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A high-throughput, ultrafast, and online three-phase electro-extraction method for analysis of trace level pharmaceuticals

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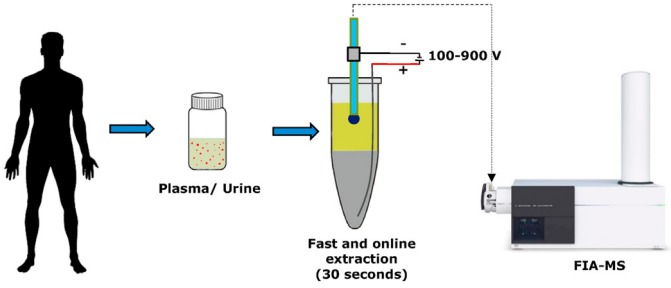
HIGHLIGHTS

- Ultrafast analyte extraction, down to 30 s.
- High enrichment factors of up to 569-fold.
- Direct hyphenation to MS.
- Simple extraction setup.
- Limit of detection as low as 360 pg/mL.

GRAPHICAL ABSTRACT



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ABSTRACT

Sample preparation is often reported as the main bottleneck of analytical processes. To meet the requirements of both high-throughput and high sensitivity, improved sample-preparation methods capable of fast analyte preconcentration are urgently needed. To this end, a new three-phase electro-extraction (EE) method is presented that allows for ultrafast electroextraction hyphenated to flow-injection analysis mass spectrometry (FIA-MS). Four model compounds, *i.e.*, propranolol, amitriptyline, bupivacaine, and oxeladin, were used to optimize and evaluate the method. Within only 30 s extraction time, enrichment factors (EF) of 105–569 and extraction recoveries (ER) of 10.2%–55.7% were achieved for these analytes, with limits of detection (LODs) ranging from 0.36 to 3.21 ng mL^{-1}, good linear response function ($R^2 > 0.99$), low relative standard deviation (0.6%–17.8%) and acceptable accuracy (73–112%). Finally, the optimized three-phase EE method was successfully applied to human urine and plasma samples. Our three-phase electroextraction method is simple to construct and offers ultrafast, online extraction of trace amounts of analytes from biological samples, and therefore has great potential for high-throughput analysis.

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1. Introduction

In the past few decades, the field of analytical chemistry has seen unprecedented development in detection and separation techniques, including ultrahigh-resolution mass spectrometry,

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matrix-assisted laser desorption/ionization, multi-dimensional liquid chromatography, ultra-performance liquid chromatography, and capillary electrophoresis–mass spectrometry. Sample preparation plays a crucial role in the removal of interferents, and the enrichment of analytes concentration in the bio-analytical workflow. However, contemporary sample-preparation methods often still form a bottleneck for the entire analytical workflow [1–6]. This is especially the case for high-throughput and low concentration level analysis [7–10] as they are time- and labor-intensive [1,11–13] and offer relatively low enrichment [14]. An ideal sample-preparation method should provide speed, simplicity, efficiency, high enrichment factors, and should be environmentally friendly [1,7,15,16].

Liquid-liquid extraction (LLE) is one of the most commonly-used sample preparation techniques [17], and solid-phase extraction (SPE) has been increasingly used in recent decades [4,18]. However, both techniques are time consuming, and use large volumes of toxic solvent [7]. In the last two decades, electro-driven extraction has gained attention for its simplicity, fast extraction, high analyte enrichment, and low sample consumption [19–22]. Electro-driven extraction is based on the active migration of charged analytes in an applied electric field. Since electro-migration of analytes is a fast one-step process, electro-driven extraction offers faster extraction and enrichment compared to LLE and SPE. The two main variants of electro-driven extraction are electroextraction (EE) and electromembrane extraction (EME), with the main difference being the addition of a membrane in the latter. EME was first reported in 2006 by Pedersen-Bjergaard et al. [23] and uses a membrane with organic solvent held in its pores, between the aqueous sample and an acceptor solution. EE was first developed for analytical purposes in 1994 by Van der Vlis et al. [24], but was not used for bioanalysis until 2010 by Lindenburg et al. [25]. Due to the omission of a membrane, EE is more straightforward in operation and necessary equipment [26]. Depending on the number of phases, EE can be categorized as two-phase or three-phase EE. In two-phase EE, the phases consist of an organic phase and an aqueous acceptor phase, and analytes have to be dissolved in the organic phase before extraction [25]. In three-phase EE, the donor phase consists of the aqueous sample and is separated from the aqueous acceptor phase by an organic phase [27]. Typically, the time needed for the three-phase electro-driven extraction process ranges from 2.5 min to 33.3 min [19,27–36]. To make electro-driven extraction more suitable for high-throughput analytical platforms, the extraction time should be further reduced.

