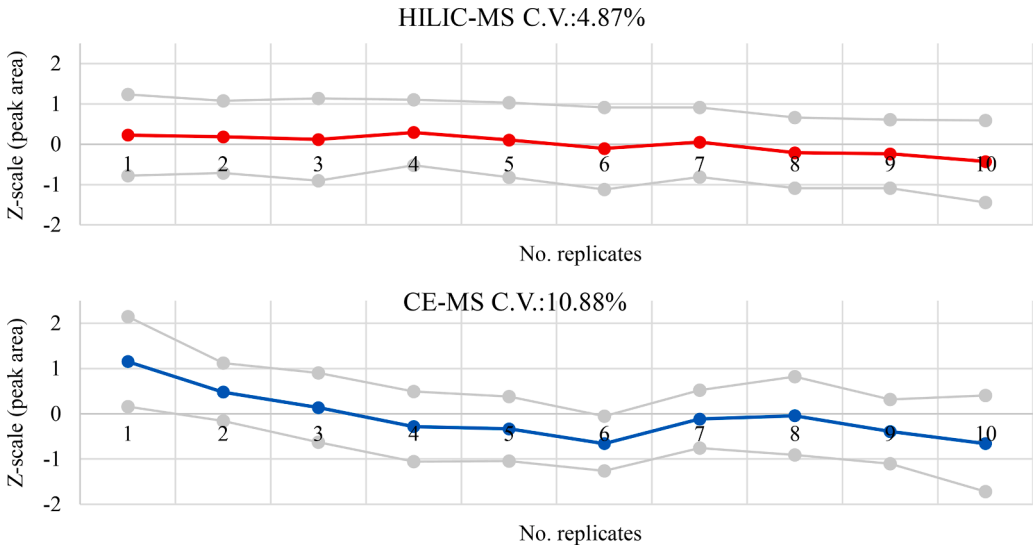

Fig. 2. Trend plot of the standardized cumulative peak area of all the compounds analyzed in this study. The trend plot and the coefficient of variation for repeatability (CV,%) were estimated from the mean and standard deviation of 10 replicates.

tendency towards a decrease in signal intensity over time points. The cumulative CV of the HILIC-MS method was lower (4.87%; with only 3 compounds with CVs above 10%, namely cysteine, decanoic acid and dodecanoic acid), than the cumulative CV of the CE-MS method (10.88%; with 14 compounds with CVs above 10%, namely 2-picolinic acid, cholic acid, decanoic acid, octanoic acid, glycine, L-citrulline, N-acetyl-glutamic acid, acetylcysteine, glutathione reduced, L-kynurenine, melatonin, serotonin, xanthosine, uridine-5′-diphosphate and also the internal standards). In this sense, compounds migrating within the EOF are reliable for quantification, since metabolites as xanthosine, aspartic acid, hydroxyproline and fructose migrating around 9 and 11 min had CVs between 7 and 10%. IS were exclusively thought to perform peak alignment and not to correct signal drift along the analysis; however, the high intra-day variability highlights the fact that QC-based correction and data normalization is strictly necessary for CE-MS data.

3.2.4. MS-DIAL automatic data analysis evaluation

MS-DIAL software was deployed to automatically analyze and extract the data from the 10 consecutive injections of each QC-mix on both analytical platforms. To our knowledge, this is the first time that MS-DIAL has been used to analyze metabolomics data produced with CE-MS. The idea was to evaluate and compare the feasibility of automatic data analysis for both methods using the different platforms. A first term of comparison was the ability of MS-DIAL to correctly detect the compounds spiked in each different QC. As shown in Table 4, in both cases, MS-DIAL was able to detect most but not all of the peaks (>80%) in any of the evaluated methods. In particular, MS-DIAL showed the highest "sympathy" with CE-ESI(+)-MS, while the lowest affinity with CE-(ESI-)-MS. Both HILIC-MS modes showed a similar affinity with MS-DIAL peak detection. In both HILIC-MS and CE-MS, peak detection was affected mainly by low peak intensity and the presence of double peaks.

