High-throughput profiling of small molecules using mass spectrometry

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Chapter 4

Three-phase Electroextraction: a new (online) sample purification and enrichment method for bioanalysis

ABSTRACT

The migration, and at the same time enrichment of analytes from a liquid aqueous sample donor phase, through an immiscible organic solvent layer acting as a filter phase, into a liquid aqueous acceptor phase is enabled by the application of an electric field between the donor and acceptor phase. The organic filter phase acts as a purification filter which prevents e.g. proteins from migrating into the acceptor phase. Moreover, the composition of the organic filter phase influences the selectivity of the extraction. We show that analytes can be rapidly enriched from a 50 µL donor phase at the bottom of a sample vial, via an immiscible organic filter phase, into a 2 µL acceptor phase which consists of a droplet that is hanging from a (conductive) pipette tip in the organic filter phase. Acylcarnitines spiked to human plasma as a donor phase were extracted reproducibly with good linearity and a 10-fold improved limit of detection and, importantly, resulted in a stable, protein-free nanoelectrospray signal. Finally, a proof of principle towards the on-line integration in an automated nanoelectrospray-Direct Infusion-Mass Spectrometry platform has been realized. This makes 3-phase electroextraction (3-phase EE) a novel sample purification and enrichment method, with straightforward on-line integration possibility. We envision that 3-phase EE will enable new possibilities using electrokinetic sample pretreatment for fully automated, high-throughput bioanalysis purposes.

Based on

R.J. Raterink, P. W. Lindenburg, R. J. Vreeken, and T. Hankemeier, *"Three-phase electroextraction: a new (online) sample purification and enrichment method for bioanalysis", Analytical Chemistry, vol. 85, no. 16, pp. 7762–8, 2013.*

Cover article.

INTRODUCTION

In the last decades the demand for rapid and accurate bioanalysis has increased. Effective sample preparation is an important step in the chemical analysis of biological samples. The main purposes of sample preparation are to (1) transport the analytes to a medium compatible with the analytical instrument, (2) remove interfering matrix components and (3) concentrate the analytes, i.e. increase the concentration in the analysis solution compared to the concentration in the sample. Commonly used sample preparation methods include protein precipitation, liquid-liquid extraction and solid phase extraction[1]. For special applications special modifications of these approaches have been developed, such as e.g. single droplet microextraction (SDME) in where a hanging immiscible organic droplet is inserted in an aqueous sample in order to perform liquid-liquid extractions[2]. Although not often used yet, electrically driven sample pretreatment methods are increasingly reported such as electromembrane extraction (EME), electroextraction (EE)[3] and online preconcentration techniques in capillary electrophoresis[4]. The latter, however, are not further considered in this article as for complex biosamples additional prior steps such as removal of plasma proteins is necessary. Sample preparation procedures based on electromigration are very promising, since a considerable part of the analytes in bioanalysis include compounds that are or can be charged (e.g. metabolites, xenobiotics and drugs) and thus are suitable for an electrophoretic approach. A recent three-phase elektrokinetic sample preparation method is EME[5]–[10]. In this technique, an organic solvent is held by capillary forces in the pores of a polymeric membrane. This membrane acts as a phase boundary between the aqueous donor and acceptor solutions. The analyte of interest is extracted from the aqueous donor phase on one side of the supported liquid membrane, through the organic membrane into the aqueous acceptor phase on the other side of the supported liquid membrane. This promising method has been successfully applied to the analysis of several pharmaceuticals in plasma and urine and also low-abundant peptides in human plasma[11], [12]. Another rapid electromigration-driven technique is 2-phase EE, which takes place in a two phase liquid-liquid system consisting of an aqueous and an organic phase, where an electric field causes the analytes to be extracted from the organic donor phase into the aqueous acceptor phase[13]–[15]. Ions in the organic phase are subjected to very high electric field strength due to low conductivity. As a consequence, ions in the organic phase will migrate at high velocity, to be concentrated just after the liquid-liquid interface since the electric field of the aqueous acceptor is much lower. 2-phase EE has been successfully applied to several endogenous metabolites like peptides and carnitines and also to standard solutions of leukotriens and catecholamines[16], [17].

Direct infusion-mass spectrometry (DI-MS) is a rapid, comprehensive analytical method without the use of chromatography prior to MS. By using nanoelectrospray (nanoESI) instead of conventional ESI, ionization efficiency is enhanced, only a small amount (sub)-µL of sample is needed and ion suppression is reduced or even eliminated[18]. However, nanoESI emitters (with internal diameters of typically a few µm) are susceptible to clogging due to protein precipitation, salt crystallization and dust/impurities which drives for an efficient sample preparation prior to analysis. Moreover, when a selective sample preparation procedure is chosen, ion suppression could be further reduced, since the enriched samples may contain less molecules when co-introduced in the MS. Additionally, coupling electrokinetic driven sample preparation techniques to nanoESI would be very suitable, since cations or anions are selectively migrating.

