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Integration of three-phase microelectroextraction sample preparation into capillary electrophoresis

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ABSTRACT

A major strength of capillary electrophoresis (CE) is its ability to inject small sample volumes. However, there is a great mismatch between injection volume (typically <100 nL) and sample volumes (typically 20–1500 µL). Electromigration-based sample preparation methods are based on similar principles as CE. The combination of these methods with capillary electrophoresis could tackle obstacles in the analysis of dilute samples.

This study demonstrates coupling of three-phase microelectroextraction (3PEE) to CE for sample preparation and preconcentration of large volume samples while requiring minimal adaptation of CE equipment. In this set-up, electroextraction takes place from an aqueous phase, through an organic filter phase, into an aqueous droplet that is hanging at the capillary inlet. The first visual proof-of-concept for this set-up showed successful extraction using the cationic dye crystal violet (CV). The potential of 3PEE for bioanalysis was demonstrated by successful extraction of the biogenic amines serotonin (5-HT), tyrosine (Tyr) and tryptophan (Trp). Under optimized conditions limits of detection (LOD) were 15 nM and 33 nM for 5-HT and Tyr respectively (with Trp as an internal standard). These LODs are comparable to other similar preconcentration methods that have been reported in conjunction with CE. Good linearity (R^2 > 0.9967) was observed for both model analytes. RSDs for peak areas in technical replicates, interday and intraday variability were all satisfactory, i.e., below 14%. 5-HT, Tyr and Trp spiked to human urine were successfully extracted and separated. These results underline the great potential of 3PEE as an integrated enrichment technique from biological samples and subsequent sensitive metabolomics analysis.

1. Introduction

Sample preparation is a crucial aspect of bioanalysis. The main objectives of sample preparation are to purify and enrich analytes prior to separation and detection. Commonly used sample preparation techniques are protein precipitation and partitioning based techniques, e.g., solid phase extraction (SPE) and liquid–liquid extraction [1,2]. In the past years, electromigration-based extraction methods have gained increased attention [3–5]. The main principle behind electromigration-based techniques is the use of an electric field to extract ions from a donor phase (optionally through intermediate phases) to an acceptor phase. The migration speed depends on the electrophoretic mobility of the analyte and the electric field. The electric field strength is typically low in the acceptor phase and thereby leads to stacking and preconcentration of analytes. Electromigration-based techniques offer several advantages over partitioning-based techniques, such as being able to handle small sample volumes, enhanced extraction speeds (due to the electric field being the driving force rather than partitioning between phases) and ease of automation [4–6].

The combination of electromigration-based sample pretreatment with CE offers two main benefits. First, both approaches are based on electromigration, so compounds that can be extracted are also suited for CE separation. Second, electromigration-based techniques can help overcome one of the drawbacks of CE: there is a great mismatch between the injected volume (typically <100 nL) and the sample volume (typically 20–1500 µL). In order to overcome this mismatch miniaturized inserts have been developed [7]. However, when samples are too dilute and compounds fall below detection limits this does not provide a solution. Electromigration-based sample preparation can help overcome the mismatch that is often present between injection volumes and sample volumes

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in CE analysis and can offer significant advantages in loadability compared to in-line stacking methods. On-line SPE-CE, where SPE is coupled to the capillary, could also serve as a solution for enhancing the loadability, but has thus far only been demonstrated for more apolar compounds on apolar cartridges [8]. Moreover, to the best of our knowledge, no commercial solutions for SPE-CE are available and setting them up is complex.

In electromembrane extraction (EME) a supported liquid membrane (SLM) is used to separate the donor from the acceptor phase. The analytes are extracted through the SLM into the acceptor phase using an electrical potential. Several set-ups in which EME was coupled off-line to CE-UV and applied to bioanalysis have been reported [6,9–15].

Off-line, at-line, as well as in-line coupling of EME to CE-UV has been reported on several occasions. An off-line process consists of two steps (e.g. sample preparation followed by separation). An at-line process combines the two steps via an automated handler (e.g. a robot). An in-line process takes place within the separation system (e.g. SPE-CE with sorbent inside the capillary). An on-line process takes place right before the sample stream enters the separation system, but does take place within the analytical instrument (e.g. SPE-CE with sorbent outside the capillary) [16]. An example of on-line coupling of EME to CE-UV, is nano-EME, for preconcentration and analysis of drugs. Here, basic drugs were extracted from a sample volume of 200 µL, through a membrane over a cracked capillary, into an acceptor of ~8 nL. A single SLM could last for more than 200 extractions. Enrichment factors (EFs) ranging between 25 and 196 were reported for basic drug compounds, corresponding to recoveries of 0.1% and 0.79%. Under different conditions, higher EFs were obtained, up to 500 for loperamide [17]. Moreover, EME-CE for preconcentration and analysis of basic drugs was reported. Here, an SLM was formed between two conical polypropylene units and extraction took place from the device, which was used as a vial insert. Extraction took place from a 40 µL donor compartment over the SLM into an acceptor compartment of 40 µL and therefore it only served for sample cleanup and not for sample preconcentration. Recoveries ranging from 37% to 84% were obtained [18]. However, the device requires reassembly for each new experiment, which hampers automated analysis. Chui et al. integrated the free liquid membrane (FLM) in an electrokinetic supercharging (EKS) method in-line to further improve detection limits in CE. In this approach, a small plug of immiscible organic solvent in the capillary was used as filter during the electrokinetic sample injection to enhance stacking efficiency. Analysis of cationic herbicides in environmental water samples were used to evaluate the on-line preconcentration efficiency and results showed detection limit enhancements of over 1500 times. EKS over an FLM using CE for analysis on real-world samples has only been demonstrated on river water samples, a relatively clean matrix. Since the donor phase is drawn partially into the capillary, protein-rich samples could cause reproducibility problems due to absorption to the fused silica capillaries [19].