In this study, a new online three-phase EE setup coupled to mass spectrometry was developed by using a switching valve, a syringe pump, an LC pump, a power supply with an electrode. A digital video-camera was utilized to record the EE process. The solvent type and composition of the organic phase, the composition of the acceptor phase and the aqueous sample, and the extraction voltage and time were optimized for four commonly used model compounds, *i.e.*, propranolol, amitriptyline, bupivacaine, and oxeladin. Finally, the three-phase EE setup was successfully applied to human urine and plasma samples. This study provides an ultrafast, simple online sample preparation setup with high enrichment factors, which has great potential for high-throughput sample analysis.

2. Experimental section

2.1. Chemicals

Propranolol, amitriptyline, bupivacaine, and oxeladin were all purchased from Sigma-Aldrich (Steinheim, Germany). Deionized (DI) water was obtained from a Millipore high-purity water dispenser (Billerica, MA, USA). Formic acid (FA) was purchased from Acros Organics BVBA (Geel, Belgium). Methanol, ethyl acetate, and toluene were purchased from Biosolve Chimime SARL (Dieuze, France). Cyclohexane, n-hexane, and n-butanol were purchased from Sigma-Aldrich (Steinheim, Germany). Bis(2-ethylhexyl) phosphate (DEHP) and tributyl phosphate (TBP) were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents were HPLC grade or higher.

2.2. Standard and sample solutions

Stock solutions of all compounds (100 μg mL^{-1}) were prepared in 1:1 MeOH: H_2O. Standard solutions were prepared by diluting stock solutions to a concentration of 500 ng mL^{-1} in 4% FA, unless stated otherwise. To evaluate the method in human plasma and urine samples, 50 ng mL^{-1} of propranolol, amitriptyline, bupivacaine, and oxeladin were firstly spiked to pure urine and plasma samples, and then 5-fold diluted samples [37–40]. Human urine samples (pooled from healthy donors) and EDTA-treated plasma samples (Sanquin, Leiden, The Netherlands) were kept frozen at −80 °C until analysis and were thawed at room temperature directly before use.

2.3. Electroextraction setup

Fig. 1A schematically depicts the online EE-MS setup, and a video of a three-phase extraction (Video 1) can be found in the Supporting Information. The installation consists of four parts: 1) an LC pump (Agilent 1200-series, Waldbronn, Germany) for FIA-MS solvent delivery; 2) a quadrupole-time-of-flight (Q-TOF) mass spectrometer (Agilent 6530 Accurate-Mass Q-TOF LC/MS, Waldbronn, Germany) for analyte detection; 3) a syringe pump (KD Scientific LEGATO 270, Holliston, MA, USA) for infusion and withdrawal of the acceptor phase droplet; and 4) the three-phase EE setup, detailed in Fig. 1B. At the heart of the system is a two-position ten-port switching valve (IDEX Health & Science, Lake Forest, IL, USA) to connect the parts. The connecting tubing consists of 120 mm fused silica (200 μm ID, 360 μm OD) between syringe pump and valve, and 200 mm PEEK tubing (150 μm ID, 360 μm OD) from the valve to the negative electrode.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.aca.2021.338204

The three-phase electroextraction was performed inside a 0.5 mL Eppendorf tube. A 250 nL droplet of aqueous acceptor phase is suspended from the tip of a fused silica capillary (100 μm ID, 365 μm OD, 70 mm length) in 300 μL of organic phase, which is on top of 200 μL of an aqueous sample. A platinum wire (280 μm diameter) with a polytetrafluorethylene (PTFE) insulating sleeve is inserted through the organic layer into the donor phase as the electrode. The tip of the electrode protrudes approximately 1 mm from the sleeve. The negative electrode was connected to a stainless steel union between two sections of fused silica capillary. The extraction voltage was delivered by a DC power supply (FUG HCN 140–3500 DC, FuG Elektronik GmbH, Schechen, Germany). The extraction process and droplet stability were monitored and recorded with a USB pen camera and Debut Video Capture (NCH Software, Greenwood Village, CO, USA).

2.4. Extraction procedure

For the three-phase EE procedure, the valve was first set in position 1 as depicted in Fig. 1C. The platinum electrode and fused silica capillary were inserted manually into the Eppendorf tube containing the organic phase and the aqueous sample. The organic phase was saturated with water to avoid the dissolution of the acceptor droplet [27]. A 250 μL syringe (Hamilton, Bonaduz,