In this research paper we present 3-phase EE which enables fast migration of analytes from an aqueous donor phase through an immiscible organic filter phase into an aqueous acceptor

phase by applying an electric field between donor and acceptor, without the use of a membrane like in EME. Moreover, in this system the analytes do not have to be dissolved and diluted in the organic phase prior to extraction, opposed to 2-phase EE. By locating the acceptor phase in a conductive pipette tip, a convenient implementation of the whole sample preparation procedure is realized, enabling easy integration with downstream applications. Extraction is realized by dispensing a hanging acceptor droplet out of the conductive pipette tip into the immiscible organic filter phase and applying an electric field between both aqueous donor and acceptor phases (see Figure 1A). Compared to SDME our method is stagnant, but moreover the driving force is an applied electrical field rather than a distribution coefficient, and by that, a more complete extraction can be achieved into such a small volume. The organic phase placed on top of the donor phase prevents proteins being transported into the acceptor phase and furthermore, by changing its composition, selectivity of the extraction can be tuned. In this way it is possible to extract analytes from a relatively large donor volume into a small droplet, since only (sub)-µL volumes are needed for performing nanoESI. In this paper, we introduce and characterize the 3-phase EE process by studying the influence of extraction time and voltage, organic phase selectivity and protein elimination, and we demonstrate its potential in combination with mass spectrometry-based bioanalysis by the extraction of acylcarnitines spiked to plasma.

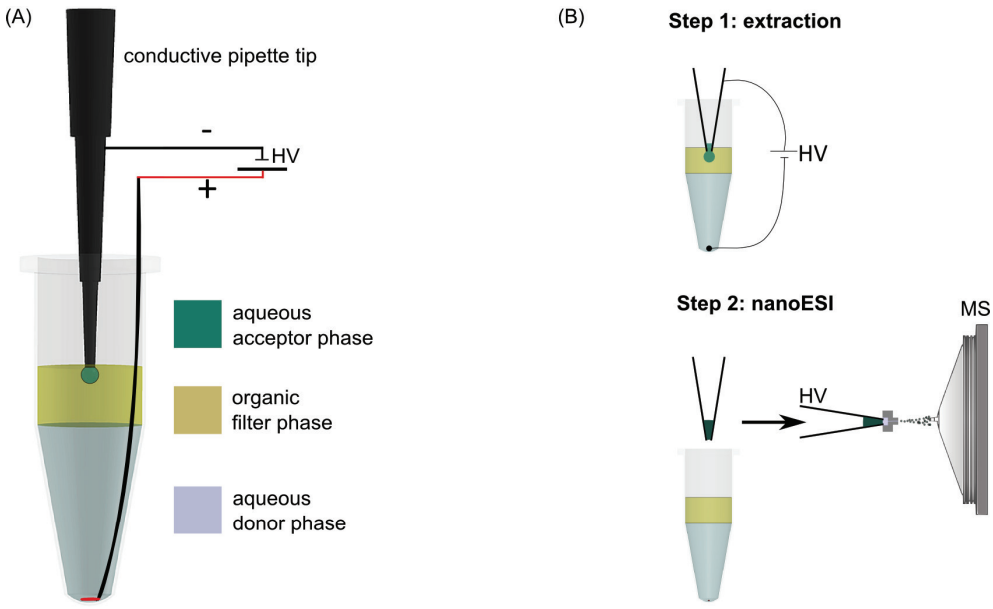

Figure 1: Schematic illustration of (A) the 3-phase EE setup and (B) 3-phase EE followed by nanoESI-DI-MS analysis

EXPERIMENTAL SECTION

Chemicals

Methanol was from Biosolve (Valkenswaard, The Netherlands). Water (dH_2O) was obtained from a Millipore high purity water dispenser (Billerica, USA). Ethylacetate (EtoAc), methylacetate (MEtoAc), bis(2-ethylhexyl)phosphate (DEHP), crystal violet, bovine serum albumine (BSA), cytochrome C (Cyt C), formic acid (FA), L-carnitine, acetyl-l-carnitine, propionyl-L-

carnitine DL-hexanoylcarnitine, DL-octanoylcarnitine, DL-decanoylcarnitine were obtained from Sigma-Aldrich (Steinheim, Germany). Deuterated butyryl-L-carnitine-d_3 was obtained from CDN Isotopes (Pointe-Claire, Canada) and was used as an internal standard. Butyryl-L-carnitine was bought from Larodan AB (Malmö, Sweden). All solvents were HPLC grade. Human plasma (EDTA) was obtained from healthy volunteers and combined in a plasma pool.

