Microchemical Journal 162 (2021) 105741

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

On-line coupling of two-phase microelectroextraction to capillary electrophoresis – Mass spectrometry for metabolomics analyses

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ARTICLE INFO

Keywords:
Electroextraction
Electromembrane extraction
Capillary electrophoresis
Single-drop microextraction
Bioanalysis
Metabolomics

ABSTRACT

We have integrated two-phase electroextraction (EE), capillary electrophoresis (CE) and mass spectrometry (MS) to combine rapid sample extraction, high performance separation and sensitive detection of metabolites. EE took place from a sample vial containing organic donor phase into a ~100 nL droplet of background electrolyte, which was hanging at the inlet of the capillary. In order to enable the EE process while the outlet of the capillary was connected to the MS several modifications were made. A modification was made to the sheath-liquid CE-MS interface, based on the use of a corrosion-resistant titanium ESI sprayer and a 6-port valve to switch between the CE-MS sheath liquid and the EE make-up liquid. Furthermore, a counter-pressure was applied, in order to prevent EOF from retracting the droplet during EE.

Then, using five model metabolites (namely, leucine, isoleucine, adenosine, phenylalanine and guanosine) and crystal violet (CV) the extraction time and voltage were optimized and found to be 2000 s and 1 kV, respectively. Using these optimized conditions, the effect of various sodium chloride concentrations was examined to assess the influence of varying salt concentrations in biological samples. A set of 9 amino acids was used to validate the method. The detection limits ranged between 5 and 100 nM. LODs were improved 50–250 times in comparison with conventional CE-MS. Finally, to demonstrate the potential of the EE-CE-MS platform for bioanalysis of volume-limited samples, a urine sample of 300 nL was analyzed. This resulted in detection of 122 putative metabolites. The results indicate that EE-CE-MS could become a powerful tool for metabolomics analyses of volume-limited samples.

1. Introduction

Metabolomics aims to measure and quantify all small molecules in a biological matrix [1]. These small molecules can for example differentiate disease state from non-diseased state or a drug naïve from a drug treated group. With this information predictions can be made with regards to drug efficacy and adverse effects as well as tailoring therapy to individual patients [2–4]. In order to measure the metabolites using sensitive mass spectrometry the metabolites of interest need to be extracted from the matrix and compounds that disturb the analysis need to be removed. Many methods capable of extracting analytes of interest exist for bioanalysis. Prominent and commonly used methods are protein precipitation, liquid–liquid extraction and solid phase extraction [5]. These methods have limitations when dealing with biomass-limited samples or volume-limited samples. Biomass-limited samples are dilute, such as tissue dialysates and the perfusate of microfluidic cell cultures [6–8] and volume-limited samples are in the sub-μL range, such as animal tissues, rodent cerebrospinal fluid and 3D microfluidic cell cultures [9,10]. Volume-limited samples often require diluting the sample in order to improve sample handling and circumvent evaporation effects. This often causes analytes to fall below the limit of detection (LOD) as only a part of the sample is injected into the analysis system. In order to circumvent this problem, various sample preconcentration techniques have been developed [11], in particular the class of liquid phase microextraction (LPME) techniques [12], which include single-drop microextraction (SDME) [13]. These techniques extract analytes from a large volume donor phase into a small volume droplet prior to analysis and thereby improve the LOD. Aside from preconcentrating compounds of interest these techniques also selectively remove interfering components, such as proteins. On-line coupling of SDME has been achieved

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https://doi.org/10.1016/j.microc.2020.105741
Received 4 September 2020; Received in revised form 8 November 2020; Accepted 8 November 2020
Available online 3 December 2020

using various analytical systems, such as SDME-CE [14–16], SDME-GC [17–20], SDME-LC [21,22] and using direct infusion (DI): SDME-DI-MS [23]. In one SDME-CE procedure [15] a three-phase system consisting of a two phase droplet with an exterior of octanol and an interior of BGE acceptor phase is reported. The third phase is the aqueous sample in which the dual phase droplet is immersed. The analytes are driven into the droplet interior due to pH difference, with the analytes becoming uncharged in the aqueous donor phase and charged in the aqueous acceptor phase in the interior of the droplet. This approach has also been used in combination with MS detection, leading to detection limits in the low nM range for cationic drug compounds, anionic herbicides and pesticides [14,16]. However, for coupling to MS the capillary outlet needs to be placed in an outlet vial filled with BGE to prevent air from entering the capillary at the outlet during droplet formation. Afterwards, the capillary needs to be manually placed back into the ESI sprayer. This makes long sequences of analyses laborious. Several preconcentration approaches in which electromigration is the driving force have been developed as well, prominently electromembrane extraction (EME) and EE [24]. In 3-phase EME, analytes are typically extracted from an aqueous donor phase via an electric field over an organic supported liquid membrane (SLM) into an aqueous acceptor phase. The technique has been most commonly applied to drug analysis [24,25], although recent efforts have been focusing more on analyses of endogenous compounds as well [26,27]. In 2-phase EE [28–31] analytes are extracted from an organic donor phase into an aqueous acceptor phase via an electric field. During EE the analytes are, after extraction, stacked beyond the liquid–liquid interface in the aqueous acceptor compartment. This stacking is caused by differences in electric field strength between the phases and thereby differences in velocity of analytes. Previously, we have addressed the mismatch of injection volume and typical sample volumes in capillary electrophoresis (CE) by integrating three-phase microelectroextraction into CE-UV [32]. While this significantly improves sample loading and detection limits, the method has limitations, stemming from its detector, in terms of selectivity.