EME can be used without SLM. This offers several advantages, such as not requiring reassembly and regeneration of the SLM. In this case the technique is referred to as EME over an FLM [20] or 3PEE [21]. Off-line coupling of a EME-FLM extraction to CE-UV was successfully demonstrated for analysis of charged basic drugs (nortriptyline, haloperidol and loperamide) in human urine and blood [22].

Two-phase electroextraction (EE) is a process where charged analytes are transferred from an organic donor phase into an aqueous acceptor phase using an electric field [23–25]. EE coupled to CE was first reported in conjunction with isotachophoresis by Van der Vlis et al. in 1994 [26].

Previously, we have reported 3PEE as a powerful preconcentration technique. Herein, we propose integration of this system into a CE set-up for on-line sample clean-up and preconcentration. Raterink et al. demonstrated the extraction of acylcarnitines from a 50 µL donor phase, through a filter phase into a 2 µL acceptor phase that was formed by a conductive pipet tip prior to direct analysis with high-resolution mass spectrometry. In 3PEE, altering the organic filter phase composition influences the selectivity and enables selective extraction of desired analytes [21].

In this article, we demonstrate a proof-of-principle of a novel on-line analytical system in which electromigration-based sample preparation technique, i.e., 3PEE, is directly hyphenated to CE-UV. Our method offers several advantages: automation, sample preconcentration and the ability to extract analytes from salt-rich matrices without sample preparation. In order to enable 3PEE the electrode configuration of a commercially available CE apparatus was modified. This modification entails placing an insulating sleeve over the electrode whilst leaving the bottom part of the electrode exposed. 3PEE-CE-UV permits automated on-line selective analyte extraction and enrichment, directly from a dilute sample. In this set-up the analytes are extracted from a sample vial containing a two-phase liquid-liquid system, i.e., the aqueous sample and an organic filter phase. Extraction takes place into a droplet of acceptor phase hanging at the capillary inlet in the organic filter phase.

In a first series of experiments the process was visualized using the cationic dye CV to assess the stability of the droplet of acceptor phase during 3PEE. Then, the proposed method was evaluated using selected biogenic amine model compounds to evaluate its potential for bioanalysis of polar metabolites. In order to enhance the CE separation, pH-mediated stacking was included in the system. Consecutively, important extraction parameters (EE voltage and EE time) and selectivity of the developed 3PEE-CE-UV method were investigated to optimize extraction. Then, the analytical performance of 3PEE-CE-UV was compared to conventional CE-UV. Finally, the performance of 3PEE-CE-UV as a sample preparation procedure for polar compounds in salt-rich biological samples was investigated by analysis of spiked human urine samples in order to show its applicability for metabolomics analyses.

2. Materials and methods

2.1. Chemicals and reagents

Sodium chloride (NaCl), CV, ammonium hydroxide (>25%), 5-HT, Tyr and Trp were obtained from Sigma-Aldrich (Steinheim, Germany). Ethyl acetate (EtOAc) was obtained from Actu-All (Oss, The Netherlands). Formic acid (FA) was obtained from Acros Organics (Geel, Belgium). Sodium hydroxide was obtained from VWR (Amsterdam, The Netherlands). All solutions were of HPLC grade or higher. Water was prepared using a Milli-Q® Advantage A10® system (Billerica, MA, USA).

2.2. Samples and stock solutions

Aqueous stock solutions of analytes (2 mM) were stored at −20 °C until use. Sample solutions were prepared at the desired concentration by dissolving the aqueous stock solutions in 1 M FA (pH 1.8) and were, at maximum, kept at 4 °C for 1 week. Human urine was provided by a healthy volunteer and stored at −20 °C. Prior to analysis, urine was thawed and centrifuged for 15 min at 10,000 rpm, the urine was spiked with the model analytes in the desired concentration and FA was added until 1 M was reached in urine.

2.3. Equipment and techniques

2.3.1. Capillary electrophoresis

Analyses were performed using a Beckman Coulter P/ACE MDQ (Fullerton, CA, USA) CE apparatus using UV diode array detection. A fused silica capillary of 50 µm I.D. and 365 µm O.D. with a total length of 60 cm was used (Polymicro Technologies, USA). New capillaries were sequentially rinsed at 1379 mbar with MeOH for 10 min, 1 M NaOH for 10 min, water for 5 min and background electrolyte (BGE) for 20 min. Between runs, the capillaries were flushed for 5 min with BGE.

Separation was performed using 1 M FA (pH 1.8) as the BGE buffer using a separation voltage of +17.5 kV. The capillary cartridge temperature was set at 20 °C. Detection was set at 195 nm to maximize the number and response of metabolites that can be detected with a reference at 400 nm.

2.3.2. Software

32 Karat (Beckman Instruments, Fullerton, CA, USA) was used for controlling the CE-UV system and for data acquisition. Injection volumes as well as the volumes of the acceptor droplet formed by reversed pressure were calculated using Sciex CE Expert V2.2 (Framingham, MA, USA).

3. Results and discussion

3.1. Modification of the CE instrument for three-phase electroextraction

In order to enable 3PEE using a Beckman Coulter CE apparatus the electrode configuration was modified by replacing the existing electrode with a longer platinum electrode of 4 cm (Fig. 1B). From the bottom 2.8 cm of the electrode was isolated using a polytetrafluoroethene (PTFE) sleeve, leaving only a tip of 2 mm of the electrode exposed. This modification enables an electric field from the donor phase through the FLM into the acceptor droplet. The septa of the inlet vials were removed to ensure that the modified electrode, which had a slightly increased thickness due to the PTFE sleeve, could still reliably enter the vial.