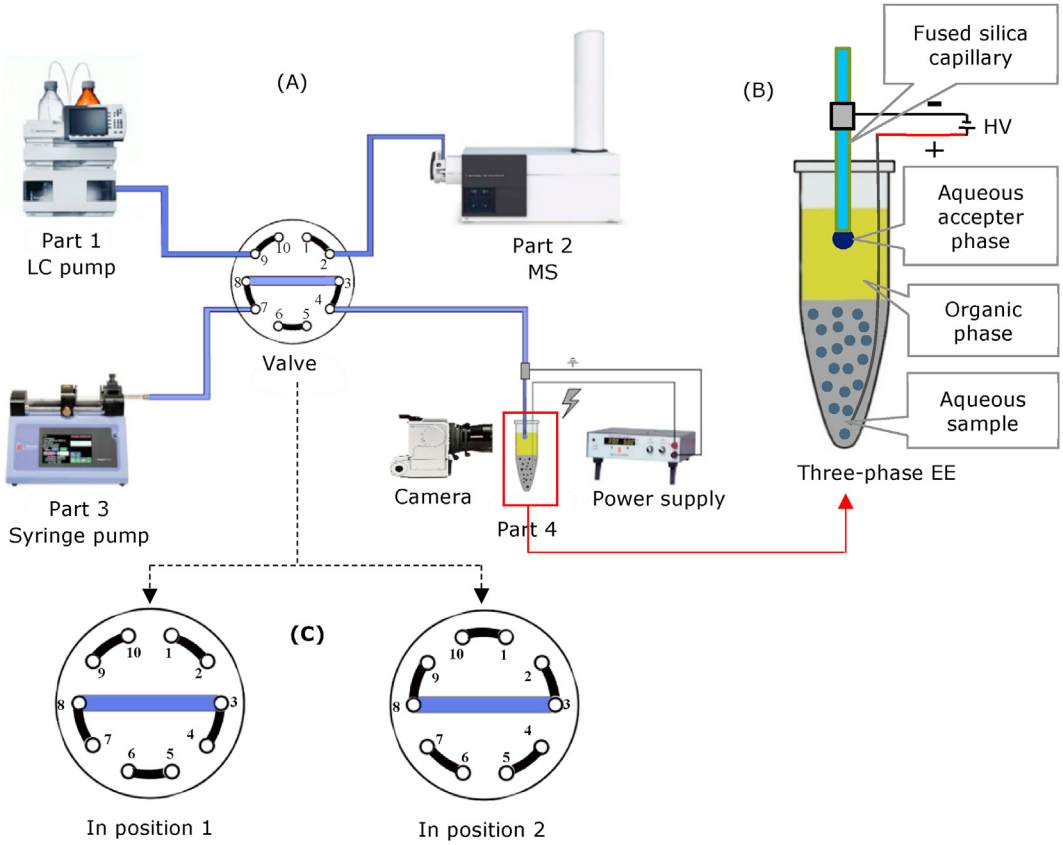

Fig. 1. (A) The schematic diagram of the online three-phase EE setup, (B) detail of the three-phase EE process inside an Eppendorf tube, and (C) the positions of the switching valve, in which position 1 is the extraction, position 2 is the injection to FIA-MS. (Schematic Video 1 can be found in SI).

Switzerland) and the programmable syringe pump were used to infuse the aqueous acceptor phase (20% MeOH and 4% FA, unless stated otherwise) to form a 0.25 μL droplet in the organic phase layer (ethyl acetate with 1% TBP, unless otherwise indicated). Subsequently, the extraction voltage was applied by manually switching on the high-voltage source to extract and concentrate analytes from the aqueous sample into the aqueous acceptor droplet. After the extraction time, the voltage was disconnected, the acceptor droplet was aspirated into the fused silica capillary, and the Eppendorf tube was replaced manually with a vial with the same solvent as the acceptor phase, and the droplet was further aspirated into the 1 μL sample loop, after which the valve was switched to the inject position (Fig. 1C, position 2). The sample plug was transferred to the MS by the continuous flow (0.4 mL min^{-1}) of the flow-injection solvent. When withdrawing the droplet, ethyl acetate from the organic phase layer is at risk of being withdrawn too. However, this would be negligible compared to the volume of the acceptor phase.

2.5. MS methods

The EE setup was hyphenated online with an Agilent 6530 quadrupole-time-of-flight mass spectrometer (Q-TOF/MS) equipped with an Agilent Jet Stream (AJS) ESI source. Electrospray ionization was operated in the positive mode. The source parameters were: drying gas temperature 350 °C, drying gas flow 8 L min^{-1}, nebulizer gas pressure 35 psi, sheath gas temperature 350 °C, sheath gas flow 11 L min^{-1}, capillary voltage 3500 V, and nozzle voltage 1500 V. The mass range of the MS experiments was 100–500 *m/z*, with an acquisition rate of 2 spectra s^{-1}. Data acquisition and instrument control were monitored using Mass Hunter version B.06.01 (Agilent, Waldbronn, Germany). MS data were processed with Mass Hunter Quantitative Analysis for Q-TOF (version B.07.00 SP1).