In this work we developed a novel online two-phase electroextraction capillary electrophoresis-mass spectrometry (EE-CE-MS) method for metabolomics analyses. The electric field is applied via a hollow anode in the inlet vial through which the capillary protrudes. Importantly, there is no need for an outlet vial, as the near vertical position of the ESI sprayer enables the formation of a pendant droplet of make-up liquid at the outlet of the capillary. Constructing a corrosion resistant system required modification of the sprayer tip material from stainless steel to titanium. In a first series of experiments, the extraction performance of the system, in particular the stability of the acceptor droplet, was examined visually using the cationic dye CV. The extraction conditions, i.e. voltage and time and the influence of salts, were investigated for model metabolites. Analytical figures of merit, including LOD, are shown as well as a comparison with conventional CE-MS and other SDME methods combined with CE-MS. Lastly, we demonstrated the potential of EE-CE-MS for volume-limited metabolomics by analyzing a 300 nL urine sample.

2. Materials and methods

2.1. Chemicals

Ethyl acetate (EtOAc) and methanol (MeOH), both analytical grade, were obtained from Actu-all Chemicals (Oss, The Netherlands). Deionized water was generated via a Merck Millipore water purification system (Billerica, MA, USA). Formic acid (FA) (98%+) was obtained from Fischer Scientific –Acros Organics (Hampton, NH, USA). Trifluoroacetic acid (TFA) 99% was obtained from Alfa Aesar (Haverhill, MA, USA). CV was obtained from Merck (Darmstadt, Germany). The following analytical grade analytes were obtained from Sigma Aldrich (St. Louis, MO, USA): adenosine, guanosine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. Cell free amino acid mix (U-^{13}C 97 – 99%, U-^{15}N 97 – 99%) was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA).

2.2. Equipment

An Agilent G1600 3D CE (Waldbronn, Germany) was used for performing the experiments. An 80 cm 50 μm I.D. / 365 μm O.D. bare fused silica capillary obtained from Polymicro Technologies – Molex LLC (Lisle, IL, USA) was used as the extraction and separation capillary. When the capillary was placed into the vial it protruded approximately 2.2 mm from the electrode. New capillaries were flushed with 1 M FA for 10 min prior to the first use. The sample tray was cooled to 20 °C using a Tamson Instruments TLC 10–3 Cooler/circulator (Bleiswijk, The Netherlands). A USB pen-camera model B005 + made by Shenzhen Supereyes Co., Ltd (Shenzhen, Guangdong, China) recording at a resolution of 640 × 480 pixels was placed inside the system to monitor the capillary inlet using Debut Video Capture by NCH Software (Greenwood Village, CO, USA), which was set to capture 1 frame every 2 s at an output framerate of 30 frames per second. The inlet vials were backlit using a white LED with an adjustable output luminance, which was controlled by a custom made LED controller. Make-up flow was delivered via an Agilent 1260 series pump (Waldbronn, Germany) at a rate of 50 μL min^{-1} for the EE process and sheath liquid (SL) at 15 μL min^{-1} post-split for CE-MS separation. Selection of SL was controlled via a contact-closure that switched a Cheminert® 6-port valve using a two position actuator controller module, both were obtained from VICI AG (Schenkon, Switzerland). A customized ESI spray tip for the Agilent CE-ESI-MS Sprayer was manufactured from titanium by the Leiden University Fine Mechanical Department. The CE-ESI-MS Sprayer was placed into a Bruker Daltonics micrOTOF mass spectrometer (Bremen, Germany).

2.3. Preparation of standard solutions

The EE acceptor phase, BGE and make-up liquid for EE all consisted of 1 M FA. Sheath liquid during CE-MS operation consisted of 50:50 MeOH:H_2O and 1 M FA.

Two standard solution mixtures at 250 μM were made in water one containing leucine, isoleucine, adenosine, phenylalanine and guanosine the other containing nine amino acids (leucine, isoleucine, proline, phenylalanine, tyrosine, valine, threonine, methionine and serine). For the lower calibration range the standard mixtures were diluted to 10 μM.

The standard samples for analysis were made prior to each analysis in 4850 μL EtOAc containing in total 3% (v/v) H_2O (i.e. standards, internal standards and additional water to saturate the EtOAc summed to 150 μL), 6.25 μL FA (0.125%) (v/v), 245 nM CV, and depending on the experiment a variable amount of either standard mixture at 250 μM or 10 μM and 30 μL ^{13}C^{15}N internal standards were added.

Salt-rich samples were made by adding valine, leucine/isoleucine, proline, phenylalanine and tyrosine to EtOAc, with the final concentration in EtOAc being 500 nM, along with their stable isotope labelled counterparts as well as CV and 0.1% (v/v) FA. The total water content for each sample was 3% (v/v). Sodium chloride was varied within the aqueous mixture prior to dilution in EtOAc at 0.1% (m/v), 0.2% (m/v), 0.5% (m/v), 1% (m/v) and 2% (m/v).

For urine analysis the sample was obtained from a healthy male volunteer and 10 μL of the urine and 30 μL cell free labelled amino acid internal standard mixture was mixed with 4850 μL EtOAc and 0.125% (v/v) FA. The EtOAc was subsequently saturated with 110 μL water. From this mixture an aliquot of 150 μL, corresponding to 300 nL urine, was transferred into a sample vial for analysis. After adding all components together, the sample was mixed thoroughly using a vortex mixer.