In order to visually monitor the extraction process a USB-pen video camera was mounted inside the CE machine and focused on the capillary inlet. Debut Video Capture (NCH Software, Greenwood Village, CO, USA) was used to record the extraction videos.

3.2. Three-phase electroextraction procedure

Prior to placing the sample vials in the CE system, 375 µL donor solution was pipetted in conventional CE vials (1.5 mL). Based on previous EE works the donor solution was acidified to 1 M FA, which has proven to be a good donor solvent [23–25]. This was followed by 725 µL organic filter phase consisting of water-saturated EtOAc, which is crucial for the electric field and thereby the transfer of ions through the organic filter layer [21]. Fig. 1 graphically depicts a typical 3PEE experiment in the CE instrument. First, the capillary was rinsed with FA. Then ammonium hydroxide solution was injected, followed by an injection of BGE. After inserting the capillary in the sample vial, a hanging droplet of 100 nL is created in the organic filter phase by applying a pressure of −69 mbar for 1 min from the BGE outlet vial. Then, electroextraction was performed by applying the extraction voltage, after which the enriched droplet was retracted into the capillary. At last, the capillary inlet was inserted into a BGE vial and separation was carried out. In order to visualize the electroextraction procedure, the cationic dye CV was added to the donor phase.

3.3. Visualization of on-line three-phase electroextraction

The setup for 3PEE hyphenated to CE-UV was based on the previously reported 3PEE-DI-MS [21]. In a visual proof-of-concept 10 µM CV was electroextracted at 3.5 kV from 375 µL donor phase

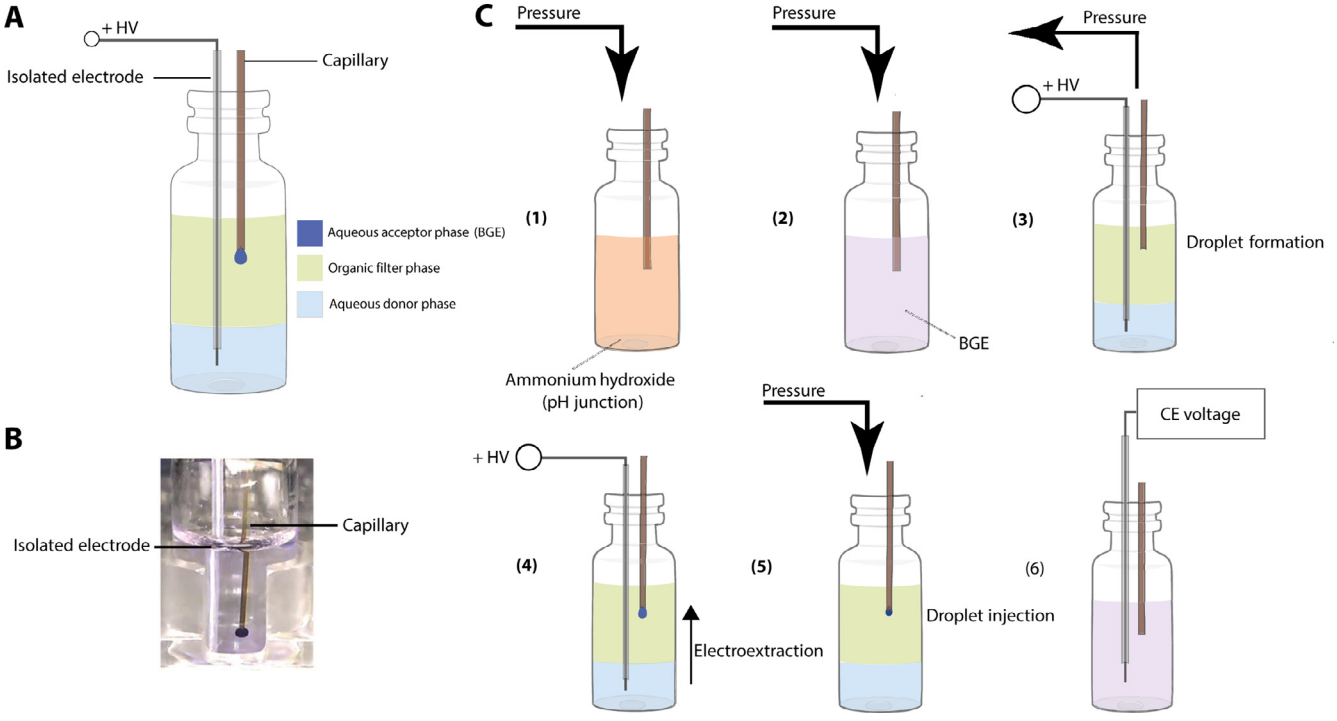

Fig. 1. (a) Schematic representation of the 3PEE setup, and (b) actual set-up incorporating the modified electrode configuration used during experiments (bottom of vial not visible). (c) Schematic representation of the key steps in the extraction procedure in the CE-UV system: (1) injection of ammonium hydroxide, (2) injection of BGE, (3) application of negative pressure, (4) application of voltage, (5) retraction of droplet using pressure, (6) vial switch to BGE and start of CE separation.

