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Development of a fast, online three-phase electroextraction hyphenated to fast liquid chromatography–mass spectrometry for analysis of trace-level acid pharmaceuticals in plasma

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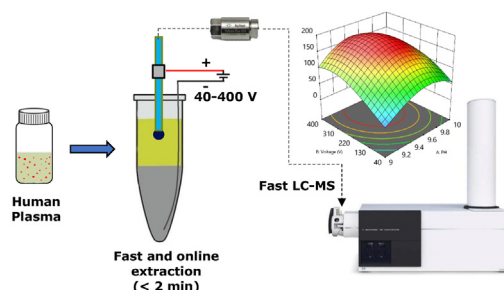
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HIGHLIGHTS

- Fast extraction of acidic analytes, less than 2 min.
- High enrichment factors, up to 190-fold.
- Low sample consumption, down to 15 μL .
- Hyphenation to fast LC-MS.
- Limit of detection as low as 1.05 ng mL^{-1} .

GRAPHICAL ABSTRACT



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ABSTRACT

Sample preparation is a challenge for high-throughput analysis, especially for volume-limited samples with low-abundant analytes. Ideally, sample preparation enriches the analytes of interest while removing the interferences to reduce the matrix effect and improve both sensitivity and quantification. In this study, a three-phase electroextraction (EE) method hyphenated to fast online liquid chromatography–mass spectrometry (LC-MS) was developed. Four model acidic drugs of relevance for drug monitoring in plasma, *i.e.* naproxen, fenoprofen, flurbiprofen, and ibuprofen, were utilized for the optimization and evaluation of the method. A Design of Experiment approach (Box-Behnken design) was used to optimize the critical parameters of the method, *i.e.*, the type of organic solvent, pH of the sample and acceptor phase, and the extraction voltage and time. Good fitness ($P < 0.02$, $R^2 > 0.95$) was observed for the developed quadratic model. Extraction could be achieved in less than 2 min (115 s) with enrichment factors (EF) up to 190 and extraction recoveries (ER) up to 38% for academic samples. Additionally, the optimized three-phase EE method was successfully applied to spiked plasma samples with low-abundant (50 ng mL^{-1}) analytes and a low sample volume of 15 μL plasma in 10-fold diluted samples. Finally, two crucial contributors to the matrix effect of three-phase EE application on plasma samples were determined. Specifically, the ion-suppression effect in the MS source was reduced by the fast LC separation, and the matrix effect during extraction was negligible for the diluted protein-precipitated plasma samples. The developed three-phase EE method is easy to operate and provides fast and on-line extraction of trace-level acidic analytes from volume-limited biological samples. Therefore, this

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method can provide a potential solution for sample-preparation bottlenecks in high-throughput bio-analysis workflows.

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1. Introduction

Sample-preparation methods remain challenges for high-throughput bio-analytical workflows [1–6] as they are often time- and labor-intensive [1,7–9] yet achieve relatively low enrichment [10], especially for volume-limited samples with low-abundant analytes [11–14]. The ideal sample-preparation method should be fast, simple, and environmentally friendly. Additionally, the method should be able to clean up and highly pre-concentrate the analytes [1,11,15–17]. At the same time, miniaturized sample-preparation methods have been an emerging topic for bio-analysis in recent years [18].

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are well established and the most commonly used sample-preparation methods [4,19,20], but both are labor intensive, not well adapted for high enrichment, and use large amounts of high-purity organic solutions which are environmentally unfriendly. Electro-driven extraction is a fast one-step sample-preparation technique developed in the last two decades and is based on the migration of charged analytes in a sample with applied voltage. Thanks to its low solvent and sample consumption, and high enrichment factor, an increasing number of researchers focus on further development and optimization of electro-driven extraction techniques [21–24]. Electro-membrane extraction (EME) and free liquid membrane electroextraction (FLM-EE) are the two main variants of this technique. The difference between them is the application of a stable solid membrane between the sample and acceptor phase in EME to hold the organic solution [24]. Three-phase electroextraction (EE) is a subtype FLM-EE and was first reported by Raterink et al. in 2013 [25]. During three-phase EE, an aqueous acceptor droplet is formed in the organic phase. Many electro-driven extraction studies have so far focused on the optimization for basic analytes by low-throughput serial sample extraction or high-throughput parallel sample extraction in in-house developed 96-well plates [25–32], in which three-phase EE was reported as a high-throughput extraction method with extraction time down to 30 s and enrichment factors up to 569 [28]. For acidic analytes, EME was reported with extraction time ranging from 5 to 25 min and enrichment factors from 0.8 to 185 [27,32–39]. To meet the requirements of high-throughput analysis for acidic analytes, the sample extraction time must be further reduced while achieving higher enrichment.