2.6. Data analysis and calculation

All MS data was collected with Agilent Masshunter Workstation Data Acquisition, analyzed with Agilent Masshunter Quantitative Analysis (for QTOF) and R (version 3.6.1). The enrichment factor (EF) [20,27] and extraction recovery (ER) [20,33] were used to evaluate the extraction performance of analytes under different conditions. EF and ER were calculated according to Equations (1) and (2):

$$EF = \frac{[Acceptor\ \ phase]_{\ after\ \ EE}}{[Aqueous\ \ sample]_{\ before\ \ EE}} \tag{1}$$

$$ER(\%) = EF \times \frac{V_d}{V_s} \cdot 100\% \tag{2}$$

Where V_d is the volume of the aqueous acceptor phase droplet. Since the acceptor phase contains 20% methanol (unless stated otherwise), part of the droplet will dissolve in the ethyl acetate. Here, we assume the worst case, *i.e.* 20% methanol was totally dissolved in the organic phase, leading to a corrected droplet volume of 0.2 μL instead of 0.25 μL V_s is the volume of the aqueous sample, *i.e.*, 200 μL.

3. Results and discussion

3.1. Qualification of EE

The three-phase EE method was first visually assessed using 50 ng mL^{-1} of the cationic dye crystal violet as a sample. The acceptor phase droplet colored dark blue within a few seconds after starting the extraction and remained stable for over 360 s (Video 2 in Supporting Information). The aspiration rate and volume of the syringe pump were then optimized to ensure the complete transfer of the acceptor droplet to the sample loop via aspiration (aspiration volume 7.0 μL, flow rate 7.5 μL min^{-1}). The transparent PTFE sample loop allowed for visual confirmation of positioning the extracted sample in the loop. For this, a series of crystal violet extractions was performed. Under the optimized conditions, 10 consecutive extractions were carried out and it was observed that extracted crystal violet zone was positioned in the middle of the sample loop each time. No carryover was observed on the electrode and fused silica capillary after washing with isopropanol:water (1:1, v/v). Additionally, no carryover was detected for the four model compounds, hence demonstrating that the capillary and electrode can be reused.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.aca.2021.338204

3.2. Optimization of the EE method

To optimize the three-phase EE method, the following four parameters were studied: 1) the solvent type of the organic phase, 2) the composition of the organic phase, 3) the percentage of MeOH and FA in the aqueous acceptor phase and the aqueous sample, and 4) the applied extraction voltage and time.

3.2.1. The selection of the solvent type for the organic phase

During extraction, the analytes selectively pass through the organic phase layer, based on properties such as polarity, electrical conductivity, or viscosity. Five common organic solvents (solubility in water), *i.e.* ethyl acetate (83 g L^{-1}), n-butanol (73 g L^{-1}), cyclohexane (immiscible), n-hexane (9.5 mg L^{-1}), and toluene (0.52 g L^{-1}) were studied as the organic phase.

As shown in Table 1, the EF of the four model compounds with ethyl acetate as the organic layer was significantly higher than those in the other tested solvents ($P < 0.05$). This might be due to the relatively higher electrical conductivity and lower viscosity of ethyl acetate (Table S1 in SI) [41], which allowed for faster migration of analytes through the organic layer. Thus, ethyl acetate was selected as the organic phase for subsequent experiments.

3.2.2. Modulation of the composition of the organic phase

The influence of the polarity of the organic phase on the extraction performance has been reported in several studies [22,27,42,43]. To further modulate the organic phase, two organic solvents were added to ethyl acetate; the well-known ion-pair reagent, di (2-ethylhexyl) phosphate (DEHP), and the more recently discussed tributyl phosphate (TBP) [42]. These two organic solvents were mixed with ethyl acetate in 1%, 3%, 5%, and 10%, respectively.

Table 1
The influence of the organic phase solvent on the EF of different compounds (n = 3).

	Propranolol	Amitriptyline	Bupivacaine	Oxeladin
ethyl acetate	61.33 ± 8.67	73.67 ± 10.17	456.67 ± 68.04	148.33 ± 21.94
n-butanol	0.06 ± 0.03	0.12 ± 0.01	0.40 ± 0.10	0.14 ± 0.01
cyclohexane	0.29 ± 0.09	0.28 ± 0.06	1.20 ± 0.37	0.29 ± 0.07
n-hexane	0.06 ± 0.01	0.22 ± 0.07	0.44 ± 0.15	0.30 ± 0.14
toluene	0.06 ± 0.05	2.04 ± 0.16	1.15 ± 0.40	0.64 ± 0.01

Fig. 2A shows that the addition of DEHP negatively affects the extraction performance. A probable cause is that its addition modulates the organic phase more favorable towards polar analytes [27], while the analytes of interest are non-polar. Also, the ionic nature of DEHP resulted in excessively high current of over 180 μA, compared to 15 nA normally during the extraction, which can induce electrolysis and an increase in pH at the cathode, *i.e.*, the acceptor phase [42,44]. The pKa of the four non-polar compounds ranges from 8.10 to 9.42, hence the increased pH decreases the charged state and increases their affinity for the organic solvent. Therefore, the non-ionic solvent TBP was applied to adjust the polarity of the organic phase.