3-phase EE

All 3-phase EE results were obtained using an off-line setup (except for the experiments as described in the last paragraph). The equipment used for the off-line 3-phase EE is illustrated in Figure 1A. The DC power supply used had a voltage range from 0-2 kV, delivering a current of maximum 0.5 mA measured with a Keithley 485 picoammeter. A platinum wire (diameter = 0.28 mm) isolated with a Teflon sleeve was stripped from the end (1 mm) resulting in the electrode at the bottom of the donor phase. The pipette tip made from a conductive polymer (Advion, Ithaca, USA) was used as the counter electrode. A 10 µL syringe was connected to the pipette tip in order to aspire and dispense the acceptor phase. Off-line 3-phase EE was performed according to the following procedures; first the platinum electrode was inserted into a 500 µL eppendorf tube. The donor phase was pipetted (50 µL) into the eppendorf tube, followed by 150 µL of the immiscible organic filter phase as a layer above. The organic phase was saturated with dH_2O in order to conduct ions and to prevent the acceptor droplet from dissolving. The pipette tip, filled with 2.5 µL aqueous acceptor phase, was inserted into the immiscible organic filter phase after which 2 µL was dispensed so that a hanging droplet at the tip was created and a voltage (typically 140V) was applied for 3 minutes (or stated otherwise). After 3-phase EE, the voltage was switched off and the 2 µL acceptor droplet was aspired back into the pipette tip and transferred for analysis (Figure 1B). The organic filter phase used was a composition of EtoAC:MEtoAC (3:2) or stated otherwise in the selectivity experiments. Using this composition, the organic filter phase was immiscible with the donor and acceptor phase. In all experiments the donor phase consisted of 33% methanol (to decrease conductivity, thus increase the electric field strength) and in case of the plasma experiments also 5% FA was added to reduce plasma protein binding of the model analytes. The acceptor phase consisted of 33% methanol (to enhance ionization efficiency in ESI) and 5% FA (to anticipate on possible pH drop due to electrochemical reactions). Moreover, using these aqueous compositions, the essential condition of immiscibility with the organic filter phase was still maintained. The internal standard was spiked to the acceptor phase prior to 3-phase EE.

Mass Spectrometry

The 3-phase electroextraction analyses were performed by DI-MS in the positive mode using an automated nanoESI (Advion Triversa NanoMate, Ithaca, USA) source coupled to a LTQ-Orbitrap XL (Thermo Fisher Scientific). In the off-line setup, the 2 µL acceptor was pipetted into Eppendorf 384 well plate in order to be infused with a back pressure of 0.25 psi and an electrospray voltage of 1.55 kV in the positive mode.

The inlet capillary temperature was 120 °C, the capillary voltage and the tube lens voltage was 30 and 100 V, respectively. Mass spectra were recorded at a resolution of 30,000 and 10-20 scans were averaged in order to further process the data.

RESULTS AND DISCUSSION

The general operation of 3-phase EE is illustrated in Figure 1A. The sample (50 µL) was pip-

peted into an Eppendorf tube and 150 µL of the immiscible organic filter phase was placed on top as a second layer. For a visual proof of concept of the general operation, an experiment was conducted using a 10 µM crystal violet (cationic dye) standard in the donor phase. The result before and after 3-phase EE is shown in Figure 2. After 3 minutes the donor phase appeared depleted of crystal violet, whereas the acceptor droplet was enriched with crystal violet (see Movie 1, Supporting Information). In addition the movie shows that in the acceptor droplet the extracted crystal violet appeared to distribute homogeneously within seconds caused by diffusion and convection: this shows that a possible problem due to improper mixing of analytes and internal standards spiked to the acceptor phase prior to 3-phase EE does not occur.

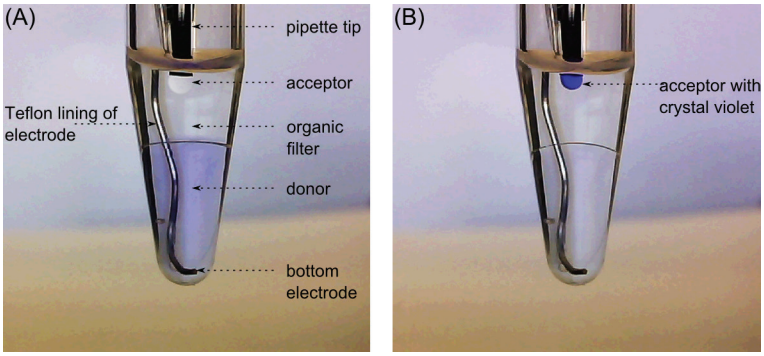

Figure 2: Video stills of crystal violet subjected to 3-phase EE at (A) t=0, no voltage applied, (B) t=3 minutes after applying the voltage.

Two processes play an important role in 3-phase EE: (1) The driving force in 3-phase EE is the applied electrical field between the donor and acceptor phase. Therefore, to enable 3-phase EE, the whole system comprising the donor, organic filter and acceptor phase should serve as an electrical circuit. Represented in a simplified manner, the electrical circuit model consists of three serial resistors representing the three phases. Since the resistance associated with the organic phase is dominant, it limits the current through the system. In order to fine-tune the system, the conductivity of the phases can be altered by changing the composition/polarity. The donor phase consisted therefore of 33% methanol: the addition of methanol decreased its conductivity and thus increased its electric field and the analyte velocity in the donor phase during 3-phase EE.