Matrix effects causing ion suppression are an important influencing factor in the case of mass-spectrometric detection, especially for high-throughput analysis where the separation of the sample is sacrificed for the sake of analysis speed. Indeed, large matrix effects were observed for biological samples in our previous electro-extraction study with flow injection [28]. To reduce the matrix effects, further investigation should be conducted on whether these effects arise from the analyte's extraction process or from ion suppression in the MS source. At the same time, a fast analyte separation method should be coupled to the three-phase EE setup to meet the requirements for high-throughput analysis.

In the present work, an online three-phase EE setup hyphenated to fast LC-MS was developed for acidic analytes. The type of organic solvent, pH of sample and acceptor phase, extraction voltage, and extraction time were optimized by using an experimental design

methodology (Box-Behnken design) for four commonly-used model acidic compounds, *i.e.* naproxen, fenoprofen, flurbiprofen, and ibuprofen [27,40]. Finally, the optimized three-phase EE set-up was successfully applied to human plasma samples with compounds spiking before, after, and without protein precipitation (PP) to investigate how to reduce the matrix effects. This study provides a fast, simple, online, and software-operated sample-preparation method coupled to fast LC separation for bioanalysis of low-abundant acidic analytes.

2. Material and methods

2.1. Chemicals

Naproxen, flurbiprofen, fenoprofen calcium salt hydrate, ibuprofen, 1-octanol, ammonium acetate, and ammonia were purchased from Sigma-Aldrich (Steinheim, Germany). MilliQ water was obtained from a Millipore high-purity water dispenser (Billerica, MA, USA). Methanol and ethyl acetate were purchased from Biosolve Chimie SARL (Dieuze, France). All solvents were HPLC grade or higher.

2.2. Standard and sample solutions

Stock solutions of all compounds were prepared in 1:1 MeOH:H₂O at a concentration of 200 µg mL⁻¹. Academic samples were prepared by diluting stock solutions to a concentration of 500 ng mL⁻¹ in 50 mM ammonium acetate which was adjusted to the desired pH with ammonium hydroxide. The pH of all samples and acceptor phase was adjusted and verified using a Metrohm 827 pH Lab pH meter (Metrohm, Herisau, Switzerland) and double-checked with pH-indicator strips 7.5–14.0 (Sigma-Aldrich, Steinheim, Germany). To evaluate the three-phase electro-extraction method in biological samples and to investigate the matrix effects, 50 ng mL⁻¹ of naproxen, fenoprofen, flurbiprofen, and ibuprofen were spiked into pure, 5-fold, and 10-fold diluted plasma samples before, after, and without protein precipitation (PP). Ice-cold MeOH was used for protein precipitation of plasma samples with a ratio MeOH: plasma of 4:1, v/v [41]. The supernatant was evaporated to dryness using a SpeedVac Vacuum concentrator (Thermo Savant SC210A, Waltham, Massachusetts, United States) and reconstituted in 50 mM ammonium acetate which was adjusted by ammonium hydroxide to the desired pH to the starting volume, then was used in the pure, 5-fold, and 10-fold diluted PP plasma samples. Human 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. Three-phase EE setup and extraction process

Fig. 1A shows the three-phase EE setup, which has been upgraded since our previous study [28]. It now includes an Agilent 1200-series G1158B valve (Agilent, Waldbronn, Germany), controlled via MS software; a VICI M50 positive-displacement pump (VICI AG, Schenkon, Switzerland) controlled via the supplied VICI software; and a 0–1000V DC power supply (RB10 1.5P, Matsusada Precision, Shiga, Japan) controlled by a custom LabView