Fig. 2 B shows that for all compounds, the enrichment factor was reduced with the addition of more than 1% TBP. Propranolol, amitriptyline, and bupivacaine showed the best extraction performance at 1% TBP, whereas for oxeladin, there was no significant difference between 0% or 1% TBP (Fig. 2B). Similar to DEHP, addition of TBP to the organic phase increases the polarity, which adjusts the selectivity towards more polar compounds. The polarity of 1% TBP in ethyl acetate may provide the best organic phase selectivity for the four compounds in this experiment. Additionally, the non-ionic nature of TBP did not induce a high current and excessive electrolysis during electroextraction. A similar result was reported in a previous publication [42]. In this study, the addition of 1% TBP was used for subsequent experiments.

3.2.3. Effects of FA and MeOH in the acceptor phase and the aqueous sample on the EF

The EF of the model compounds was at its highest with 4% FA in the acceptor phase and the aqueous sample (Fig. 3A). The addition of FA to the acceptor phase and the sample reduces the pH, which increases the charge state and migration of the non-polar model analytes. Higher percentages of FA in the acceptor phase would most likely be beneficial, as further reduction of the pH increases the solubility of the compounds [42,44]. However, with more than 4% FA, the acceptor droplet was unstable during the extraction, most likely due to excessive positive charge in the droplet and, as a consequence, electrostatic repulsion. Therefore, all further experiments were conducted with 4% FA in the acceptor phase and the sample. Furthermore, the addition of MeOH to the acceptor droplet enhanced evaporation and ionization in the ESI source and also increased the Galvani potential difference between phases [27], hence improved extraction performance. Fig. 3B shows that the optimum point was at 20% MeOH in the acceptor phase, which was used in subsequent experiments.

3.2.4. Effects of the applied voltage and extraction time on EF

The applied voltage and extraction time are two critical parameters of electroextraction and were studied thoroughly. Four extraction voltages (100, 300, 600, and 900 V) at eight extraction times (15 s, 30 s, 45 s, 60 s, 90 s, 120 s, 180 s, and 360 s) were studied to find the optimal combination of these parameters. Since the extraction profiles are similar for all four compounds, only the data for propranolol is shown here. The results (Fig. 4 and Figs. S1–3 in the SI) showed that an extraction time with maximum EF was reached for each compound, independent of the voltage applied. This maximum was reached faster as the voltage was increased. However, at extraction voltages over 900 V the acceptor droplet became unstable, and extractions regularly failed. The profile showed a declining trend if the extraction was continued after reaching the maximum EF. This can be explained by a change in pH due to electrolysis during the extraction process [22]. The acceptor droplet acts as the cathode, and the pH increases on this side as an effect of electrolysis. As a result, the charge state equilibrium of the

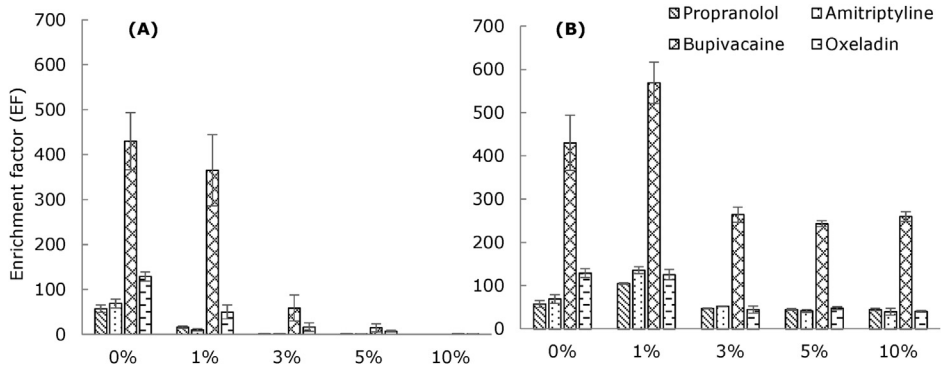

Fig. 2. The effects of DEHP (A) and TBP (B) in the organic phase on the EF of four compounds (n = 3). Extraction conditions: applied voltage, 900 V; extraction time, 30 s; organic phase, ethyl acetate; acceptor phase, 20% MeOH with 4% FA; aqueous sample, 4% FA with 500 ng mL^{-1} analyte.