Extractions for each experiment took place from 1.5 mL vials with 4.6 mm flat-bottom inserts obtained from Sigma-Aldrich (St. Louis, MO, USA) containing 150 μL of sample unless stated otherwise. In order to

ensure that the capillary would be consistently aligned with the electrode the flat-bottom inserts were immobilized in the vial by adding tape spacers around the inserts to minimize lateral movement in the vials and maintain a vertical position.

2.4. Data analysis

Estimations of the droplet volume during extraction were made by using image analysis. This was done by counting the pixels of the droplet and converting the area into cubic micrometers. After converting the area to cubic micrometers the droplet volume was estimated by assuming the shape of the droplet to be a perfect sphere. As this is not a validated method, it will only give an indication of changes in droplet size and not the actual volume. This is still useful for monitoring droplet stability.

Results obtained from the analysis of urine were analyzed for quantifiable molecular features using Bruker Daltonics Data Analysis software. The settings for the 'find molecular features' function were more strict then reported elsewhere [33,34] in order to assure true peaks: signal-to-noise ratio of 10, correlation coefficient threshold of 0.7, minimum compound length of 11 and a smoothing width of 2. The identified features were verified by visual inspection of the electropherograms.

2.5. Method characterization

The optimized EE-CE-MS method was tested for linearity using different concentrations (0, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 nM, n = 3) of 9 amino acids . Analyses were reported after being corrected with $^{13}C^{15}N$-labelled internal standards. The 500 nM samples were analyzed on separate days in order to determine the intraday (n = 3) and interday repeatability (n = 6). The analytes were also examined with conventional CE-MS in a concentration range of 1 – 100 μM using a hydrodynamic injection of 50 mbar for 40 s in order to determine enrichment factor (EF) and extraction recovery (ER). Furthermore, this enabled the comparison of EE-CE-MS with CE-MS with regards to linearity, sensitivity (i.e. slope of the calibration curve) and LOD. LODs were estimated by using the signal-to-noise (S/N) ratio of calibration measurements at the lowest detectable concentration and extrapolated to S/N = 3. EF and ER were determined according the following equations:

$$EF_i = \frac{Cacceptor_i}{Cdonor_i} \quad (1)$$

$$EF_{max} = \frac{Vdonor_i}{Vacceptor_i} \quad (2)$$

$$ER_i = \frac{EF_i}{EF_{max}} \times 100\% \quad (3)$$

3. Results and discussion

3.1. Development of EE set-up

3.1.1. Procedure for electroextraction coupled to CE-MS

The development steps leading to the EE-CE-MS procedure are described in Supporting Information S1. After several EE runs it was noted that the ESI sprayer tip suffered from chemical corrosion due to the 1 M FA SL that was flowing past the stainless steel tip at elevated temperatures. This corrosion severely affected spray performance. A new corrosion resistant spray tip was built out of titanium to replace the corroded tip (Supporting Information S5).

In order to couple EE to MS several additional steps needed to be added to the procedure. The EE procedure is shown schematically in Fig. 1. and described in Table 1.