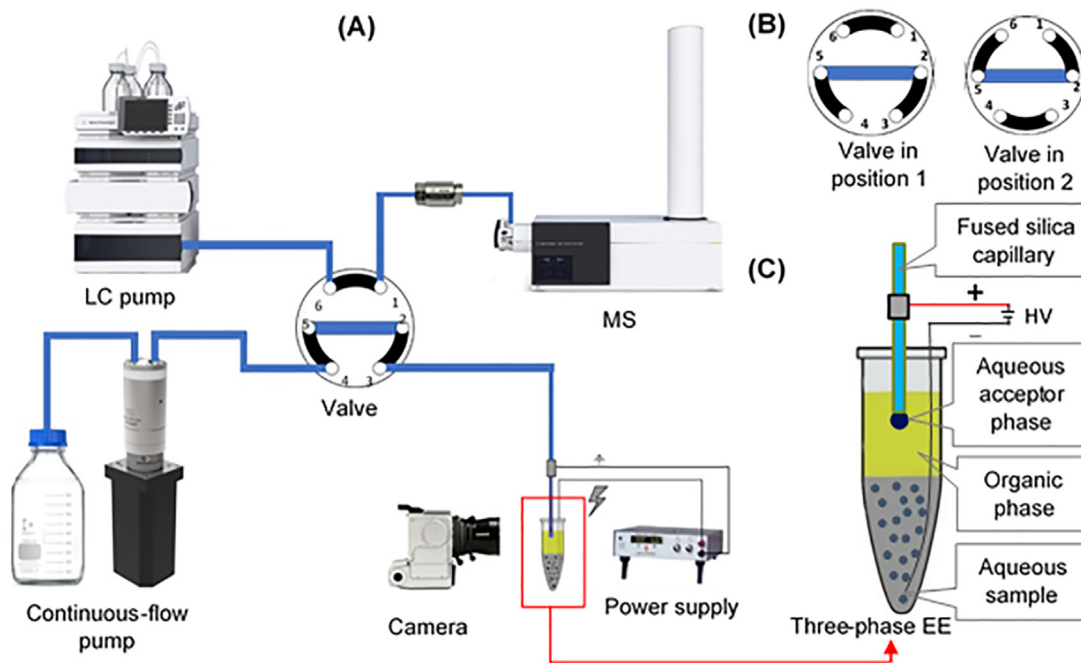


Fig. 1. (A) The schematic diagram of the online three-phase EE setup, (B) the positions of the switching valve, in which position 1 is the extraction, position 2 is the injection to fast LC-MS, and (C) detail of the three-phase EE process inside an Eppendorf tube.

script (LabView 2020, National Instruments, Austin, Texas, USA).

The three-phase EE procedure was similar as in our previous study [28]: 150 μL aqueous sample and the organic phase was added in an Eppendorf tube (Fig. 1C). A 0.3 μL acceptor droplet (50 mM ammonium acetate in stated pH) was formed in the organic phase by using the continuous-flow pump in valve position 1 (Fig. 1B). Then, an extraction voltage of 40–400 V was applied for a specified period of time of 10–150 s. Subsequently, the acceptor droplet was aspirated into the fused-silica capillary with a volume of 12.2 μL and a flow rate of 40 $\mu\text{L min}^{-1}$, the Eppendorf tube was replaced with a vial of acceptor solvent, and the droplet was further aspirated into the 1 μL sample loop with a volume of 4.4 μL and a flow rate of 40 $\mu\text{L min}^{-1}$. Finally, the sample was injected into the LC-MS after the valve was switched to position 2 (Fig. 1B). The electrode and fused-silica capillary were washed with isopropanol:water (1:1, v/v) after each extraction.

2.4. Fast LC-MS methods

The three-phase EE setup was coupled online to an Agilent ZORBAX Extend-C₁₈ LC column (2.1 $\times$ 5 mm, 1.8 μm) and an Agilent 1200 Series G1312B pump (Agilent, Waldbronn, Germany) tandem an Agilent 6530 quadrupole-time-of-flight mass spectrometer (Q-TOF/MS) equipped with an Agilent Jet Stream (AJS) ESI source. Pure methanol and MilliQ water were used as the mobile phase in a gradient method starting with 95% water (0–0.4 min) to flush out the salts in the acceptor phase, then increased to 80% of MeOH in 1.0 min and kept constant until 2 min to elute the model compounds. The flow rate was 0.4 mL min^{-1} . Electrospray ionization was set in the negative mode with the following parameters: drying gas temperature 350 $^{\circ}\text{C}$, drying gas flow 8 L min^{-1} , nebulizer gas pressure 35 psi, sheath gas temperature 350 $^{\circ}\text{C}$, sheath gas flow 11 L min^{-1} , capillary voltage 3500 V, nozzle voltage 1500 V, mass range 100–1000 m/z , and an acquisition rate of 2 Hz. The MS was turned and calibrated by ESI-L Low Concentration Tuning Mix (G1969-85000, Agilent) every day before and after the experiment.

LC-MS was controlled by Mass Hunter version B.06.01 (Agilent).