Initially, during 3-phase EE, ions migrate from the donor phase towards the organic filter interface at a velocity imposed by their electrophoretic mobility (μ) and the electric field strength (E) in the donor phase[19]–[21]. Once arrived in the organic filter phase, ions are subjected to a much higher electric field strength due to the low conductivity. As a consequence, ions in the organic phase migrate at a higher velocity and concentrate just after the organic filter-acceptor interface, as the electric field strength in the aqueous acceptor phase is much lower. A practical limitation of the applied electric field strength is the stability of the hanging droplet. In our experiments at voltages higher than 250 V the droplet is pulled off due to electrostatic force. Therefore we used 140 V to obtain reproducible results, to be sure of not losing the droplet even at extraction times up to 10 minutes.

(2) The distribution of the analytes based on the partition coefficient K_1 at the donor-organic filter interface plays an important role in 3-phase EE as well. Due to the low electric field strength in the donor phase the ions will arrive at the interface at moderate velocity, therefore K_1 determines the transport rate of the ions from the donor into the organic filter phase. In

addition, in our setup the total migration length (thus migration time) in the donor is only a few millimeters therefore further minimizes the influence of electrophoretic mobility on the transport into the organic filter phase. The partition coefficient K_2 at the organic filter-acceptor interface does only play a role when the analytes are rather apolar since ions are crossing this interface at high velocity due the high electric field strength in the organic filter phase. As a consequence for the rather polar analytes K_1 and the rather apolar analytes K_2 causes the selectivity of the system, which is elaborated in the next paragraphs.
Electrochemical reactions of analytes could occur at the surfaces of both electrodes. In all the experiments no significant gas formation was observed with the extraction voltage used, except during the longest extraction times of 10 minutes. This was tackled by aspiring the formed gas bubble into the pipette tip, but was not required at the conditions finally chosen.

Calculation of Enrichment.
The enrichment factor EF of 3-phase EE was calculated according to equation 1 for each analyte:

$$EF = \frac{[acceptor]_{normalized\ intensity}}{[donor]_{beforeEE,normalized\ intensity}} \quad (1)$$

where the normalized intensity was obtained by dividing the absolute intensity of the extracted analyte by the absolute intensity of the internal standard spiked to the acceptor phase prior to 3-phase EE. The normalized response of the donor phase was obtained by spiking that phase with internal standard and infuse it without the 3-phase EE step. To justify these calculations, the absolute intensities of the internal standard were checked; they did not significantly change during experiments because of possible electrochemical degradation or diffusion into the organic filter phase. For a good comparison, the donor was analyzed under the same conditions as the acceptor.

Effect of extraction time and voltage on enrichment
Seven carnitines with increasing size (and decreasing electrophoretic mobility[22]) and decreasing polarity were selected as model analytes (Supporting Table 1). Using these model analytes the dominant influence of the polarity of the analytes and thus the partition coefficient K_1 and K_2, in 3-phase EE could be demonstrated. In these experiments, the enrichment factor of the model analytes was studied as a function of 3-phase EE time and voltage. Enrichment in this setup was achieved by extracting the analytes from 50 µL donor to a 2 µL acceptor droplet. As a consequence the theoretical maximum enrichment factor was 25. The carnitines were always charged, since they are quaternary ammonium compounds. The acceptor phase was spiked with 1 µM butyrylcarnitine D_3 which was used as an internal standard for normalization. Experiments were performed with extraction times of 0.5, 1, 2, 3, 5 and 10 minutes at a voltage of 70 (Figure 3A) and 140 V (Figure 3B).
Applying an electrical potential difference of 140 V, several analytes were enriched close to the maximum enrichment factor, already within 3 minutes. In order to explore the depletion of the donor phase, three subsequent extractions were performed on the same donor and organic phase, refreshing only the acceptor phase after each extraction. These results show that decanoyl and octanoylcarnitine were hardly present after the first extraction indicating that for these analytes the donor was almost depleted. Another control experiment was conducted for transport based on 3 minutes of passive diffusion only, without applying the electrical potential difference. In this case none of the model analytes were detected. This proves that the

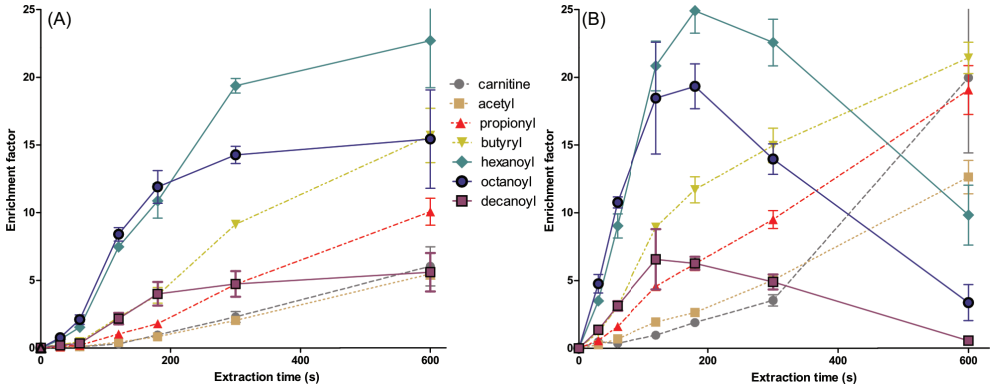

Figure 3: Average enrichment factor (n=3) versus extraction time of the 7 model analytes applying (A) 70 V and (B) 140 V.

transport speed from donor to acceptor phase was improved dramatically upon application of the voltage.