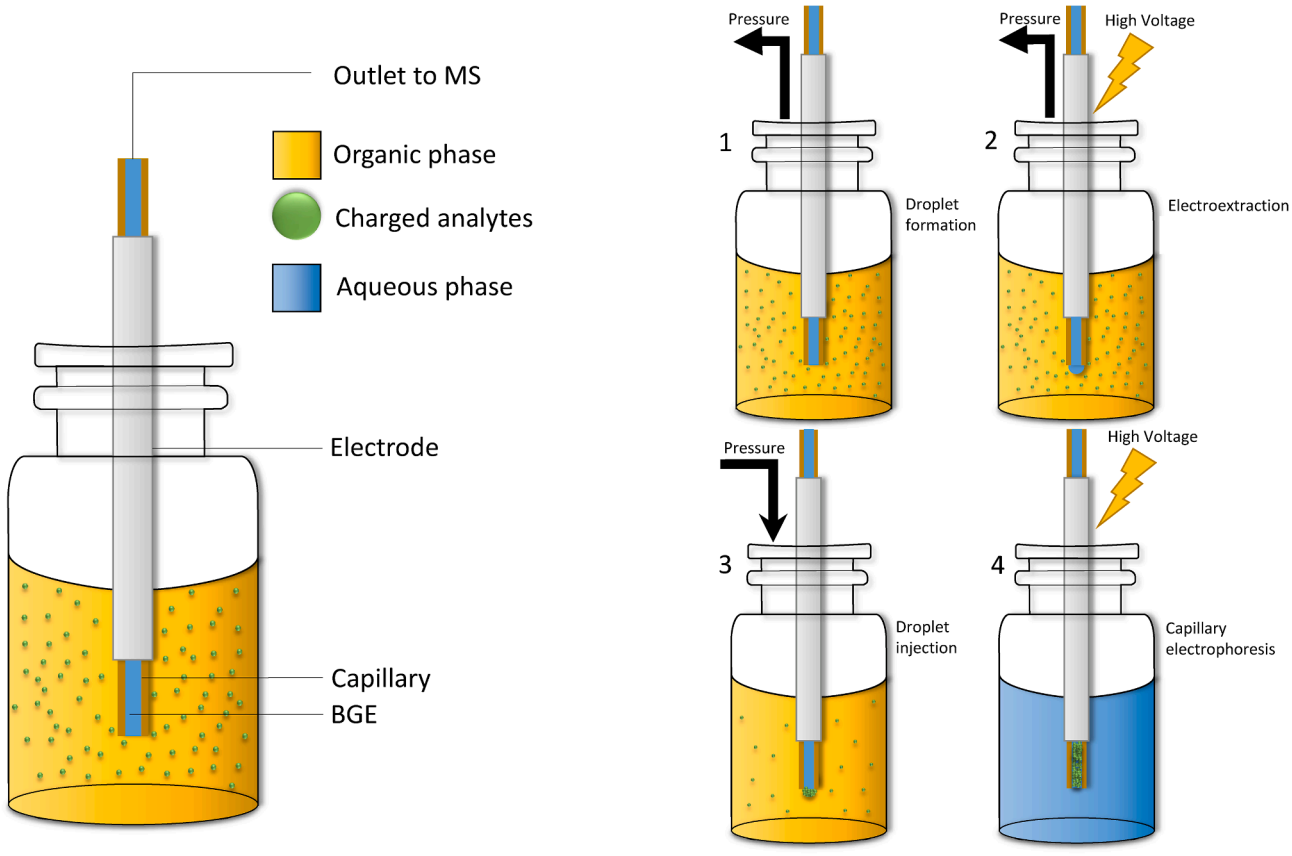

Fig. 1. Schematic representation of the EE procedure. (1) Droplet formation. (2) Electroextraction. (3) Droplet injection. (4) Capillary electrophoresis.

Table 1
EE-CE-MS procedure steps over time.

Time (min)	Step	Purpose	Fig. 1 step
0	Flush capillary with 1 M FA	Rinse capillary; allows SL of previous run to switch to make-up flow	
9.75	Switch to vial with organic phase	Sample vial selection	Fig. 1 – 1
10.00	−50 mbar for 90 s	Droplet formation	Fig. 1 – 1
11.50	Reduction in counter pressure ranging from −10 to −20 mbar. Start application of voltage ramp.	Start extraction slowly; keep the droplet stable during extraction; prevent perturbations to droplet	Figs. 1 – 2
11.60	Voltage at set value	EE	Figs. 1 – 2
11.60 + x	Depending on the extraction, voltage is applied for up to × min followed by droplet retraction at + 50 mbar for 40 s	EE	Figs. 1 - 3
12.26 + x	Vial switch back to 1 M FA	Separation	Figs. 1 – 4
12.28 + x	Application of 20 kV for separation	Separation	Figs. 1 – 4
42.28 + x	End of separation and data collection	Separation	

In the set-up the CE is connected to a contact closure. Upon starting a CE method the contact closure switches a connected six-port switching valve to a different position and sends a start signal to the MS. The activation of the contact closure enables the EE make-up liquid of 1 M FA (instead of CE-MS sheath liquid) to flow past the ESI sprayer during the EE procedure. The method starts with a roughly 10 min waiting time to enable the EE make-up liquid to enter into the capillary outlet during droplet formation whilst the nebulizer and other relevant MS parameters are set to 0. This EE make-up liquid prevents an air gap at the outlet during the next step, which starts after 10 min: the formation of a droplet of acceptor phase at the inlet through application of negative pressure (Fig. 1, step 1). After the droplet is formed for 90 s a voltage is applied whilst applying a counter pressure to electroextract the analytes in to the droplet (Fig. 1, step 2). The droplet is then injected for 40 s at 50 mbar (Fig. 1, step 3). After EE was completed, a new method is started for separation and the sample vial is switched to the vial containing BGE (Fig. 1, step 4). The start of the separation method again switches the six-port switching valve from the EE make-up liquid to CE-MS sheath liquid consisting of MeOH:H_2O + 1 M FA. See also Supporting Information S6 for further details on the operation of the switching valve.

The contact closure activation also initiates data collection of the TOF-MS. In the first minutes, the sprayer voltage, the nebulizer and the end plate offset were all switched off to enable EE make-up flow past the spray tip and prevent air from entering the capillary during droplet formation. Moreover, this prevented the suction effect [35,36] from retracting the droplet. Once the CE separation method is started the second MS segment starts. In this segment the end plate offset is set to −500 V, the capillary voltage is set to −4600 V and the nebulizer pressure is set to 0.4 bar.

3.1.2. Optimization of extraction voltage and time

First, the extraction voltage of the EE system was optimized using a set of test metabolites (leucine, isoleucine, adenosine, phenylalanine and guanosine) and CV was added in order to visually monitor the process.

The results of the peak areas for the analytes during the voltage optimization are shown in Fig. 2a. Leucine and isoleucine are reported together as the analytes were not baseline separated.

As can be seen in Fig. 2a increasing the extraction voltage leads to higher peak areas. However, it was also noted that at 1500 V the droplet was not always stable. The droplet often became deformed and at times was removed from the capillary (Supporting Information S7). Since the aim of this work is to have the highest possible electric field without compromising the stability of the system 1 kV was chosen as the most suitable extraction voltage.

The results of the extraction time optimization are shown in Fig. 2b-f.

Four metabolites, namely phenylalanine, leucine/isoleucine and guanosine, increased proportionally with extraction times up to 1600 s. Extraction times beyond 2400 s yielded instable droplets and were not repeatable. CV and adenosine appear to behave in a non-linear fashion and appear to reach a plateau sooner than the other analytes, indicating that they are extracted much faster than the other metabolites. Observations of CV in this experiment are in agreement with previous visual observations. CV is a quaternary ammonium cation with a permanent charge and has multiple cationic charges at lower pH; this strongly favors fast electroextraction. For adenosine no explanation with respect to its physicochemical parameters such as solubility, pKa, log P, size and electrophoretic mobility can be made and this warrants further investigation. In order to maximize extraction efficiency for all test compounds, the optimum extraction voltage was defined to be 1 kV and the optimum extraction time to be 2000 s. The latter was chosen as it appears that lengthening the extraction yields higher peak areas.