2.5. Data analysis and calculation

All MS data were collected with Agilent Masshunter Workstation Data Acquisition, analyzed by Agilent Masshunter Quantitative Analysis (for QTOF) and SPSS (IBM Statistics 25). Design-Expert (version 12.0, Stat-Ease, Minneapolis, USA) was utilized for the optimization of the parameters of the three-phase EE by using a Box-Behnken design (BBD) of experiment. The enrichment factor (EF) [22,25,28] and extraction recovery (ER) [22,42] were calculated by equations (1) and (2):

$$EF = \frac{[\text{Acceptor phase}]_{\text{after EE}}}{[\text{Aqueous sample}]_{\text{before EE}}} \quad (1)$$

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

where V_d and V_s are the volumes of the acceptor droplet (0.3 μL) and aqueous sample (150 μL), respectively.

3. Results and discussion

3.1. LC-MS optimization and performance

To investigate the effects of ion suppression in the MS source on the model compounds, a C18 column was used between the three-phase EE setup and MS given the high logP value (2.9–3.9) of the model compounds. The fast gradient LC method was compared with a flow-injection method without an LC column by using four model compounds (Fig. 2). With the short LC separation, the peak intensity of the model compounds was 2.5–12.2 fold higher compared to without LC column separation. This shows that the extracted ions and ions in acceptor phase notably impact the ionization of model compounds, and that the ion suppression can

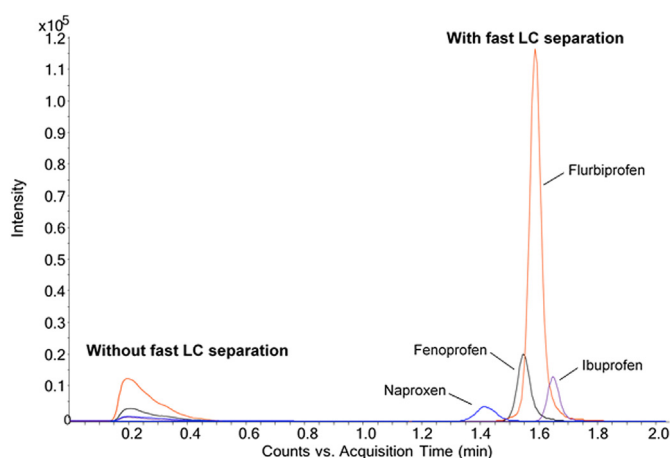


Fig. 2. The mass spectrum of model compounds with and without fast LC separation. Blue: naproxen; Black: fenoprofen; Orange: flurbiprofen; Purple: ibuprofen. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

be strongly reduced by removing salts and separating the target analytes. At the same time, the sharper peaks with LC column separation demonstrated that the compounds were focused by the LC column compared with the flow-injection peaks. Similar result was also reported in Van der Laan et al.'s study [43]. To obtain sensitivity of model compounds and further investigate the matrix effects during the three-phase EE process, the fast LC method was used in the subsequent experiments.

3.2. Optimization of the three-phase EE method

3.2.1. Design of the three-phase EE optimization model

The parameters of the three-phase EE setup were optimized for maximum enrichment factor using Box-Behnken design (BBD), based on previous studies [26,28,44] and the following reported theoretical model of analytes flux (J_i) [45–47].

$$J_i = \frac{-D_i}{h} \left(1 + \frac{\nu}{\ln \chi} \right) \left(\frac{\chi - 1}{\chi - \exp(-\nu)} \right) (C_{ih} - C_{i0} \exp(-\nu)) \quad (3)$$

where D_i is the diffusion coefficient for the analytes, h is the thickness of the membrane, C_{ih} is the analyte concentration at the organic phase/sample interface, C_{i0} is the analyte concentration at the acceptor/organic phase interface, ν is a function of electrical potential, and χ is the ion balance, i.e., the ratio of the total ionic concentration in the sample solution to that in the acceptor solution.

The flux of analyte (J_i) can be improved by increasing the applied voltage. At the same time, extraction time can also increase the flux of analytes. However, there is an antagonistic effect between extraction time and voltage, i.e., an increase in extraction voltage limits the extraction time and vice versa. Furthermore, a decreased ion balance (χ) theoretically also contributes to an increased flux of analytes [45–47]. Therefore, these three crucial parameters, pH of sample and acceptor phase (A), applied voltage (B), and extraction time (C), were selected for the simultaneous optimization and evaluation of the three-phase EE method.

A quadratic model was adopted in BBD, and seventeen experiments were conducted in triplicate. The investigating range for each parameter is listed in Table 1. Code –1, 0, and 1 were used to represent low, middle, and high levels of parameters. The maximum voltage was set to 400 V because of instability of the acceptor droplets at extraction voltages above this value. This

Table 1

Investigated parameters for Box-Behnken design.