A second term of comparison was the ability of MS-DIAL to correctly integrate the detected peaks. This was measured by a correlation analysis between the peak area detected by MassHunter quantitative software and MS-DIAL in 10 consecutive injections (Table 4). The results show that MS-DIAL was able to correctly integrate most of the peaks in HILIC-ESI(-)-MS (>90%), while its performance dropped in HILIC-ESI (+)-MS (<70%), likely due to lower peak intensity and worse peak shapes. In CE-MS data, in both ESI+ and ESI- ionization modes, this correlation was less than 70%, similarly to HILIC-(ESI+)-MS. Given that in the metabolomics workflow the statistical analysis of the data is performed on the dataset resulting from this data processing step, it is important that this last step is as efficient as possible, which depends mainly on the ability of the software to correctly integrate chromatographic/electrophoretic peaks. Thus, this finding appears overall disappointing for CE-MS data and rather acceptable for HILIC-MS data, simply because the negative mode is where most compounds are detected, and it is also the one that is best analyzed by MS-DIAL.A third term of comparison of the automatic inspection of the data was the principal component analysis (PCA) [36] performed by MS-DIAL after data analysis. The idea was to evaluate the capability of the PCA to separate the different QC classes according to the classes of standards added in each of them. As shown in Fig. 3, separation of QC classes was possible for data obtained in all analytical methods. It is evident that HILIC-MS performed better in both positive and negative modes, as consecutive analysis of the QC from the same chemical classes showed a more compact plot. Using this CE-MS method, the groups are less compact and present an analytical drift injection after injection, reflecting the high variability already encountered in the previous test. This is a major drawback in metabolomics analysis because its correction may be challenging. Boxplot analysis on all the spiked standards was performed, to confirm that the separation observed in the PCA was due to the difference in concentration of the compounds in the different QC classes. The boxplot analysis confirmed that the spiked compounds could be retrieved at different concentrations between the various QC classes, accordingly. An example is reported in the supplementary material, **Fig. S2**. This example shows that methionine was detected by CE-MS in serum samples spiked with and without QC-AA, providing insight into the real sample amount.

Table 4
Number of peaks detected and correctly integrated by MS-DIAL compared to the number of peaks integrated through targeted data analysis performed by MassHunter Quantitative software (Agilent). Peak area correlation was considered acceptable with a p-value < 0.05.

Method	MS-DIAL vs MassHunter qualitative – peak detection	MS-DIAL vs MassHunter quantitative – quantification (peak area correlation)
HILIC -	42/49 (85.7%)	38/42 (90.5%)
HILIC +	30/33 (90.9%)	20/30 (66.7%)
CE -	16/20 (80%)	11/16 (68.8%)
CE +	36/38 (94.7%)	25/36 (69.4%)

3.2.5. Migration time correction

Migration time fluctuation in CE-MS strongly affects peak alignment and identification, making migration time correction an essential step in CE-based metabolomics. Recently, the effective electrophoretic mobility (μ_{eff}) has been introduced as an effective parameter able to increase the reproducibility and identification capability of CE-MS compared to the relative migration time (RMT). An specific software (i.e., ROMANCE) has been developed by González-Ruíz et al. to correctly return μ_{eff} in open format files (.mzML) [19,37]. The results revealed that conversion of migration times into μ_{eff} reduced variability from 10.9% in RMT to 3.1% on the μ_{eff} scale for 20 compounds using the same BGE composition [28]. A similar μ_{eff} variability was achieved in this study (see supplementary material, **Table S5**). Unfortunately, the resulting .mzML files from ROMANCE retain only the MS data, losing all information about the MS/MS acquisitions. This is a very limiting factor in automatic data analysis, because MS/MS data is necessary to automatically interrogate MS/MS libraries and in-silico fragmentation tools; therefore, increasing the odds of compound identification. Despite being very promising, ROMANCE-based μ_{eff} has been set aside in this study.