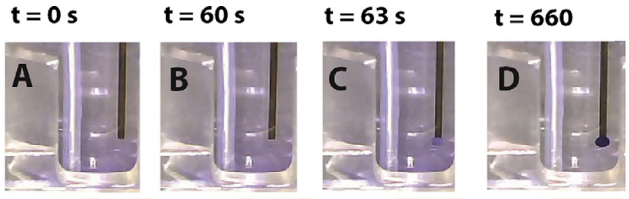

Fig. 2. Visualization of 3PEE coupled CE using CV. Video stills of: (A) initial conditions with sample vial containing 375 µL crystal violet (10 µM) and 725 µL water-saturated ethyl acetate; (B) end of droplet formation (~100 nL, 1 M FA), outline of droplet barely visible; (C) start 3PEE by application of 3 kV; (D) end of 3PEE after 10 min.

into ~100 nL acceptor phase (Fig. 2A–C). The liquid-liquid interface is not visible in the sample tray (see Supporting Information S1 for vial outside of sample tray). Prior to application of the extraction voltage, no CV can be observed in the droplet. This indicates that the contribution of partitioning to the process is minimal. After 10 min electroextraction, the droplet was enriched dramatically with CV (Fig. 2D).

These results indicate that the developed set-up is successfully extracting CV from the donor phase into the pendant acceptor phase.

3.4. On-line three-phase electroextraction coupled to capillary electrophoresis

3.4.1. Effect of pH-mediated stacking

The 3PEE-CE-UV was investigated using the biogenic amines Tyr, Trp and 5-HT. The BGE consisted of 1 M FA (pH 1.8) to ensure compounds were cationic during analysis. In a first experiment, 500 nM model analytes were extracted for 6 min at 3 kV and the droplet was partially retracted at 34 mbar for 5 s, followed by CE separation at 17.5 kV for 30 min. In Fig. 3A it can be observed that Trp and Tyr have poorly resolved peaks, with 5-HT overlapping. The peak areas are higher despite reduced injection time compared to Fig. 3B. This is caused by migration of analytes from the donor phase through the droplet into the capillary (see Supporting Information S2). This can possibly be explained by peak broadening during 3PEE caused by electrophoresis and EOF, which is still present to some extent, even at a low pH. In order to focus the broad sample zone and thereby improve separation, a pH-mediated stacking was included. By adding a plug of basic BGE after to the acceptor phase, the acidic BGE titrates the sample solution to create a neutral zone. In this zone a higher field is present causing increased migration speed of analytes and eventually stacking at the interface between the neutral zone and BGE. This pH-mediated stacking was created by injecting 15% ammonium hydroxide solution for 17 s at 34 mbar and followed by 1 M FA for 1.1 min at 69 mbar to ensure that the droplet consisted fully of BGE. The biogenic amines were extracted at a concentration of 500 nM for 8 min at 3 kV. The droplet could now be retracted much longer (1.5 min at 34 mbar) while the separation resolution improved as shown in Fig. 3B.

3.4.2. Optimization of extraction voltage and extraction time

The method was optimized in order to obtain the highest possible area under the curve (AUC) for the analytes. In the first series of experiments ($n = 3$) the extraction time was kept constant at 5 min and the extraction voltage was varied (1, 1.5, 2, 2.5, 3, 4 and 5 kV). In these experiments 250 nM Trp, Tyr and 5-HT were used. When 0 kV was used no analytes were detected (Fig. 4A). This indicates that analyte migration from the donor phase to the acceptor phase is solely driven by electric potential. Moreover, increasing the voltage up to 3 kV significantly enhanced signals for Tyr, Trp and 5-HT compared to lower voltages (Fig. 4B and C). Voltages beyond 3 kV resulted in loss of current and droplet instability (data not shown).

Subsequently, the extraction time was optimized while keeping the extraction voltage constant at the optimal value of 3 kV. Extractions were performed for 2, 4, 6, 8 and 10 min. It was shown that increasing the extraction time increased enrichment and thereby peak areas of the analytes. Beyond 8 min of extraction caused frequent current losses during CE.

In summary, the optimized 3PEE procedure was as follows. First, the capillary was flushed with 1 M FA for 5 min at 1378 mbar, followed by a 17 s 34 mbar injection of 15% ammonium hydroxide and subsequent 1.1 min 69 mbar injection of 1 M FA. Then, a droplet was formed using 1 min 69 mbar, after which electroextraction was carried out at 3 kV for 8 min. Finally, the enriched droplet was retracted using 1.5 min 34 mbar and CE-UV separation was performed for 35 min at 17.5 kV.

3.4.3. Analytical figures of merit

Table 1 shows the analytical performance of the optimized method for the biogenic amines 5-HT and Tyr in comparison with conventional CE-UV. Trp was used as internal standard.

The aforementioned conditions were used to evaluate the extraction of 5-HT and Tyr using different concentrations (0, 0.05, 0.1, 0.5, 1, 5 µM; $n = 3$) resulting in a linear range of 0.01–5 µM, yielding regression coefficients (R^2) of 0.9967 and 0.9995, respectively (Table 1). LODs were estimated using signal-to-noise (S/N) ratios of triplicate measurements at 50 nM and extrapolated to $S/N = 3$. Detection limits of 15 nM and 33 nM were observed for 5-HT and Tyr, respectively.

For comparison, calibration curves were constructed with identical electrophoresis conditions using hydrodynamic injection without pH-mediated stacking injecting 80 nL (similar to the retracted volume using the optimized 3PEE-CE-UV method) using different concentrations (0, 1, 5, 10, 25, 50 µM; $n = 3$) of 5-HT and Tyr with 25 µM Trp as internal standard. Since CE-UV could not reach the nM range of 3PEE-CE-UV the examined range was adjusted to a micromolar range to be able to construct a calibration curve (Table 1). Regression analysis yielded high R^2 values (exceeding >0.999) for 5-HT and Tyr with conventional CE-UV and observed LODs for 5-HT and Tyr were 5 µM and 1 µM, respectively. The LODs for the 3PEE-CE-UV method were improved ~333 × and ~30 × for 5-HT and Tyr, respectively. Moreover, compared to conventional CE-UV, linear range of 3PEE-CE-UV was extended an order of magnitude downwards to the 50–100 nM range.