Code level	A: pH	B: Voltage (V)	C: Extraction time (S)
–1	9	40	10
0	9.5	220	80
1	10	400	150

Note: Code –1, 0, and 1 were used to represent low, middle, and high levels of parameters.

maximum was determined as the voltage at which the acceptor droplet was kept stable for at least 150 s with an academic sample (Video 1 of the Supplementary Information). Two previously used organic solvents for electro-driven extraction of acidic compounds were adopted and compared in this study, i.e., 1-octanol and ethyl acetate [25,27,28]. BBD was applied to both organic solvents for the optimization of the three-phase EE methods.

3.2.2. Model quality

Table 2 highlights that the developed models were significant for all model compounds with $P < 0.02$ in both 1-octanol and ethyl acetate. The lack of fit of these models was insignificant with P -values higher than 0.08, demonstrating that the developed models fit well with the parameters used for optimization for all compounds. The statistical evaluation of the coefficients also revealed the good fit of the models with the determination coefficient, with R^2 and adjusted R^2 higher than 0.87 and 0.70, respectively, regardless of the compounds and organic solvents (Table 2). Overall, the developed models fit well with the experimental values for the subsequent three-phase EE optimizations.

3.2.3. Optimization of the three-phase EE method

The optimum enrichment factors of model compounds in 1-octanol and ethyl acetate were compared to determine the best organic phase for the three-phase EE method. Table 3 suggested that the optimized EF in 1-octanol was significantly higher compared to ethyl acetate. This could be explained by two factors. Firstly, 1-octanol has a higher electrical conductivity (851 nS m^{-1} [48]) than ethyl acetate (200 nS m^{-1} [28]). Secondly, 1-octanol can act as a hydrogen bond donor in contrast to ethyl acetate, which contributes to a more efficient charged-ion transfer during EE. The good electro-extraction performance of 1-octanol for acidic compounds was also observed in the publication from Hasheminasab et al. [33] and Hansen et al. [27]. Therefore, 1-octanol was adopted for the subsequent parameter optimization.

The pH of sample and acceptor phase, applied voltage, and extraction time are three critical parameters of electro-extraction and were studied thoroughly. The quadratic models depending on these three parameters for each compound in 1-octanol are visualized in Fig. 3. The optimal EF of the model compounds was obtained at a pH of 9.7, extraction voltage of 310 V, and extraction time of 115 s. A higher pH in aqueous samples improves the charge state of the acidic model compounds and increases the solubility of the acidic compounds in the acceptor phase [49,50]. However, the EF of model compounds decreased when the pH was above 9.7. These findings were also reported for similar drugs at pH above 11.5 [51] and other acidic analytes at pH above 11 [34]. The declining trend in the enrichment factor at a pH above the optimum may be due to the lower electric field, increased electrolysis during extraction, and a too high ion balance (χ in Equation (3)) induced by the high anion concentration in the sample solution at these conditions [32,33].

A higher extraction voltage contributes to faster migration of the charged model compounds during the electro extraction and improves the extraction efficiency. However, for voltages exceeding

Table 2
p-values and R^2 of response surface quadratic models for EF of model compounds in 1-octanol and ethyl acetate.

Source	1-octanol				Ethyl acetate				
	Naproxen	Fenoprofen	Flurbiprofen	Ibuprofen	Naproxen	Fenoprofen	Flurbiprofen	Ibuprofen	
Model	<0.0001	<0.0001	0.0004	0.0002	<0.0001	0.0158	0.0206	0.0191	Significant
A: pH	<0.0001	0.0003	0.1217	0.0011	<0.0001	0.0113	0.1656	0.0048	
B: Voltage	<0.0001	0.0003	0.0022	0.0056	<0.0001	0.3895	0.6718	0.5914	
C: Time	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0052	0.0065	0.0095	
AB	0.1113	0.1398	0.1965	0.2945	0.0600	0.4454	0.4455	0.4946	
AC	0.0004	0.7105	0.5430	0.0657	0.0004	0.0210	0.0280	0.0302	
BC	0.0044	0.9779	0.6058	0.8506	0.0003	0.9322	0.6454	0.4269	
A²	<0.0001	<0.0001	0.0015	0.0005	<0.0001	0.7933	0.0883	0.2121	Not significant
B²	<0.0001	0.0006	0.0065	0.0047	0.3590	0.8512	0.9318	0.1903	
C²	0.0027	0.0050	0.0189	0.0038	<0.0001	0.0082	0.0051	0.0408	
Lack of Fit	0.9649	0.0758	0.0843	0.1604	0.1821	0.1035	0.1992	0.6335	
R²	0.9964	0.9834	0.9594	0.9674	0.9940	0.8802	0.8695	0.8727	
Adjusted R²	0.9917	0.9621	0.9072	0.9254	0.9862	0.7261	0.7016	0.7091	

Table 3
The EF $\pm$ standard error (ER, %) of model compounds under optimized conditions (n = 3).