3.1.3. Influence of salt on EE

The method was tested to see the effects of salts, as these vary between biological matrices and can influence extraction efficiencies, potentially resulting in a method that is not sufficiently quantitative. Therefore, experiments were performed with samples containing between 0.1% (m/v) and 2% (m/v) sodium chloride. This range was chosen as 0.9% (m/v) sodium chloride is often referred to as physiological salt and mimics biological fluids. EE was performed by forming a droplet for 90 s at −50 mbar followed by extraction at 1 kV for 2000 s with an adapted counter pressure of −20 mbar. Finally, the droplet was retracted for 40 s at 50 mbar. Since the salt concentration influenced the absolute peak areas and extraction recoveries the analyses were internal standard corrected. The effects of these salt concentrations on the normalized signal intensities and compared to normalized internal standard corrected analytes are shown in Fig. 3.

In Fig. 3 it can clearly be seen that signal intensities drop with increasing salt concentrations. This can be explained due to the increase in conductivity of the organic donor phase with increasing sodium chloride concentrations. These increased concentrations lead to a decreased electric field over the organic phase and thereby conditions for extraction become less favorable, as the velocity of analytes is dependent on the electric field and electrophoretic mobility. This would be detrimental to the application of EE in bioanalysis as variations in salt concentrations and conductivity are likely to be expected between different biological matrices and even samples. Furthermore, it was observed that the droplet was not fully stable, leading to greater fluctuations in the volume of the droplet during and between measurements. This can be attributed to the changing distribution of the electric field during EE under increasing sodium chloride concentrations and therefore varying conductivities. As the outlet is open-ended, a counter pressure needs to be applied in order to keep the droplet stable. However, this does not fully address fluctuations in the droplet as can be seen in Fig. S3c.

However, as can be seen, correction with internal standards can reduce the variability between salt concentrations up to 0.5% (m/v). Nevertheless, the non-corrected response decreases strongly with increased salt concentrations beyond 0.5% (m/v) and that adversely affects detection limits. Moreover, peaks became wider and less repeatable with increasing salt concentrations. A possible solution would be to dilute salt-rich samples prior to electroextraction, but this requires a set-up that is capable of extracting from a larger volume of donor phase than the equipment in this study could accommodate.