3.4.4. Repeatability and technical replicates

Intra- and inter-day variability of the method were determined using optimized conditions at 500 nM. As shown in Table 2, intra-day variability analysis showed good repeatability, as RSDs for AUC values ranged from 4.7% for Tyr up to 6.9% for 5-HT. For inter-day variability, RSD values ranged between 7.9% for Tyr and 13.8% for 5-HT, indicating good repeatability of the developed method. The increased RSD values obtained compared to conventional CE can be partially explained by the added steps, including droplet formation and extraction vs. hydrodynamic injection.

Technical replicates of a single sample vial showed similar variability. It was shown that five consecutive extractions could be performed successfully and resulted in RSD values of 6.6% for 5-HT and 10% for Tyr, comparable to the 3PEE-CE-UV inter- and intra-day variability (Table 2). Migration time repeatability of the new method was comparable to hydrodynamic CE (Supporting Information S4).

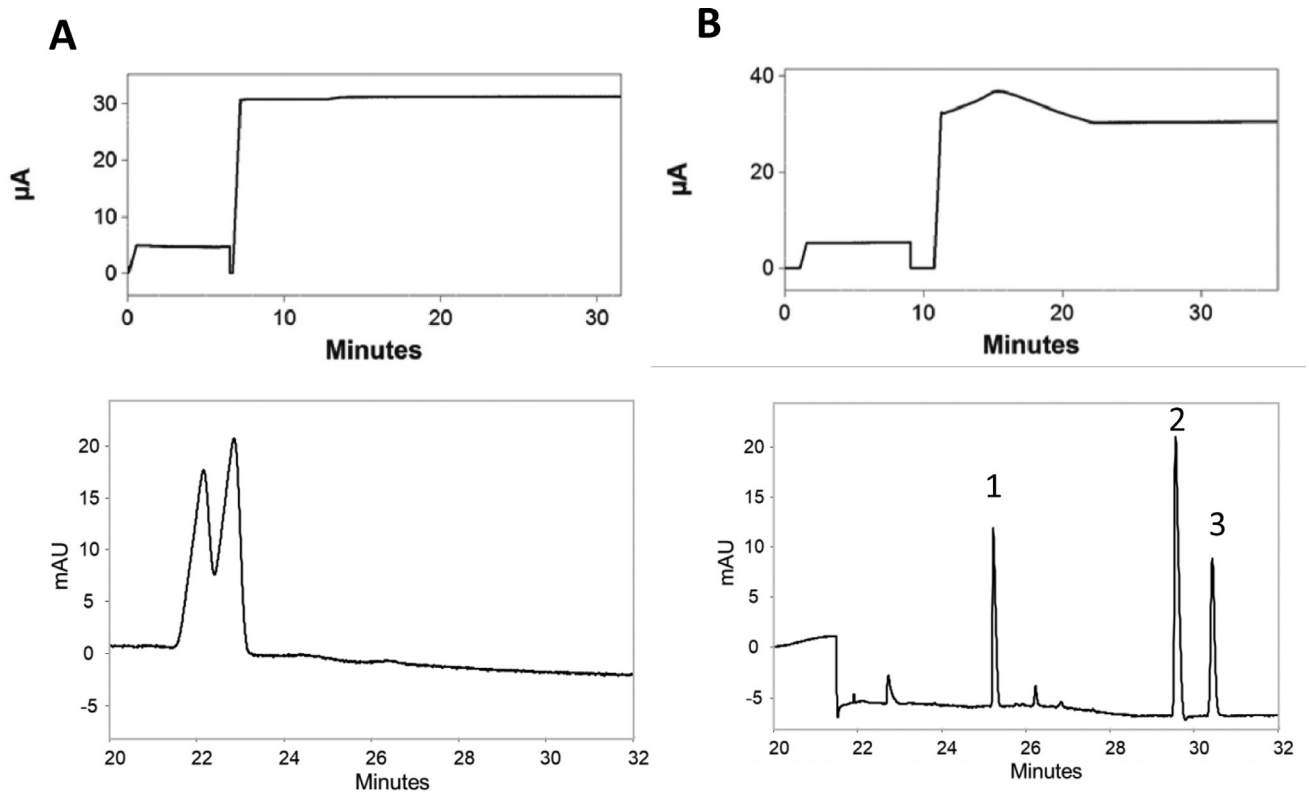

Fig. 3. Stacking effects in 3PEE coupled to CE-UV. Top figures show the current profile of extraction of 500 nM 5-HT (1), Trp (2) and Tyr (3) from 375 µl donor phase using 3PEE-CE-UV as well as the corresponding electropherograms. (A) retracting for 0.5 min at 6.9 mbar and (B) retracting for 1.5 min at 34 mbar with pH-mediated stacking.