	Naproxen	Fenoprofen	Flurbiprofen	Ibuprofen
1-octanol	69.85 $\pm$ 5.51 (13.97%)	189.63 $\pm$ 14.87 (37.93%)	159.53 $\pm$ 16.79 (31.91%)	69.88 $\pm$ 7.53 (13.98%)
Ethyl acetate	38.19 $\pm$ 4.34 (7.64%)	150.74 $\pm$ 13.37 (30.15%)	26.97 $\pm$ 3.98 (5.39%)	22.16 $\pm$ 2.67 (4.43%)

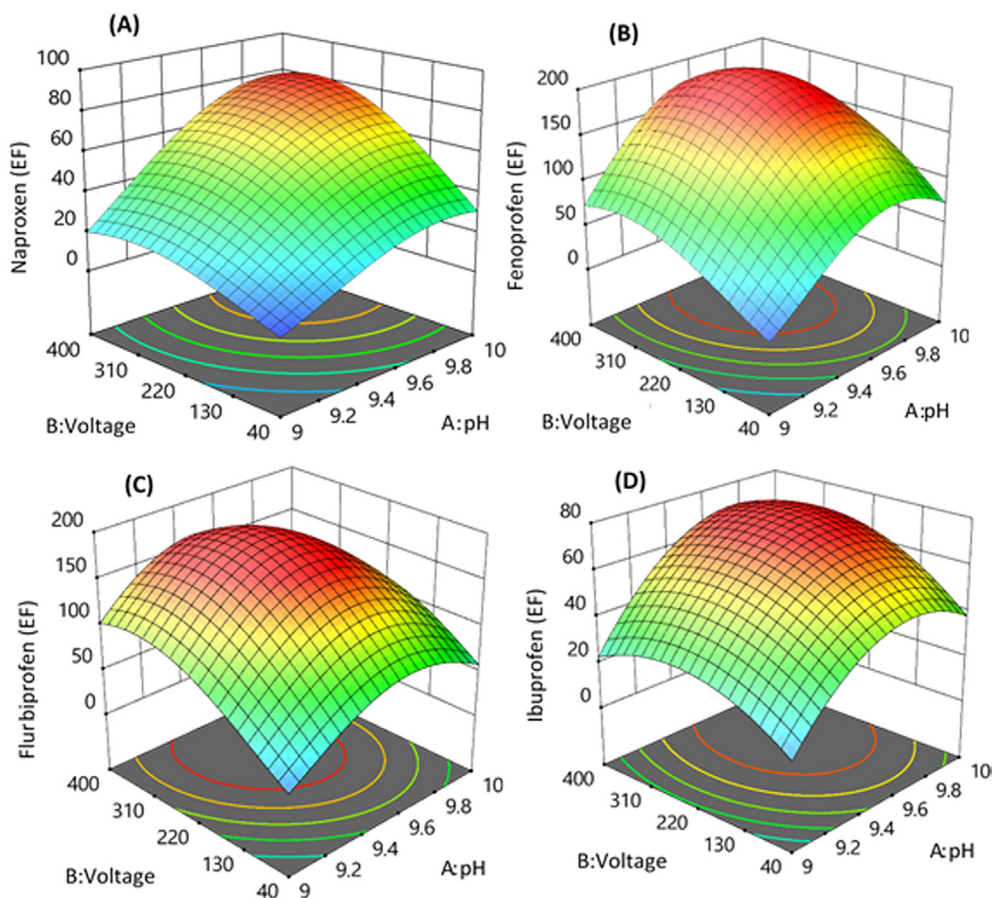


Fig. 3. Surface profiles of the developed Quadratic models for naproxen (A), fenoprofen (B), flurbiprofen (C), and ibuprofen (D) as function of pH in sample and acceptor phase (x-axis) and extraction voltage (y-axis) at the optimum extraction time of 115 s.