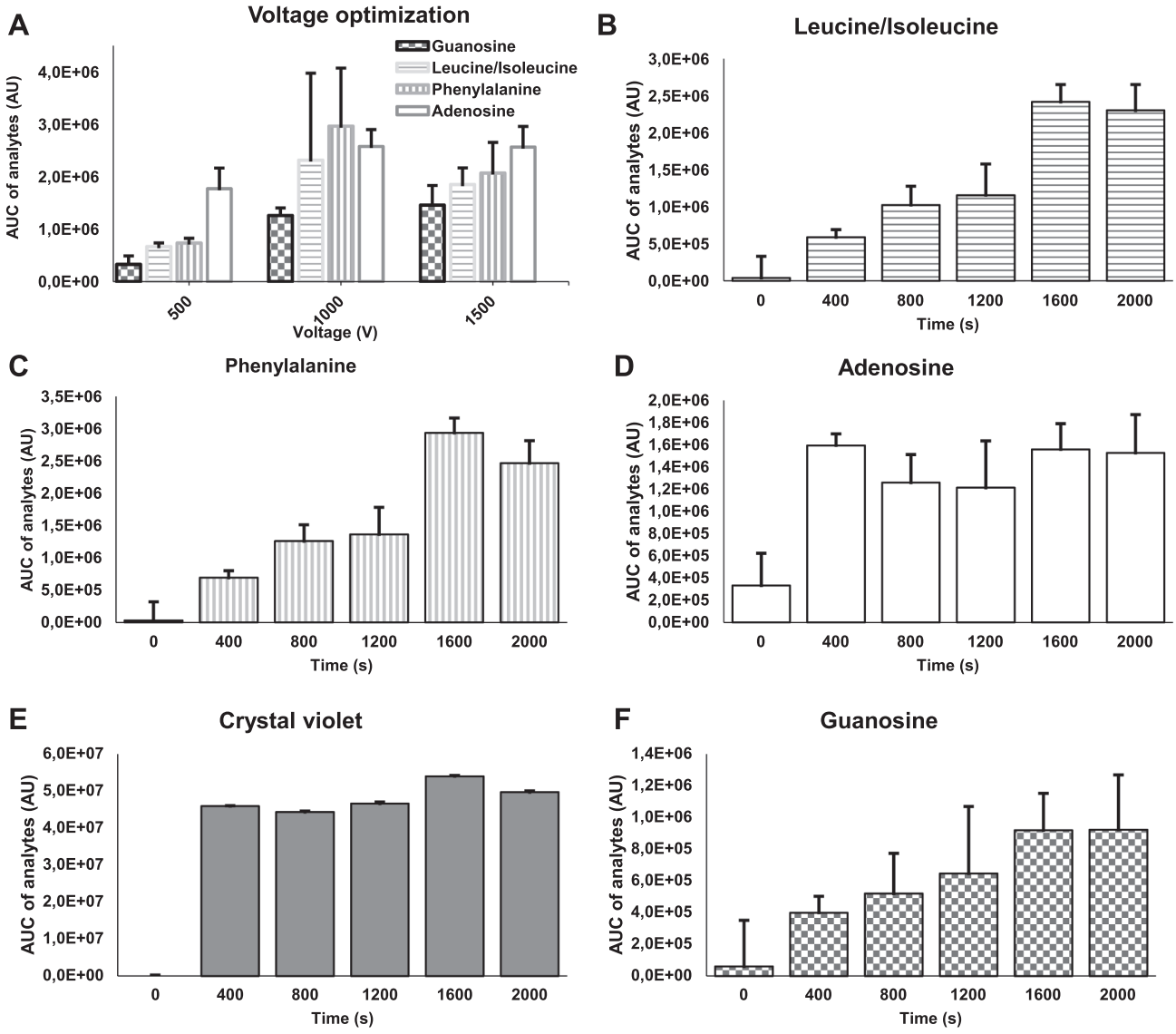

Fig. 2. Extractions were performed with all analytes at 1000 nM except CV, which was present at 245 nM. (a) Optimization of EE voltage using droplet formation of 90 s at −50 mbar at an extraction voltage (n = 3) of either 500, 1000 or 1500 V and extraction time at 1000 s with an appropriate counter pressure, and retraction for 40 s at 50 mbar. (b-f) Optimization of EE time using droplet formation of 90 s at −50 mbar at an extraction voltage of 1000 V and extraction time (n = 3) of either 0, 400, 800, 1200, 1600, 2000 s with an appropriate counter pressure and retraction for 40 s at 50 mbar.

Another possible solution would be to utilize a neutral coated capillary to suppress the effects of EOF. This would enable stable extractions for a wide range of biological samples at the cost of a reduced extraction efficiency.

3.2. Analytical performance of EE-CE-MS

3.2.1. Analytical figures of merit

Amino acids were chosen to characterize the method as they play an important role in metabolomics analyses. Nine amino acids were used to characterize the EE-CE-MS method and to compare the method with conventional CE-MS. An overlay of extracted electropherograms of these nine amino acids for conventional CE-MS and EE-CE-MS can be seen in Supporting Information S8. The results of this characterization can be seen in Table 2.

Determination coefficients (R^2) of >0.99 were obtained, indicating good linearity, with the exception of proline and serine which had an R^2 value of >0.95. Linear ranges for the compounds went up to 2000 nM. Using higher concentrations such as 5000 nM frequently caused clogging of the capillary, likely due to precipitation of analytes due to the high concentration present within the extraction droplet, underlining the concentrating power of EE.

3.2.2. Enrichment factors and extraction recovery

In order to assess the performance of the new method the ER and EF were calculated for the analytes [24]. EF_{max} from the organic phase was determined to be 3000× as extraction took place from 150 μL donor phase to approximately 50 nL acceptor phase. EF_{max} from the initial aqueous sample was determined to be 90× this is equal to the sample size prior to dilution (4.5 μL) which was extracted into a 50 nL acceptor droplet. Therefore a 2 μM analyte in EE should yield the same response as a 180 μM analyte in conventional CE, given full droplet retraction and complete recovery. ER_i was determined by comparing the response of 2 μM during EE to the theoretical response at 180 μM via conventional CE by extrapolating the calibration curve and expressing ER_i as the fraction of the expected response. Using ER_i both the EFi from the organic phase and the aqueous phase could be determined.