During the first 3 minutes the extraction rate (Δenrichment factor/Δextraction time) was higher at 140 V than when applying 70 V (Figure 3) which can be explained by the difference in the driving electrical field strength. In the first 3 minutes also the influence of both K_1 and K_2 is revealed: the most polar acylcarnitines (carnitine to hexanoylcarnitine) are extracted with an increased extraction rate as the polarity of the analyte increases, imposed by K_1. These results confirm that the partition coefficient K_1 across the aqueous-organic filter interface is the limiting factor for this settings, since hexanoyl-and octanoylcarntine were extracted faster than the smaller carnitines, while their electrophoretic mobilities are lower. For the most apolar carnitine (decanoylcarnitine) the extraction rate decreased with an increase in apolarity, which is caused by the influence of K_2, which becomes important for more apolar compounds. After 3 minutes the three most apolar carnitines started to decrease over time. This decrease was caused by back extraction from the acceptor phase into the organic filter phase, imposed by K_2. This is explained by the fact that the electrical field density is not homogeneously distributed around the acceptor droplet being significantly less dense at the top surfaces of the acceptor droplet (see finite element simulation, Supporting Figure 1). Analytes could reach the top of the acceptor droplet by diffusion and/or possible convection. To test this hypothesis, two follow-up experiments were performed. First we spiked 10 µM of the carnitines to the acceptor phase and using a blank donor when no electrical field was applied, more than 95% of the three most apolar carnitines were diffused into the organic filter phase after 10 minutes of extraction, solely caused by partition coefficient K_2. Secondly this experiment was repeated, but an electrical field was applied. Now the three most apolar carnitines were back extracted into the organic filter phase to about 50% of their total amount, which is comparable with the actual decrease we observed during 3-phase EE after 10 minutes of extraction. Other possible causes for the decrease such as electrochemical degradation and adhesion to the pipette tip were excluded (data not shown).

These characterization results indicate that for each application with its associated target analytes an optimal organic filter phase should be chosen (see next paragraph). Probably, possible back-extraction can be reduced by increasing the surface of the bottom electrode of the well and optimizing the geometry of the setup.

Influence of the organic filter phase composition on enrichment

To further show the influence of the partition coefficient K_1 for 3-phase EE, experiments with different organic filter phase compositions were conducted and the enrichment factor was calculated. By mixing organic solvents and by adding an ion pair modifier, the polarity of the organic filter phase, therefore K_1 and K_2, can be changed to tune selectivity of 3-phase EE. By adding an ion pair modifier (e.g. DEHP), the organic filter phase becomes more accessible for ions and therefore making K_1 more favorable towards polar analytes. Organic filter phases with increasing polarity were tested: EtoAC, EtoAC:MEtoAC (3:2), EtoAC+1% DEHP and EtoAC+5% DEHP. The acceptor phase was spiked with 1 µM butyrylcarnitine d_3 as an internal standard for normalization purposes. Figure 4 shows that by increasing the polarity of the organic filter phase, the optimum analyte apolarity shifts to lower values while seemingly narrowing the polarity window, and hence increasing selectivity. It can be observed that by using pure EtoAC the more apolar carnitines were most enriched, while transport of the smallest polar analytes was (almost) disabled at the same time due to the partition coefficient K_1. In contrast, by adding 5% of the modifier DEHP, the extraction was more selective towards the polar carnitines and the largest (apolar) decanoylcarnitine was hardly enriched. These findings lead to the important conclusion that selectivity in 3-phase EE can be tuned. As a consequence, the selection of the composition of the organic filter phase should be tuned according the analytes of interest. These observations are in agreement with applications of EME which reported that the chemical composition of the membrane influenced selectivity[3].

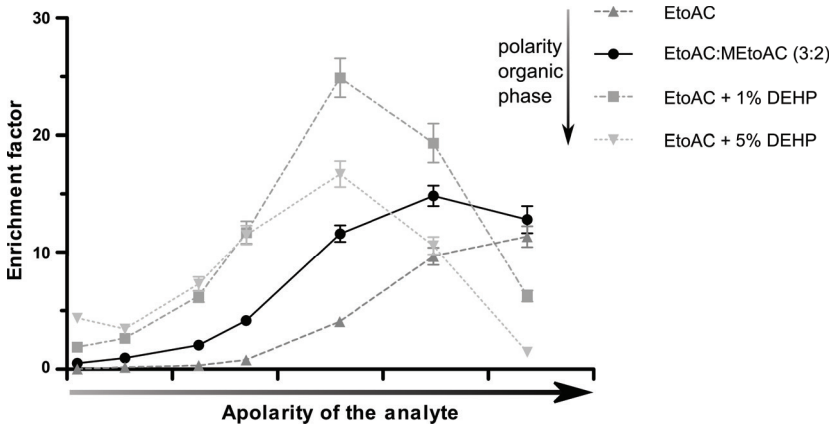

Figure 4: Average enrichment factor versus apolarity (expressed as log P) of the analyte and the influence of the organic filter phase ((n = 3), all RSD<15%).