310 V, the EF of model compounds decreased. Similar results were also observed by Hasheminasab et al. [33] and Hansen et al. [27] when the extraction voltage exceeded the optimal value. The EF

also showed a declining trend if the extraction was continued after reaching the optimal extraction time; 115 s. These declining EF trends in EE optimization might be due to excessive electrolysis

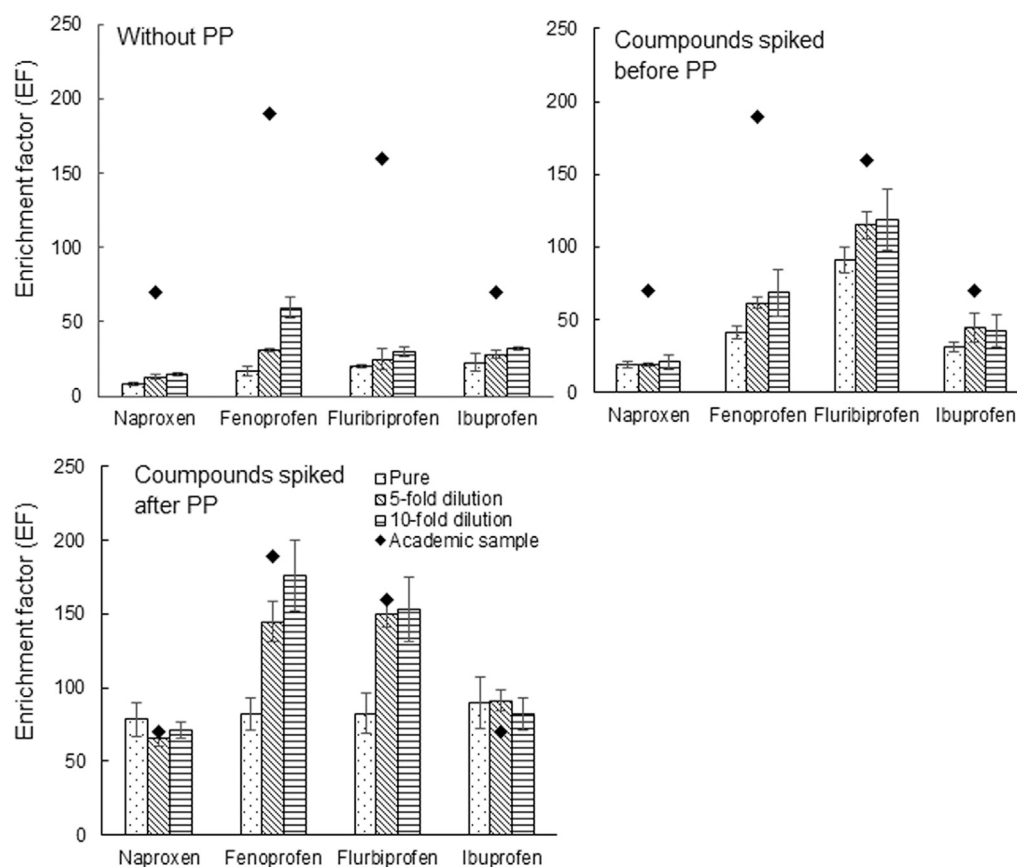


Fig. 4. The EF of the model compounds spiked in pure, 5-fold and 10-fold diluted plasma samples before, after, and without protein precipitation ($n = 3$).

Table 4

Calibration curve and precision (RSD) of the model compounds in plasma samples by using the optimum three-phase EE method ($n = 3$).

	Linear range (ng mL ⁻¹)	Linearity (R ²)	LODs (ng mL ⁻¹) (S/N = 3)	LOQs (ng mL ⁻¹) (S/N = 10)	Accuracy (%) (50 ng mL ⁻¹)	RSD (50 ng mL ⁻¹)	
						Intraday	Interday
Naproxen	25–1000	0.9920	2.97	9.90	92	11.6%	10.5%
Fenoprofen	10–1000	0.9916	1.28	4.27	77	10.3%	18.1%
Flurbiprofen	10–1000	0.9928	1.05	3.50	90	21.4%	21.1%
Ibuprofen	25–1000	0.9923	5.88	19.61	101	11.6%	16.0%

[33,37,52,53]. The acceptor phase acts as the anode during three-phase EE and excessive electrolysis in the anode decreases the pH of the acceptor phase, leading to a shift in charge state equilibrium of the model compounds. This reduces their polarity and increases back extraction into the organic phase through a passive liquid-liquid extraction mechanism, which has been reported in numerous publications [21,25,28,29,33,37]. This was consistent with the theoretical model of analyte flux (J_i in Equation (3)), which states that there is an antagonistic effect between extraction time and voltage [46].