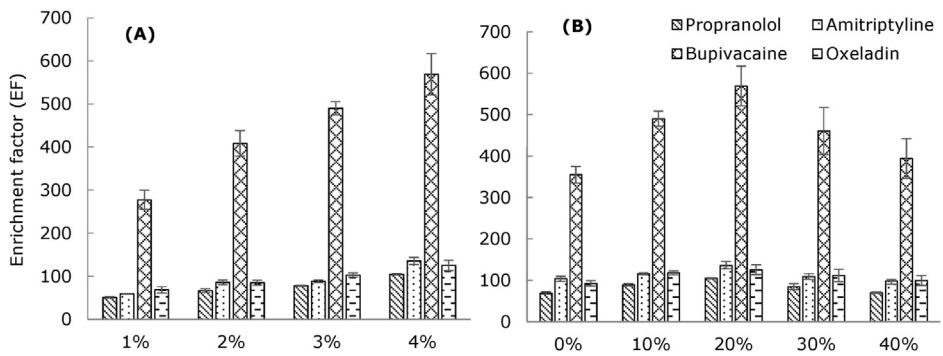

Fig. 3. The effects of FA (A) and MeOH (B) in the acceptor phase and the aqueous sample on the EF (n = 3). Extraction conditions (A): extraction voltage, 900 V; extraction time, 30 s; organic phase, ethyl acetate with 1% TBP; acceptor phase, 20% MeOH; aqueous sample, stated percentage of FA with 500 ng mL^{-1} analytes. Extraction condition (B): extraction voltage, 900 V; extraction time, 30 s; organic phase, ethyl acetate with 1% TBP; acceptor phase, 4% FA; aqueous sample, 4% FA with 500 ng mL^{-1} analytes.

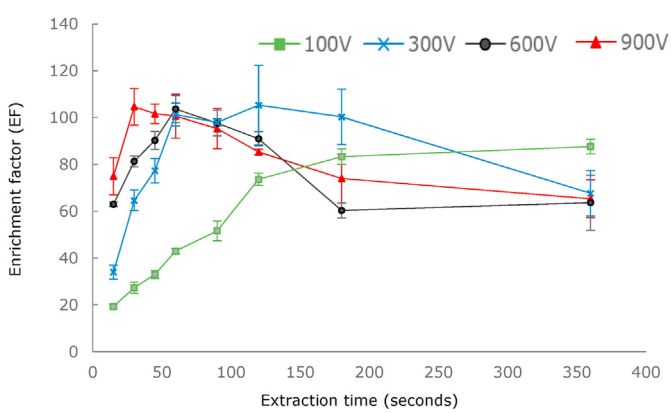

Fig. 4. The effects of voltage and extraction time on the EF of propranolol (n = 3). Extraction conditions: organic phase, ethyl acetate with 1% TBP; acceptor phase, 20% MeOH with 4% FA; aqueous sample, 4% FA with 500 ng mL^{-1} analytes.

analytes shifts towards a more neutral state, reducing their polarity and giving rise to back-extraction into the organic phase. The competition between extraction and back-extraction leads to an optimum point in accordance with observations from various publications [19,27,28].

The optimum extraction time of 30 s for propranolol (at 900 V) is much shorter than the 2.5–33.3 min reported in other publications [19,27–36]. Despite that the ER values are lower for some compounds (from 10.2% to 55.7%, Table S2), the EF of the four compounds (105–569) is higher than comparable non-polar and basic compounds in other studies. For instance, the EF of apolar carnitines ranged from 6 to 25 [27], and from 69 to 363 for atenolol and betaxolol [34]. The biggest improvement of this method with respect to the three-phase EE method described in Ref. [27] is the improved fast extraction time from 3 min to 30 s, and higher EF for the compounds, from 6 to 25 to 105–569. The shorter extraction time and higher extraction performance of the model compounds might be due to the higher extraction voltage and the small-volume droplet as the acceptor phase. Spherical droplets have a high surface-area-to-volume-ratio and the electric field is denser around the droplet perimeter, which all contribute to fast migration of the analytes. Avelar et al. reported a novel multiphase electroextraction setup in which a chromatographic paper was located in the aqueous acceptor phase, allowing for direct coupling with paper spray mass spectrometry. They demonstrated the setup by extracting five tricyclic antidepressants from saliva and obtained extraction efficiencies ranging from 42 to 63% and low matrix effect. The extraction efficiency is probably enhanced by the high absorption performance of the chromatographic paper and thorough desorption of the extracted analytes [45]. The total time for the extraction procedure is 2–3 min, including adding the sample and organic phase, forming acceptor droplet and injecting.3 min, including adding the sample and organic phase, forming acceptor droplet and injecting.

3.3. Application of the three-phase EE method to human urine and plasma samples

3.3.1. Performance of EE method in urine and plasma samples

To further evaluate the EE method with biological samples, the

Table 2
The EF of compounds before and after ion suppression correction in pure and diluted urine and plasma samples (n = 3).