Sample purification

In the following experiments an academic test solution (500 µg/mL) of a large model protein BSA (MW = 66.5 kDa) and and a smaller model protein Cyt C (MW = 12 kDa) was tested. BSA was chosen because albumine is the most predominant (55%) blood plasma protein[23] and Cyt C was added to assess the behavior of small proteins in the system. The protein standard solutions were subjected to 3-phase EE-DI-MS and was compared to DI-MS of the donor phase before 3-phase EE to which 5% FA added. Supporting Figure 2A shows that the MS spectrum of the BSA standard consists of a broad BSA envelope centered around 1500 *m/z*, obtained for the analysis of the donor phase which is not observed with 3-phase EE-DI-MS (Supporting Figure 2B). Supporting Figure 2C shows the MS spectrum of the Cyt C

standard consists of two smaller envelopes centered around 1537 *m/z* and 1752 *m/z*, opposed to the MS spectrum of the 3-phase EE extract (Supporting Figure 2D). These results show that the large BSA as well as the much smaller Cyt C are not transported into the acceptor phase during 3-phase EE. These results confirm the same selectivity principle (partition coefficient K_1) discussed in the previous paragraph: since the proteins do not dissolve (precipitation) in the used organic filter phase, they did not pass the donor-organic filter interface during 3-phase EE. Additionally, in the next paragraph the same mass range was scanned for the presence of proteins in spiked plasma. In these experiments protein precipitation was observed between the donor and organic filter phase.

Spiked plasma

To finish the evaluation of 3-phase EE, the acylcarnitines were spiked into samples of a pool of human blood plasma. By spiking the carnitines the plasma was diluted 10-fold in 33% methanol and 5% FA (pH=2.0), in order to make a valid comparison with the academic test mixture experiments. After dilution no protein precipitation was observed at this level of MeOH and FA. Subsequently the samples were subjected to 3-phase EE. The model analytes were effectively extracted from human plasma (Table 1). These results show that 3-phase EE can be used for the enrichment of complex samples. For all model analytes slightly lower enrichment values were obtained from human plasma samples compared with those of the academic test solution (Figure 4). This can possibly be explained by the plasma protein binding of the model analytes. Marzo *et al.* reported that carnitine and its short-chain esters did not interact with plasma proteins while octanoylcarnitine interacted to a poor extent (12-30%), and the larger carnitines were completely bound to plasma proteins[24]. Acidification of the donor phase with 5% FA appeared to be necessary, as without little enrichment was observed: upon acidification proteins are protonated, resulting in less binding of the model analytes. Since the plasma samples had high viscosity, high protein content and are more complex than the previously examined standard standard solutions, longer extraction times were needed to obtain higher enrichment (Figure 4). After 9 minutes of extraction, the smallest carnitines were enriched approximately 3x more (Table 1), whereas the largest carnitine did not benefit from the longer extraction time, due to the back extraction process into the organic phase as discussed earlier (Figure 3B).

Table 1: Enrichment values of the carnitines (500 nM equimolar) spiked in human plasma, (n = 3)[a],

analyte	EF_{3min}	EF_{9min}
carnitine	1.0*	2.8*
acetylcarnitine	0.7*	2.0*
propionylcarnitine	1.8	4.4
butyrylcarnitine	2.5	5.4
hexanoylcarnitine	3.9	7.0
octanoylcarnitine	5.1	6.1
decanoylcarnitine	4.7	3.1

[a]All RSD <15%; * = corrected for the endogenous level (Table 2).

As a reference the plasma donor phase spiked with 500 nM of carnitines was analyzed directly. This resulted in an unstable nanoelectrospray signal possibly caused by the abundant plasma proteins which was also visible as a broad envelope in the 1000-2000 *m/z* range, and moreover only a few model analytes could be detected close to their limit of detection. These findings demonstrated the potential of 3-phase EE as a rapid and effective sample cleanup and enrichment technique for bioanalysis.
Calibration curves were obtained by spiking the acylcarnitines (0, 10, 50, 100, 500 and 1000 nM) and 1 μM butyrylcarnitine d_3 as an internal standard to the plasma and for carnitine (0, 10, 50, 100, 500, 1000 and 5000 nM). The performance results are summarized in Table 2.