ER figures ranged between 6.4% and 82%, corresponding to EF of

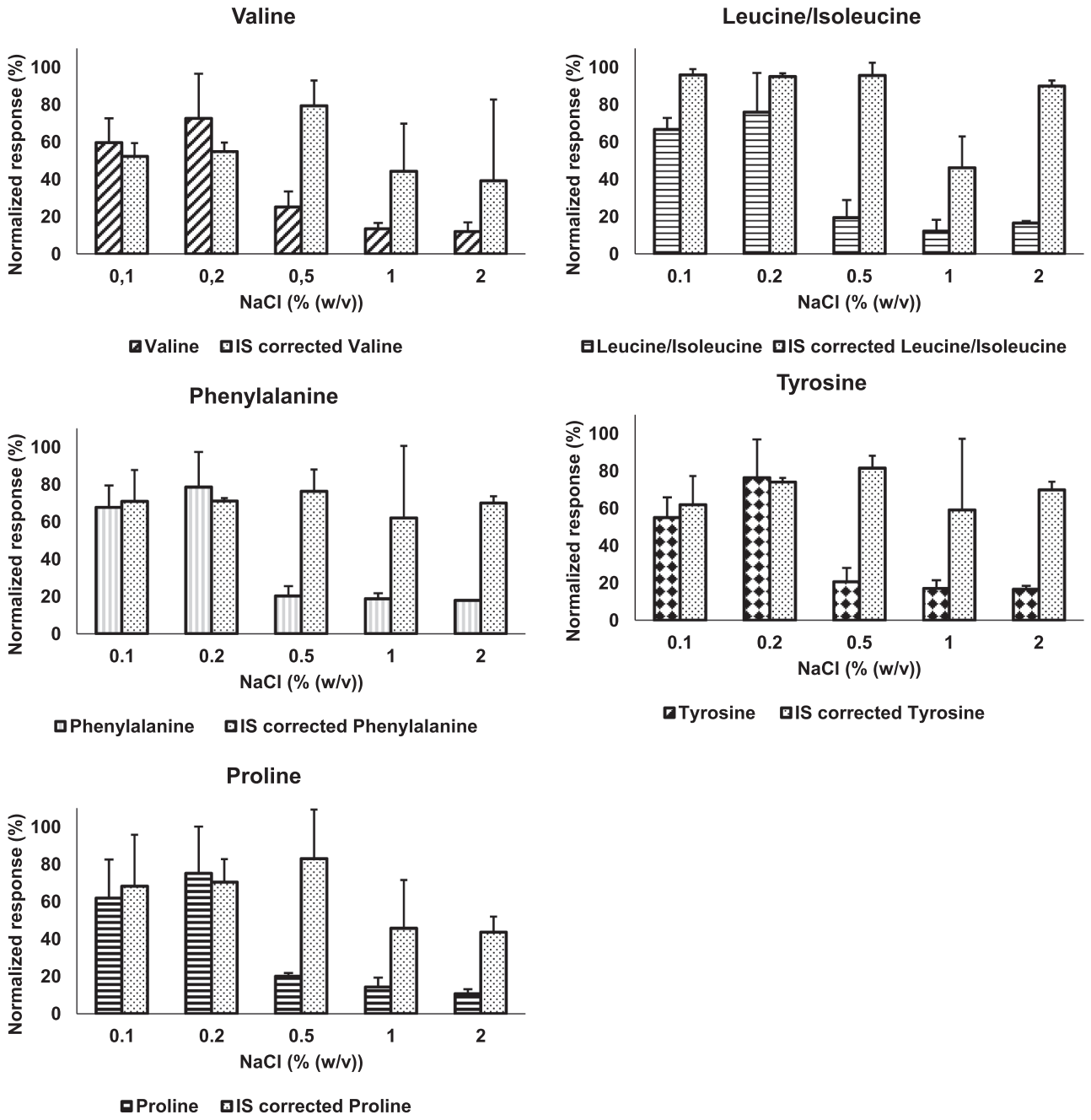

Fig. 3. The influence of salt on analyte extraction during EE without internal standard correction and with internal standard correction. Analyte concentrations are normalized to the highest signal of a specific analyte or specific analyte:internal standard ratio.

192× and 2472× from the organic donor phase. Methionine and serine had a much lower ER and EF than the rest of the metabolites, indicating poor extraction for these amino acids. It should be noted that the LODs in conventional CE-MS were relatively poor as well at 5 and 20 μM for methionine and serine respectively. The obtained ER for the other amino acids indicate that the technique is not fully exhaustive. This can be partly explained by the fact that the extraction procedure has some selectivity towards certain amino acids. The type of organic phase as well as additives that play a role as ionic carriers are known to influence selectivity based on polarity in EME [37–40] and both are likely to influence selectivity in EE as well.

3.2.3. Detection limits

LODs were calculated by measuring analytes at the lowest concentration in the linear range and extrapolating to a S/N of 3. LODs of 5 – 100 nM were obtained for the analytes with EE-CE-MS compared to 1000 – 20,000 nM using conventional CE-MS with hydrodynamic injection, corresponding to LOD improvements between 50 and 250 times.

3.2.4. Repeatability

Repeatability was determined using internal standard correction. Intraday repeatability, which was measured at 500 nM, varies between the various analytes between 0.50% RSD and 32% RSD. Good intraday repeatability (<15% RSD) was obtained for leucine/isoleucine (6.3%), phenylalanine (1.9%), tyrosine (4.9%), threonine (0.50%), methionine (1.4%); poor intraday repeatability was obtained for valine (27%), proline (28%) and serine (18%). This reduced intraday repeatability is likely caused by the lower absolute response (i.e. before IS correction) of proline, valine and serine which were already low at the high end of the calibration curve: between 10^4 and low 10^5 AU at the 2000 nM level

Table 2
Analytical figures of merit for EE-CE-MS and CE-MS.

Compound	EE: Lin. Range (nM)	EE: Slope (AU/nM)	EE: LOD (nM)	EE: R^2	EE: Intra-day rep. (n = 3) (%)	EE: Inter-day rep. (n = 6) (%)	ER (%)	EF from organic	EF from aqueous
Leucine/ Isoleucine	5 – 2000	3700	5	0.9949	6.3	4.4	75.3	2259	67.8
Proline	100 – 2000	1900	20	0.9587	28	21	59.5	1785	53.5
Phenylalanine	10 – 2000	24,000	5	0.9986	1.9	4.9	82.4	2472	74.2
Tyrosine	20 – 2000	31,000	5	0.9972	4.9	11	79.8	2394	71.8
Valine	20 – 2000	5400	20	0.990	27	19	64.6	1937	58.1
Threonine	5 – 2000	93,200	5	0.9995	0.50	1.1	55.3	1659	49.8
Methionine	500 – 2000	50	100	N/A	1.4	n.d.	6.4	192	5.8
Serine	200 – 2000	70	100	0.9582	32	42	38.2	1146	34.4

Compound	CE: Lin. Range (nM)	CE: Slope (AU/nM)	CE: LOD (nM)	CE: R^2
Leucine/Isoleucine	$1000 – 1\times10^5$	53.1	1000	0.9997
Proline	$2000 – 1\times10^5$	35.9	2000	0.9995
Phenylalanine	$1000 – 1\times10^5$	320	1000	0.9999
Tyrosine	$1000 – 1\times10^5$	431	1000	0.9999
Valine	$5000 – 1\times10^5$	92.9	5000	0.9999
Threonine	$1000 – 1\times10^5$	694	1000	0.9999
Methionine	$5000 – 1\times10^5$	96.4	5000	0.999
Serine	$2\times10^4 – 1\times10^5$	2	20,000	N/A

while the other analytes had responses in the low 10^6 to low 10^7 peak areas at the same concentration. A relatively small change in peak area response affects the analytes with the lowest response the most. The interday variability is also the largest for these analytes as is shown by the RSDs for proline (21%), valine (19%) and serine (42%). For the other analytes, the interday repeatability shows good repeatability: leucine/isoleucine (4.4%), phenylalanine (4.9%), tyrosine (11%), and threonine (1.1%). The main reason for the analytes showing poor repeatability is the large variation in the droplet size at the end of the extraction leading to variations in the fraction of the droplet being retracted.