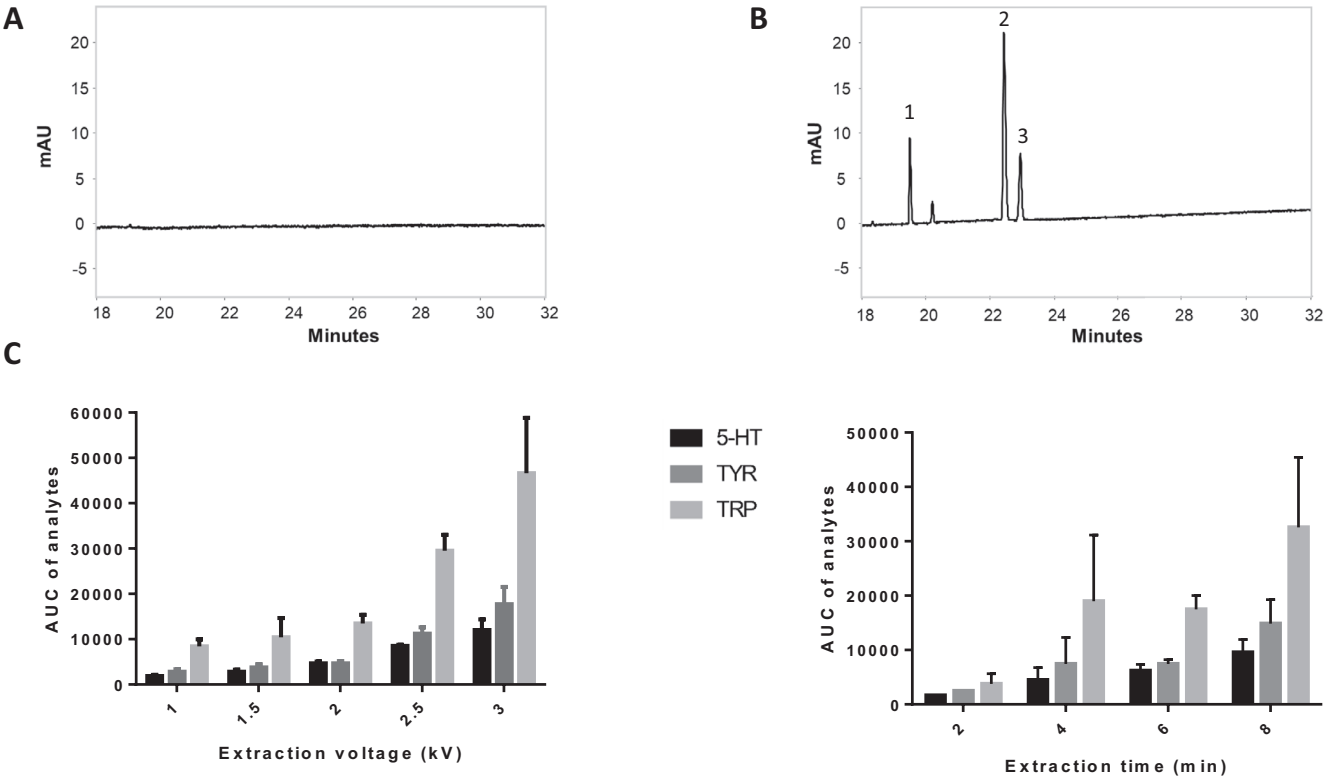

Fig. 4. 3PEE voltage and time optimization. Extractions were performed using 250 nM 5-HT (1), Trp (2) and Tyr (3) in 1 M FA. Electropherograms are shown for: (A) 0 kV 3PEE voltage and (B) 3 kV 3PEE voltage. (C) Results of voltage and time optimization for 3PEE coupled online to CE-UV. Error bars represent standard deviation of $n = 3$.

Table 1
Comparison the analytical performance of 3PEE-CE-UV and conventional CE-UV in neat solutions. AUCs were corrected using Trp as internal standard.

Analyte	Linear range (µM)		Sensitivity ± SD ($\times 10^{-2}$ AU/µM)		Intercept ± 95% CI[a] ($\times 10^{-2}$ AU/µM)		Linearity (R^2)		LOD[b] (µM)	
	CE	3PEE	CE	3PEE	CE	3PEE	CE	3PEE	CE	3PEE
5-HT	5–50	0.05–5	2.54 ± 0.01	102.2 ± 1.3	−0.40 (−2.5–2.5)	−2.10 (−11.6–7.4)	>0.9999	0.9995	5	0.015
Tyr	1–50	0.1–5	9.65 ± 0.06	76.8 ± 2.6	1.67 (−3.5–6.9)	5.98 (−12.8–24.8)	0.9999	0.9967	1	0.033

Note: for repeatability see Table 2.
[a] No significant intercept values were observed ($p < 0.05$).
[b] Extrapolation towards S/N of 3 from lowest measured concentration.

Table 2
Intra- and interday repeatability and technical replicates of 3PEE-CE-UV analysis of target compounds. AUCs were corrected using Trp as internal standard.

Analyte	3PEE-CE-UV intraday ($n = 3$)		CE-UV intraday ($n = 3$)		3PEE-CE-UV interday ($n = 6$)		3PEE-CE-UV technical replicates[a] ($n = 5$)	
	Mean AUC ratio	RSD area ratio (%)	Mean AUC ratio	RSD area ratio (%)	Mean AUC ratio	RSD area ratio (%)	Mean AUC ratio	RSD area ratio (%)
5-HT	0.34	6.9	0.12	1.85	0.35	13.8	0.41	6.6
Tyr	0.44	4.7	0.50	0.87	0.43	7.9	0.43	10.0

[a] Obtained from 5 consecutive extractions from a single sample vial.

3.4.5. Enrichment and recovery

In order to assess the performance of 3PEE-CE-UV correctly, the extraction recovery (ER) and EF were calculated [6].