Table 2: Analytical performance of 3-phase EE-DI-MS of carnitines spiked to human plasma[a]

analyte	Linearity (R^2) 10-1000 nM	LOD donor solution (nM)	LOD blood* (nM)	Concentration blood* (μM)
carnitine	0.999	-	-	26
acetylcarnitine	0.997	-	-	0.8
propionylcarnitine	0.994	9	90	x
butyrylcarnitine	0.989	29	290	x
hexanoylcarnitine	0.987	33	33	x
octanoylcarnitine	0.995	28	280	x
decanoylcarnitine	0.992	29	290	x

[a] x< LOD; - = not possible to determine; * the donor solution was obtained by diluting 1:10, the LOD for blood and the concentration in blood was obtained by multiplying the concentrations obtained in the donor with 10. LOD was determined by $(Y_{blank} + 3SD_{blank} - intercept_{response\ curve})/slope_{response\ curve.}$

Linear behavior of 3-phase EE was observed by measuring the response of the analyte vs. their concentration over 2 orders of magnitude. The R^2-values of the linear regression line ranged from 0.987 to 0.999 for the compounds tested. Limits of detection[25] were in the nM range for most of the tested compounds and were improved 10-fold compared to the DI-MS analysis of the donor phase. The limit of detection of carnitine and acetylcarnitine could not be accurately determined, since no measurements could be done at levels close to the detection limit due to the relatively high endogenous levels[26]. In the future the overall limit of detection can easily be improved by using a larger donor volume and/or using a smaller acceptor volume, possibly at the cost of a longer extraction time. Also, future research will be directed to optimizing the organic filter phase composition to achieve a more efficient extraction. The repeatability of the 3-phase EE measurements in plasma showed RSD values below 15%, and in the future this can most likely be improved by automation.
The endogenous levels of free carnitine and acetylcarnitine were in agreement with literature[27], [28]. Interestingly, and also as expected, many other plasma components were observed in the MS spectra and by putative identification (based on exact mass), among others, several amino acids were annotated such as leucine and phenylalanine. This suggests that the used organic filter phase was also suitable for the 3-phase EE analysis of many amino acids. Future research has to be done in order to characterize 3-phase EE for nonpermanently charged compounds like amino acids and drugs.

Toward online 3-phase EE-nanoESI-DI-MS: proof of principle.
In the final experiments the first steps of the integration of 3-phase EE into a fully automated system using an automated nanoESI robot (Triversa NanoMate) were made.
A 96 polypropylene well plate was modified by removing the bottom of some of the wells and replacing it by a stainless steel plate which functioned as the anode (Supporting Figure 3). The mandrel of the NanoMate, which grabs the pipette tip and which is also electrically connected to the grabbed pipette tip, functioned as the cathode. Both electrodes were connected to a voltage source.
150 µL of the donor academic test mixture of 500 nM carnitines was pipetted in one of the wells containing a bottom electrode, followed by 250 µL of the organic filter phase on top. A sequence was programmed in the NanoMate Chipsoft software which (1) aspired 1 µL of the acceptor phase from a certain well, (2) moved the pipette tip to the extraction well in which it was positioned in the organic filter phase, (3) dispensed a 1 µL droplet and waited 3 minutes to perform the extraction, (4) subconsequently aspired the acceptor droplet back into the pipette tip and (5) performed nanoESI. The required extraction voltage was only 55 V in order to get the same extraction currents as for the off-line experiments and was manually turned on and off using the external voltage source, but in the future this can also be automated. The first proof of principle result is shown in Supporting Table 2 which revealed an about 5-fold increase of the enrichment factor, compared to the off-line results in Figure 4. This increase in the enrichment factor can be explained by the 2-fold smaller acceptor volume and the 3-fold larger donor volume. These results demonstrate the potential of 3-phase EE as an easy automated, high-throughput sample preparation method. Further research and development has to be done on improving the on-line setup into a fully automated, high-throughput screening platform.

CONCLUSIONS AND OUTLOOK

In this paper, we demonstrated the fast and selective electromigration of analytes from an aqueous sample through an immiscible organic filter phase into an aqueous acceptor phase. We demonstrated that this technique, which we described as 3-phase electroextraction (3-phase EE), can be efficiently used for sample purification in bioanalysis, since the organic phase prevents proteins from being transported into the acceptor phase. Selectivity can be tuned by proper selection of the composition of the organic filter phase, being an attractive feature of 3-phase EE. Enrichment of the analytes was demonstrated by extracting them from a 50 µL sample into a small 2 µL acceptor droplet. Using academic test solutions, several of the model compounds were enriched close to the expected enrichment maximum, already within 3 minutes. Moreover, 3-phase EE was found to be suitable for extracting and enriching spiked carnitines from human plasma samples. 3-phase EE coupled to direct infusion-MS of plasma showed a good linear response over two orders of magnitude and improved detection limits and importantly, resulted in a stable protein-free nanoelectrospray-MS signal. Since the maximum enrichment factor is proportional to the volume of the donor phase and inversely proportional to the volume of the acceptor phase, 3-phase EE could easily be used to achieve a 100-fold or more enrichment. In addition, future research will be done on optimizing the composition of the organic filter phase, on 3-phase EE geometries in order to maximize fast enrichment and on extending the type of analytes.
Finally, proof of principle of the integration of 3-phase EE in a commercially available on-line robotic nanoESI-DI-MS system was achieved. Next to coupling to DI-MS, 3-phase EE can also be integrated in other downstream (separation) applications such as LC and CE. Thanks

to its combination of sample purification, selective enrichment of analytes from biofluids to an aqueous phase and the simplicity of the setup, 3-phase EE holds the promise to become a core sample preparation module in fully automated, high-throughput bioanalysis.