At the optimum settings, the extraction time of 115 s was faster, and the EF (70–190) was higher, for the four model compounds than reported in other electro-driven extraction studies [27,32–36,54], despite the relatively low ER values (14%–38%, Table 3). In comparison, Balchen et al. achieved an EF of 2.5–10 for naproxen, fenoprofen, flurbiprofen, and ibuprofen under 5 min extraction [37], and Payán et al. reported an EF of 32 and 38 for respectively naproxen and ibuprofen with 10 min extraction time [36]. The high EF of naproxen (188) and ibuprofen (180) in Hasheminasab et al. might be due to the application of carbon

nanotubes in their support liquid membrane, which increased the analyte partition coefficient in the membrane [33]. In this study the higher EF with shorter extraction time could be attributed to the combination of higher extraction voltage, smaller volume of the acceptor droplet, and the high surface-area-to-volume ratio of the near-spherical acceptor droplet [28]. The total time for the electro-extraction procedure and fast LC-MS detection is 5–6 min, including the addition of the sample and organic solvent, forming, aspirating, and injecting the acceptor droplet, fast LC separation, and MS detection.

3.3. Application and performance evaluation of the three-phase EE

To further investigate the applicability of the optimal three-phase EE method for biological samples, sub-therapeutic concentrations (50 ng mL⁻¹) of model compounds were spiked into pure, 5-fold, and 10-fold diluted human plasma samples without protein precipitation (pH 9.7) [55–58]. The EF for samples without PP notably ($P < 0.05$) increased with dilution (Fig. 4A) and was dramatically lower compared to the academic samples (Table 3). A

plausible cause is that proteins from the plasma are present at the interface between sample and organic layer, and effectively form a barrier for the analytes to migrate.

The same electro-extraction method was applied to plasma samples with compounds spiking before protein precipitation to increase the performance. The significantly larger EF of compounds spiking before PP (Fig. 4B) than that without PP demonstrates that the proteins in plasma samples hamper the migration of analytes in the electro-extraction thus lowering the EF in samples without PP. However, the notably lower EF than that achieved in academic samples was still observed in plasma samples with compounds spiking before PP. To further investigate whether this lower EF arises from the compound loss during protein precipitation process or from the matrix effects during analyte's extraction process, the electro-extraction method was applied to plasma samples with compounds spiking after protein precipitation. The compared EF with academic samples in diluted plasma samples (Fig. 4C) demonstrates that compound loss occurred during protein precipitation process, and the negligible matrix effects during the three-phase EE procedure and the ion suppression in the MS source in plasma samples with compounds spiking after PP.

Finally, the method was characterized for response function, repeatability, sensitivity, limits of detection (LODs), and limits of quantification (LOQs) using the optimum extraction conditions that yielded the highest enrichment factor, *i.e.*, 10-fold diluted plasma with compounds spiking before protein precipitation, to evaluate the performance of develop three-phase EE method. All model compounds exhibited linear response ($R^2 > 0.99$) within the concentration range of 10–1000 ng mL⁻¹ (Table 4). The LODs and LOQs are in the range of 1.05–5.88 ng mL⁻¹ and 3.50–19.61 ng mL⁻¹, respectively. The accuracy was between 77 and 101%, and intra- and inter-day relative standard deviation (RSD) was below 25% for all compounds. These results demonstrate that this three-phase electro-extraction method is both stable and repeatable for bioanalysis of plasma.

4. Conclusion

An online three-phase EE method for acidic analytes was developed and optimized by using a Box–Behnken design methodology for four model compounds, *i.e.* naproxen, fenoprofen, flurbiprofen, and ibuprofen. The optimum extraction time was less than 2 min with EFs ranging from 70 to 190. The optimized three-phase EE method was successfully applied to human plasma samples, achieving LODs down to 1.05 ng mL⁻¹ from an equivalent of 15 µL plasma with a linear response function of $R^2 > 0.99$. We demonstrated that proteins in the plasma affect the electro-extraction and the enrichment factors can be significantly increased with a simple protein precipitation step. Additionally, a fast C18 LC separation reduced ion suppression in the MS and further improved the analysis.

In summary, a fast, simple, and online three-phase EE method coupled to fast LC-MS for acidic analytes was presented in this study. For future development, we envision that this method can be integrated with our previous electroextraction method for basic analytes to further improve the range of analytes the method can be applied to, and thoroughly optimize the integrated method by decreasing the ion balance, and simultaneously optimizing the types and thickness of the liquid membrane, extraction time, and voltage. As in our previous work, the method can be fully automated to meet the requirements for high-throughput analysis. We believe that this technique can further advance the solutions for sample-preparation bottlenecks in high-throughput bioanalysis workflows.

CRedit authorship contribution statement

Yupeng He: Investigation, Conceptualization, Formal analysis, Writing – original draft. **Nicolas Drouin:** Conceptualization, Investigation, Writing – original draft. **Bert Wouters:** Conceptualization, Writing – original draft. **Paul Miggiels:** Conceptualization, Writing – original draft. **Thomas Hankemeier:** Conceptualization, Supervision. **Peter 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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