3.3. Urine analysis

As a proof of concept for bioanalysis the EE method was applied on urine that was obtained from a healthy male volunteer. In order to mimic a biomass-limited sample, only a fraction of urine (300 nL) was spiked to the water-saturated EtOAc donor phase along with $^{13}C^{15}N$-labelled internal standards, and 0.125% (v/v) FA.

Fig. 4 shows the electropherogram of the urine sample after EE-CE-MS. Here it can be seen that many potential molecular features are present in the sample. The data analysis identified 235 molecular features, after visual examination 122 features that represent putative metabolites remained.

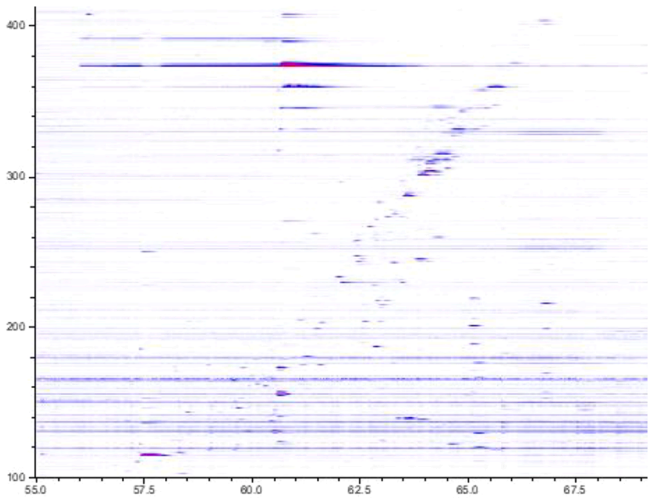

Fig. 4. 2D electropherogram of 300 nL urine analyzed by EE-CE-MS.

3.4. Comparison to other techniques

The developed EE-CE-MS technique can be categorized as a SDME technique. These involve the use of a pendant droplet to extract and preconcentrate analytes from a large donor phase into a small, typically sub-μL droplet.

Compared to 3PEE-CE-UV [32], which achieved LODs of 15 and 33 nM for serotonin and tyrosine respectively, there has been a large increase in ER. This is likely caused due to the difference in donor volume amount as in EE-CE-MS the donor volume consisted of 150 μL of EtOAc of which ~ 2.5% (v/v) (~3.75 μL) was water-based sample. While in both techniques the acceptor droplet was approximately the same the volume of the aqueous component of the donor phase volume differed greatly (4.5 μL vs. 375 μL). When taking into account the maximum recoveries: 82.4% of 4.5 μL was enriched in the droplet (~3.7 μL) with the current set-up and 1.1% of 375 μL (~4.1 μL) was enriched in the droplet with 3PEE-CE-UV – explaining why both techniques achieve good LODs, both in nM range, despite having dissimilar recoveries.

SDME has been coupled to CE-MS using an outlet vial, to prevent an air gap from forming at the outlet of the capillary during the formation of the extraction droplet [14,16]. Here, LODs of 2 – 5 nM were obtained for both acidic herbicides and pesticides as well as basic drugs. Using an in-line approach and coupling to CE-UV, Purgat et al. were able to detect homocysteine thiolactone in urine at a LOD of 25 nM [41]. In another work the use of in-line SDME yielded LODs in the low μM range (4.3 – 12 μM) for several non-steroidal anti-inflammatory drugs [42].

In our work, a make-up flow of 1 M FA was sent past the sheath liquid sprayer to prevent the air gap from forming during droplet formation. An unforeseen consequence of the use of 1 M FA at elevated temperatures inside the source was corrosion of the sprayer tip. In order to address this the stainless steel sprayer tip was replaced by a machined titanium sprayer tip.

Compared to the other discussed SDME techniques [14,16,41,42], our work makes use of a different driving force for extraction, namely an electric field. Once the variability in the droplet size and injected fraction is resolved we expect the developed technique to be orthogonal to SDME. As the electromigration-based EE-CE-MS technique and partition-based SDME techniques both have different selectivities. These differences in selectivities enable a single sample to be analyzed by both EE-CE-MS and SDME-LC-MS, which will likely yield enhanced information regarding metabolite profiles [43]. The donor phase and acceptor phase in this work both contained FA. By combining a steep pH gradient, as is typically found in SDME, and an electric field faster extraction should be attainable with EE.

4. Conclusion

In this paper we have demonstrated the integration of EE with CE-MS for metabolomics analyses. A minimal instrumental change, namely re-machining the sprayer tip out of titanium, was required to facilitate the fully automated EE process to take place. With this EE-CE-MS system LODs were enhanced 50 – 250-fold compared to conventional CE-MS. The next step to unlock the potential of this method would be to utilize neutral capillaries for the extraction of analytes. This would suppress EOF effects and thereby increase repeatability and reduce the effects of varying salt concentrations. The technique was successfully applied to a volume-restricted urine sample to extract amino acids along with more than a hundred other molecular features, thus signifying the method's utility in the metabolomics toolbox for the analysis volume-restricted samples.

CRediT authorship contribution statement

Amar Oedit: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Thomas Hankemeier:** Resources, Writing - review & editing, Supervision, Funding acquisition, Project administration. **Peter W. Lindenburg:** Conceptualization, Writing - review & editing, Supervision.

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.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under Grant Agreement No.306000 (STATegra) and Grant Agreement No.602783 (CAM-PaC). The authors would like to acknowledge Raphaël Zwier for machining the sprayer tip out of titanium.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105741.

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