		Propranolol	Amitriptyline	Bupivacaine	Oxeladin
Urine	Pure	3.5 ± 0.2	2.9 ± 0.1	28.7 ± 1.2	13.1 ± 0.5
	Diluted	18.8 ± 1.8	14.7 ± 0.9	87.1 ± 3.5	28.2 ± 0.1
Plasma	Pure	4.0 ± 0.7	2.5 ± 0.8	n.a.	11.9 ± 2.1
	Diluted	22.6 ± 0.1	14.8 ± 1.0	156.6 ± 2.2	64.9 ± 3.8
*After ion suppression correction (**EF_c**)*					
Urine	Pure	28.6	22.5	330.1	76.4
	Diluted	61.4	60.0	400.7	98.7
Plasma	Pure	19.6	9.7	n.a.	35.9
	Diluted	58.3	37.0	423.7	108.2

Note: n.a. means nothing was detected.

model compounds were spiked at therapeutic concentrations (50 ng mL^{-1}) into pure and 5-fold diluted samples of human urine and plasma (with 4% FA added, no precipitation) [37–44]. The mass spectra of the model compounds can be found in Fig. S4 of the Supporting Information. The EF in pure urine and plasma was significantly lower than that in diluted samples ($P < 0.05$), as shown in Table 2. These pure matrices suffered from severer ion suppression than diluted samples in the MS analysis. This was confirmed by comparing to the academic sample (50 ng mL^{-1} model compounds with 4% FA in MilliQ water), which showed significantly higher EF and ER (Table S2), and do not suffer from ion suppression.

3.3.2. Ion suppression evaluation and correction

A continuous-flow injection analysis was set up to qualitatively assess and quantitively correct the ion suppression effects. The model compounds (50 ng mL^{-1}) were added in the FIA carrier flow and the EE acceptor phase. This provides a continuous high signal for the model compounds. The optimized EE method was then applied to an academic sample, diluted and pure urine and plasma samples, without addition of the model compounds. The ion suppression effect can then be quantified as the decrease in signal of the model compounds. As shown in Fig. 5 and S5 – 7, the ion suppression effect increases with the concentration of the urine and plasma samples. This observation confirms the hypothesis that the decreased EF of the compounds from urine and plasma is mainly caused by ion suppression in the MS source, and not by lower extraction efficiency.

The corrected enrichment factor (EF_c) is then calculated by dividing the enrichment factor (EF) by the ion suppression ratio (R), ergo $EF_c = EF/R$ [46]. The R here is defined as the lowest point of the model compound's spectrum in the biological matrix over the baseline signal of model compound (in percentage, Fig. 5 and S5 – 7). Also, the extraction recovery of the analytes was corrected by EF_c and Equation (2). The high EF_c (Table 2) and corrected ER results (Table S2) showed good enrichment and recovery of analytes from urine and plasma samples, demonstrating that the developed EE method is suitable to the analysis of plasma and urine samples. The results demonstrate that dilution increases the EF and ER, which was consistent with [47]. However, the gain in EF is lower than the dilution factor, thus there is still a net loss of signal, and it can be concluded that diluting the biological samples improves the extraction recovery, but is not advantageous for reducing the matrix effect.

3.4. Validation of the EE method

Higher EF and ER results indicate better performance of the EE method in 5-fold diluted samples. Therefore, the performance of the EE method in diluted plasma and urine were evaluated by determining the response function, limits of detection (LODs), limits of quantification (LOQs), intra- and inter-day EF. All compounds showed good linear response ($R^2 > 0.9903$) within a concentration range of two orders of magnitude, from 10 to 1000 ng mL^{-1} (Table 3). The LODs and LOQs of the four compounds were in the range of 0.36–3.21 ng mL^{-1} and 1.20–10.71 ng mL^{-1}, respectively. The accuracy (73–112%), obtained intra- and inter-day EF and RSD (Table 3), and all the validated results indicate the robustness, stability and sensitivity of the EE method in both plasma and urine samples.