In this framework, migration time correction has been performed using the retention time correction wizard of the MS-DIAL software (ver. 4.92) [23]. MT correction was executed applying the following settings: linear interpolation method, extrapolation method to MT beginning at 0 min and ending at linear extrapolation, and calculation of MT difference by "sample average" method. Then, for positive CE files, information of three reference compounds (4-aminobutyric acid-2,2,3,3,4,4-d6, L-tryptophan-(indole-d5) and paracetamol) was introduced (MT in min, MT tolerance of 2 min, *m/z, m/z* tolerance of 0.05 Da and minimum height of 1000 counts). After MT correction of reference compounds (Fig. 4A, B & C), the software uses this correction to align the rest of the peaks. As an example, Fig. 4D shows L-citrulline peak before and after the MT correction for 10 electropherograms. Further examples for other compounds (fructose, uric acid, allantoin and serine) are reported in the supplementary material (**Fig. S3**). For negative CE files, also two internal standards (paracetamol and ethyl sulfate) were used as reference compounds for MT correction. Apparently, MS-DIAL is able to overcome migration time fluctuations using as reference at least two internal standards.

3.2.6. Time & cost analysis

To increase comparability of the methods applied, several steps of the analysis have been kept equal, including the same type of samples obtained from the same sample-set, the same extraction method (with the exception of a necessary filtering step in CE-MS at these conditions), the instrumental brand (Agilent), the MS system, the calibration mixes, the standards, and internal standards used, the open-source software (MS-DIAL). Nevertheless, there were small adjustments that remained different between the two methods and that increase the time-cost

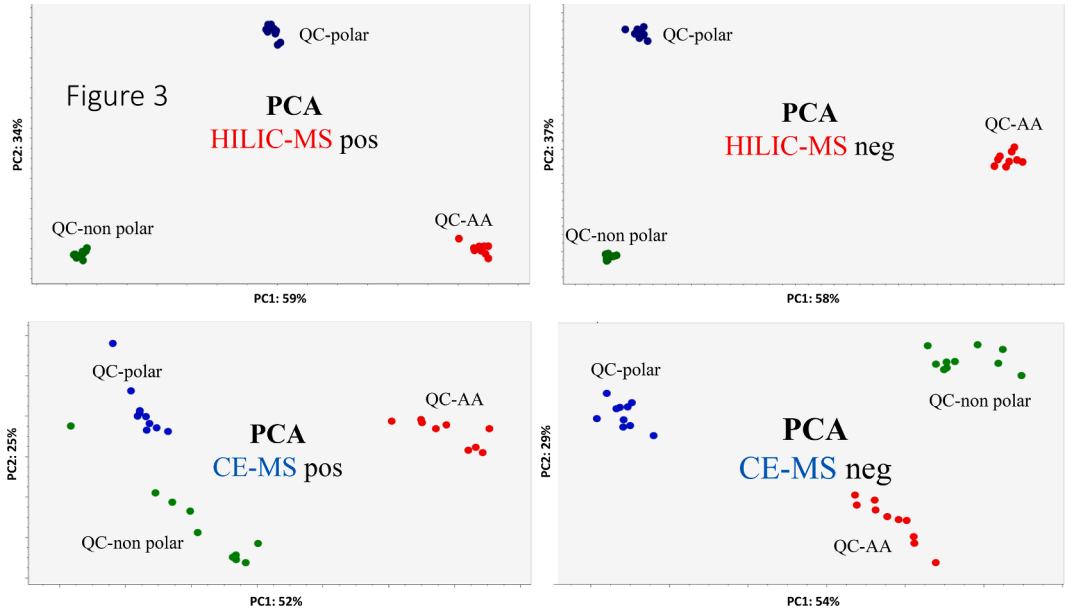

Fig. 3. Principal component analysis (PCA) of the HILIC-MS and CE-MS data in both positive and negative modes.