These results show that even though the EF_{max} is much greater, enrichment was limited. Tyr in the acceptor phase was around 8 times more concentrated than the donor phase after extraction (Supporting information S3). It was observed that the enrichment factor of 5-HT was 41.4 and therefore five times higher than for Tyr. A possible explanation for this is the lack of the carboxylic acid moiety ($pK_a = 2.38$) in 5-HT, thereby enabling more efficient transfer from the FA containing donor phase. Likewise, a lower recovery was observed for Tyr (0.2%) than for 5-HT (1.1%) after extraction which correlates with the EF values. The improvements in LOD in Table 1 differ from the obtained EF values as the developed method included both stacking through on-line electroextraction (increasing loading) and in-line stacking through a dynamic pH-mediated stacking (improving peak shapes). Both techniques are essential to the final method and therefore the final method was compared to a simple hydrodynamic injection method (thus without dynamic pH-mediated stacking). These results show that the extraction process is not exhaustive and is a soft extraction method, which offers several advantages such as opening up the possibility of studying (bio)chemical reactions and concentration-time monitoring without disturbing the overall system. EF and ER can be further improved by reducing the volume (and thereby height) of the organic phase to enhance the electric field distribution to be more favorable towards analyte extraction. Moreover, the composition of the organic phase can be modified to enhance EF and ER as well [21].

3.4.6. Comparison to other set-ups

A comparison of 3PEE-CE-UV method to other sample extraction techniques that were hyphenated directly to CE and reported in literature is shown in Table 3 and Supporting Information S5.

Single drop micro-extraction (SDME) techniques have been coupled to CE [27,28] with EFs ranging between 130–150 and EME has been coupled on-line to capillaries [17] with reported EFs ranging between 25–196, with loperamide reaching an EF of up to 500 under optimal conditions. On-line back extraction field amplified sample injection relies on both partitioning between an organic chloroform donor phase and an aqueous acceptor phase and simultaneous depletion of the acceptor phase via electrokinetic injection into the capillary [29]. 3PEE-CE-UV bears similarities to the electrokinetic supercharging over FLM set-up, but differs in two ways: (1) preconcentration takes place at the capillary inlet into a pendant droplet rather than inside the capillary, and (2) the electrokinetic supercharging over FLM set-up incorporates a t-ITP step to further enhance stacking of the analytes rather than pH-mediated stacking. Most preconcentration set-ups in Table 3 are coupled to CE are reported for analysis of apolar basic drug compounds, with the exception electrokinetic supercharging over an FLM [19], which was used polar herbicides. Unlike electrokinetic supercharging over an FLM, 3PEE-CE-UV does not require removal of FLM from the capillary, which can be a convoluted procedure and requires re-optimization for each new organic FLM phase.

3PEE-CE-UV has a relatively long total analysis time compared to the discussed set-ups. However, as this is a proof-of-principle, separation parameters such as separation voltage and capillary length could still be optimized to yield shorter analysis times. The obtained EFs of 3PEE-CE-UV were 1–2 orders of magnitude lower than other reported methods. However, due to the combination with dynamic pH-mediated stacking similar LODs were obtained. A probable explanation for this is the fact that we studied the potential of our method for polar metabolites, while in other work apolar drugs, which are more easily extracted are studied. Transfer of polar molecules, such as the biogenic amines in this work, has always been a challenging endeavor in EME and tuning of composition and size of the organic phase remains at the forefront of interest [10]. The developed 3PEE-CE-UV has LODs in the low nM range, which is similar to other techniques in Table 2, despite having lower EFs likely due to its higher injection volume combined with in-capillary stacking. The low recoveries of 3PEE-CE-UV make it suitable as a soft extraction technique. Moreover, on-line methods such as described in Table 3 are more suited for analysis of large dilute samples as these can handle larger sample sizes compared to high nL range samples in in-line methods. Finally, as 3PEE-CE-UV is not exhaustive it can be used to measure technical replicates (i.e. repeat analyses of the same sample vial).

3.4.7. Proof of concept: urine bioanalysis

The potential of on-line 3PEE as sample cleanup procedure for metabolomics analyses was investigated by analyzing human urine with 5-HT, Trp and Tyr spiked to it. Analyte stocks were dissolved in urine and acidified to 1 M FA (pH 1.8) prior to electroextraction. Field amplified stacking is reduced when analytes are dissolved in a highly conductive matrix and effective dynamic pH-mediated stacking requires a shorter plug of ammonium hydroxide for optimal stacking [30]. As urine is a highly conductive matrix the length of the ammonium hydroxide plug was reduced (8 s at 34 mbar). In order to further increase the stacking efficiency, the droplet retraction time was reduced (68 s at 34 mbar). This shorter retraction resulted in an improved stability and comparable extrac-

Table 3
Comparison of the newly developed 3PEE-CE-UV method to other methods that were hyphenated to CE.

Set-up	Ref.	Compounds	EF (range)	ER (range)	LOD (range)	Total analysis time (min)
3PEE-CE-UV	This paper	Serotonin, *Tyrosine*, (Phenylalanine)	*7.8*–42	*0.2*–1.1%	15–*33* nM	52
Inline SDME-CE-MS	[27]	*Methoxyphenamine*, Methamphetamine, Amphetamine, Phenetylamine	*130*–150	Not reported	2–*5* nM	62
Nano-EME coupled to CE-UV	[17]	Pethidine, Nortiptyline, Methadone, Haloperidol, *Loperamide*	25–*196*	0.1%–*0.79%*	8–*31* nM (0.2–*15* ng mL^{-1})	>29[d]
FLM – electrokinetic supercharging coupled to CE-UV	[19]	Paraquat, Diquat	Not reported	(Relative recovery[a]: 97.0–97.5%[b])	58 – 58 nM (0.15–0.20 ng mL^{-1})	>20[d]
On-line back extraction field amplified sample injection (coupled to CE-UV)	[28]	Cocaine, Thebaine, (Metamphetamine)	Not reported	(Relative recovery[a]: 94.71–98.65%[c]):	16 – 16 nM (0.005–0.005 µg mL^{-1})	18

Underlined compounds and values are those with the lowest LOD, compounds in *italics* are those with the highest LOD.
[a] Relative recoveries were reported by measuring a spiked sample and comparing to the calibration curve.
[b] Spiked at 20 ng mL^{-1}.
[c] Spiked at 0.5 µg mL^{-1}.
[d] Duration of initial flush prior to each analysis not specified.