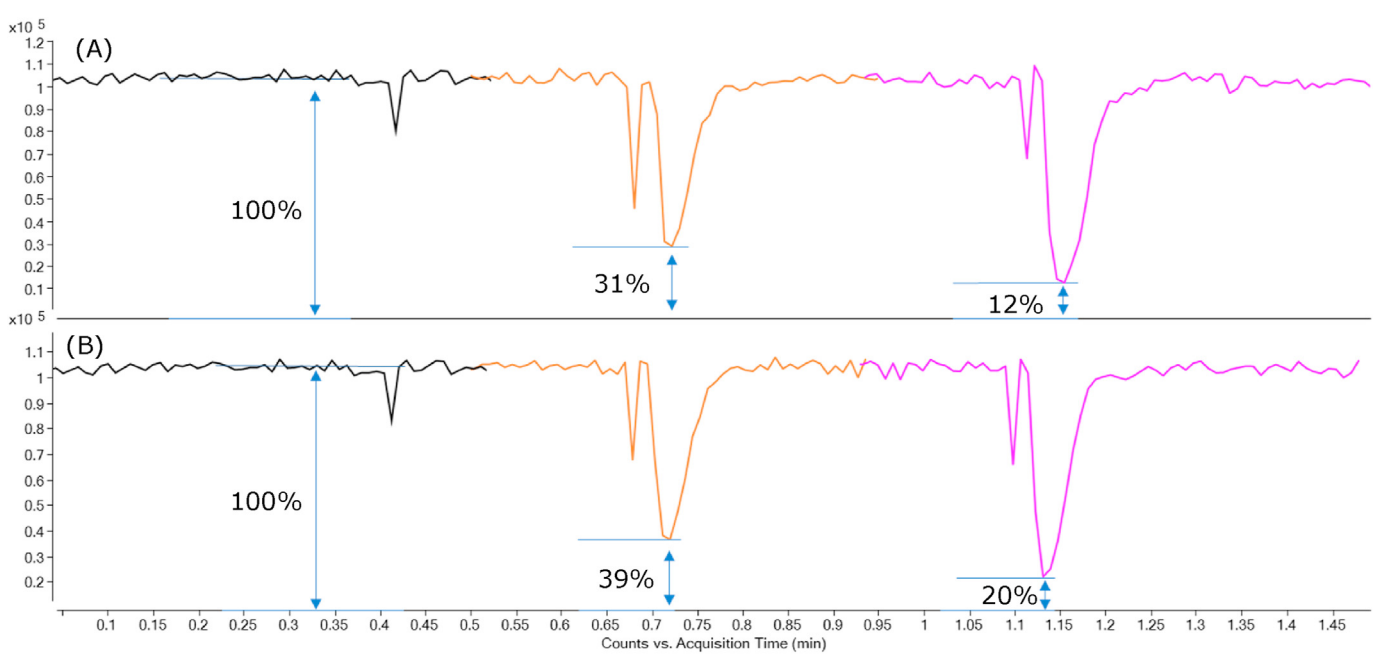

Fig. 5. The ion suppression effect and ratio of propranolol in urine (A) and plasma (B) samples. Academic sample (black color); Diluted sample (orange color); Pure sample (Pink color). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Calibration curve and precisions (RSD) of the model compounds in diluted plasma and urine samples by using the optimized EE method (n = 3).

		Linear range (ng mL^{-1})	R^2	LODs (ng mL^{-1}) (S N^{-1} = 3)	LOQs (ng mL^{-1}) (S N^{-1} = 10)	Accuracy (%)	EF (RSD) (50 ng mL^{-1})	
						(50 ng mL^{-1})	Intraday	Interday
Propranolol	Plasma	10–1000	0.9953	1.6	5.3	86	22.6 (0.8%)	21.0 (10.1%)
	Urine	10–1000	0.9996	3.2	10.7	100	18.8 (6.0%)	17.4 (17.8%)
Amitriptyline	Plasma	10–1000	0.9986	0.6	2.0	96	14.8 (9.8%)	13.7 (1.5%)
	Urine	10–1000	0.9950	1.4	4.8	107	14.7 (9.0%)	14.0 (16.7%)
Bupivacaine	Plasma	10–1000	0.9924	1.7	5.6	112	156.6 (2.0%)	142.4 (11.9%)
	Urine	10–1000	0.9954	1.9	6.5	86	87.1 (5.7%)	80.8 (17.3%)
Oxeladin	Plasma	10–1000	0.9915	0.4	1.2	90	64.9 (8.3%)	58.4 (6.3%)
	Urine	10–1000	0.9903	0.7	2.4	73	28.2 (0.6%)	27.3 (5.2%)

4. Conclusion

A three-phase EE setup was developed and optimized with propranolol, amitriptyline, bupivacaine, and oxeladin in aqueous samples. For the first time, the extraction time can be as short as 30 s while achieving enrichment factors of 105–569 and LODs of 360 pg/mL. The optimized three-phase EE method was successfully applied to human urine and plasma samples, with enrichment factors ranging from 37 to 424 and extraction recovery from 3.7% to 42.4% in diluted samples with good accuracy (73–112%). Future research will be focused on integrating a separation method, *i.e.* ultra-high pressure liquid chromatography (UHPLC), to reduce ion suppression effects.

In summary, we provided a fast, simple, and online three-phase EE setup with high enrichment factors. For future perspectives, the setup can be automated to increase the throughput. We believe that this technique has great potential to overcome the sample preparation bottleneck to enable high-throughput bioanalysis workflows.

CRediT authorship contribution statement

Yupeng He: Investigation, Conceptualization, Formal analysis, Writing - original draft. **Paul Miggiels:** Conceptualization, Writing - original draft. **Bert Wouters:** Conceptualization, Writing - original draft. **Nicolas Drouin:** Conceptualization, Investigation, Writing - original draft. **Faisa Guled:** Methodology. **Thomas Hankemeier:** Conceptualization, Supervision. **Petrus W. Lindenburg:** Conceptualization, 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.

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Appendix A. Supplementary data

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