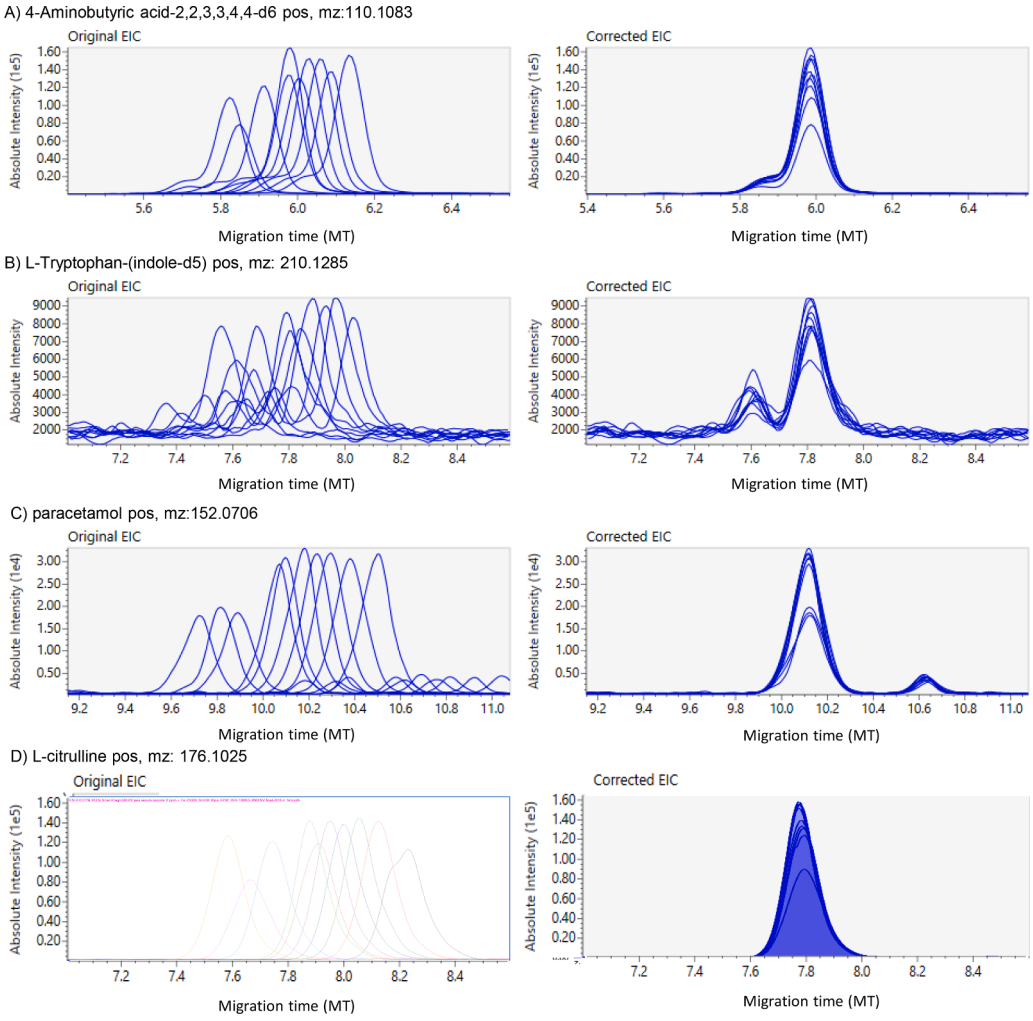

Fig. 4. (A, B and C) Internal standard peaks before and after MT correction for 10 consecutive injections; D) L-citrulline peaks before and after MT correction for 10 consecutive injections, as an example of how the retention time correction wizard applies the correction on the other compounds.

tradeoff of each of them (Table 5).

From an instrumental point of view, there is no doubt about the fact that the use of a CE system is advisable, due to the lower cost of both the instrument and maintenance. Nevertheless, a comparison of the costs in terms of consumables is necessary to understand the differences between the two techniques. The main costs for the HILIC-MS method are the chromatographic column, its filters, and solvents. In this study, one chromatographic column and 2 filters were used for about 500 injections. Problems with the filter can arise for several reasons and are difficult to predict, but mainly associated with sample complexity and low sample preparation in metabolomics. Furthermore, for the 500 injections, about 3.5 L of chromatographic solvents were used.

The main cost of the CE-MS method is related to the sample filters (about 4 euros per sample). Taking into account an average metabolomics experiment of at least 100 samples, the analytical price can be quite high compared to the sample preparation required for HILIC-MS analysis. The dedicated platinum needle for ionization in CE-MS analysis is expensive but very robust, while the silica capillary is cheap but may break after few hundred of injections. However, there is no doubt about the fact that CE is a lot greener technique than HILIC (and LC in general) and its use is advised in the future.

On the other end, the HILIC methodology requires shorter time for the preparation of the samples and shorter analytical time (Table 5). From a timely point of view, the CE-MS method requires longer extraction time (+2 h centrifugation for the filtering step is mandatory, at least at the conditions of the selected method) and a longer electrophoretic run (about 30 min) compared to the 16 min chromatographic run of HILIC.