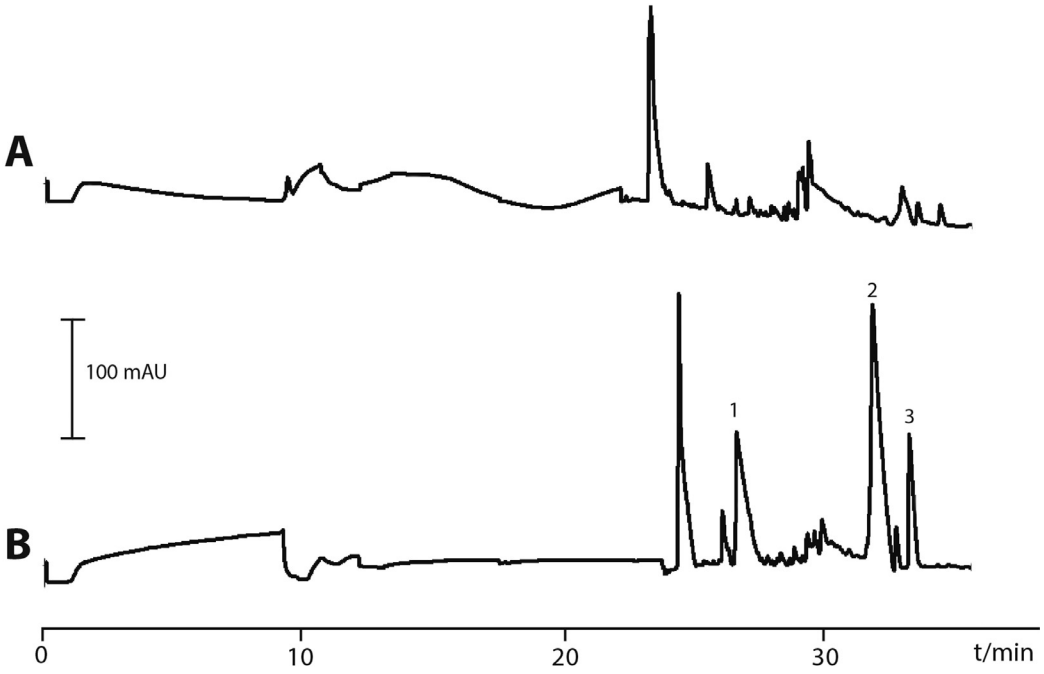

Fig. 5. Proof of concept showing urine bioanalysis using 3PEE-CE-UV. Electropherograms obtained from (A) non-spiked urine and (B) spiked urine samples extracted by 3PEE prior to CE-UV detection. Urine was spiked with 5-HT (1), Tyr (3) and Trp (2; 50 µM). Extraction and analytical conditions can be found in Section 3.6.

tion and separation current profiles to extractions performed in neat solutions (data not shown). Before analysis of the target analytes, 3PEE-CE-UV was first applied to a non-spiked urine sample. In the corresponding electropherogram at the non-specific wavelength 195 nm, many unidentified endogenous compounds were observed after the extraction procedure showing its ability to analyze a urine sample without requiring prior dilution (Fig. 5A). Then, 3PEE-CE-UV was employed for the analysis of 5-HT, Trp and Tyr (50 µM), spiked to urine of the same origin. The electropherogram in Fig. 5B, shows detection of 5-HT, Trp and Tyr. In order to confirm the identities of the endogenous compounds in urine a more selective detector is required. These preliminary results show the potential of 3PEE-CE-UV as an easy on-line sample preparation method that could be applied for the analysis of small polar metabolites, e.g., in a metabolomics setting.

4. Conclusion

In this work we have successfully developed a new approach for simultaneous sample preconcentration and cleanup. This was achieved through integration of 3PEE with CE-UV, which required a simple modification in the electrode configuration of a commer-

cially available CE instrument. By placing an immiscible organic filter phase on top of an aqueous sample, cationic analytes were extracted into an aqueous acceptor droplet formed at the capillary inlet, when an electrical field was applied. In order to enable full droplet retraction without loss of resolution after extraction, pH-mediated stacking was introduced to efficiently stack analytes. The performance of on-line 3PEE-CE-UV was evaluated by extracting 5-HT, Trp and Tyr from a 375 µL neat solution into a pendant droplet of 100 nL BGE. Low extraction recoveries were obtained, demonstrating that the technique is a soft extraction technique. To the best of our knowledge, EME over an FLM of polar metabolites was never reported. It was demonstrated that detection limits improved to 15 nM 5-HT and 33 nM Tyr with 3PEE, compared to 5 µM 5-HT and 1 µM Tyr in CE with hydrodynamic sample injection. As proof-of-concept, the on-line 3PEE-CE-UV procedure was evaluated for the analysis of human urine. It was demonstrated that 5-HT, Trp and Tyr were successfully extracted from spiked urine, thus signifying the potential of the developed procedure for urine bioanalysis and metabolomics.

Declaration of Competing Interest

None.

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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.2019.460570.

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