Table 5
Time and cost comparison of the HILIC-MS and CE-MS analytical methods. * Unpredictable.

Consumables	Platform	Amount	Cost	Constrains
Chromatographic column	HILIC-MS	ND	1100 euros	Valid at least 2000 injections
Column filter	HILIC-MS	ND	200 euros	Valid until problems arise*
CE-MS filters	CE-MS	+2 h	Min 4 euros each	Necessary
Platinum ES needle	CE-MS	ND	1200 euros	Necessary for negative mode [20]
Silica capillary	CE-MS	ND	12 euros	Valid until capillary breaks*
Analytical constrains	Platform	Amount	Cost	Constrains
Chromatographic solvents	HILIC-MS	24 mL/h	30 euros per day	Mobile phases A & B (reported in Section 2.3)
Chromatographic run	HILIC-MS	16 min		3.5 injections per hour
Sheath liquid	CE-MS	0.36 mL/h	Negligible cost (**green technique**)	Reported in Section 2.4
Electrophoretic run	CE-MS	30 min		2 injections per hour

4. Conclusions

If the interest of characterizing the polar metabolome is no longer to be demonstrated, its implementation is a complex task. Several analytical methods have been developed to analyze it in different matrices, but none has demonstrated the ability to perform a complete characterization of the polar metabolome. Thus, a plethora of analytical platforms are being tested to extend its coverage [31,38]. In this study, a direct comparison has been carried out between CE-MS and HILIC-MS based methods. At the current state-of-the-art, CE-MS shows some advantages over HILIC-MS in terms of compound detectability and overall peak shape, but also lower precision across injections is obtained, even the values could be acceptable. This issue, coupled with CE-MS data limited capacity to be automatically analyzed by MS-DIAL restrain its applicability into metabolomics studies, when using this software.

Among the five terms of comparison applied in this study, CE-MS showed a slight superior compounds' detectability and equaled HILIC-MS in the Pezzatti score (a specific rate precisely developed for comparison of metabolomics methods) but underperformed on the precision test (intra-day precision) and in the automatic analysis of the data. Despite the similarity of CE-MS peak shapes and HILIC-MS peak shapes, MS-DIAL seems to be more suitable for the deconvolution of HILIC-MS data. This cannot be considered surprising as this software has been developed primarily for LC data processing. On the other end, migration time fluctuations, a longstanding problem of CE-MS analysis, seems to be easily manageable via the retention time correction wizard of MS-DIAL. Last but not least, both methods have shown to have advantages and disadvantages in time and cost analysis, hence the simplicity of the HILIC-MS extraction method is preferable (due to the high number of samples in each metabolomics experiment). In contrast with HILIC-MS, CE-MS method is more environmentally friendly, with an extremely low volume of residues generated, considering the use of cheap and non-contaminant buffer solutions as BGE.

In the studied conditions, HILIC-MS seems to be a more suitable alternative to investigate the polar metabolome, especially from repeatability and data analysis points of view. Future developments in CE-MS in terms of analytical methods and dedicated software may reduce the current gap between the two techniques. However, CE-MS showed interesting detection abilities, including similar (or slightly superior) sensitivity, especially in the positive mode, that may allow complementing HILIC-MS in the exploration of the polar metabolome, and it is worthwhile to try to use and implement this technique in metabolomics studies.

Supplementary figure 1: a comparison between the tren do fht signal of ornithine across 100 injections of the same spiked sample before and after data correction using the internal standard 4-aminobutyric acid-d6

CRediT authorship contribution statement

Luca Narduzzi: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Visualization, Software, Writing – original draft. **María del Mar Delgado-Povedano:** Data curation, Formal analysis, Investigation, Writing – original draft. **Francisco J. Lara:** Methodology, Supervision, Validation, Visualization. **Bruno Le Bizec:** Project administration, Validation. **Ana María García-Campaña:** Funding acquisition, Resources, Writing – review & editing. **Gaud Dervilly:** Validation, Writing – review & editing. **Maykel Hernández-Mesa:** Conceptualization, Funding acquisition, Project administration, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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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.2023.464239.

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