ACKNOWLEDGEMENT

This work is financed by the Netherlands Metabolomics Centre (NMC) which is a part of The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research. Advion BioSciences is acknowledged for supplying software and technical knowledge needed for the on-line experiments.

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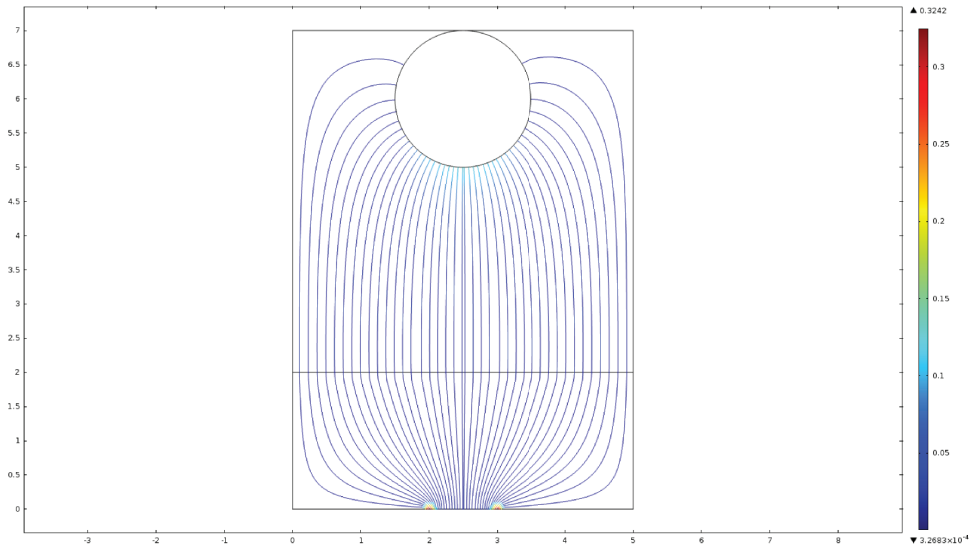
SUPPORTING INFORMATION

Supporting Table 1: Carnitines with increasing mass and increasing apolarity.

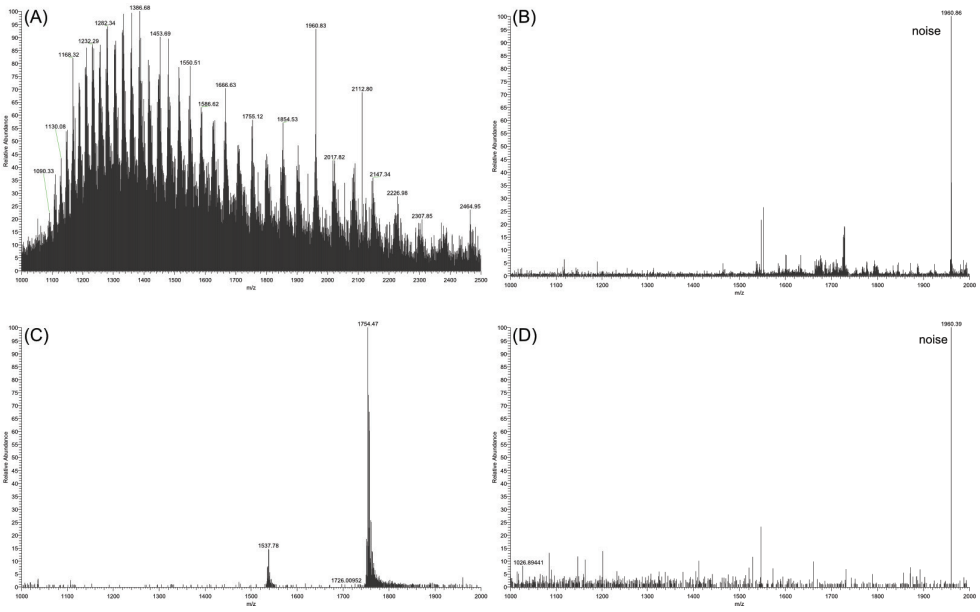
analyte	MW
carnitine	161.2
acetylcarnitine	203.2
propionylcarnitine	217.2
butyrylcarnitine	231.3
hexanoylcarnitine	259.3
octanoylcarnitine	287.4
decanoylcarnitine	315.1

Supporting Table 2: Enrichment factor of online 3-phase EE on an academic test mixture of 500 nM acylcarnitines (n = 1).

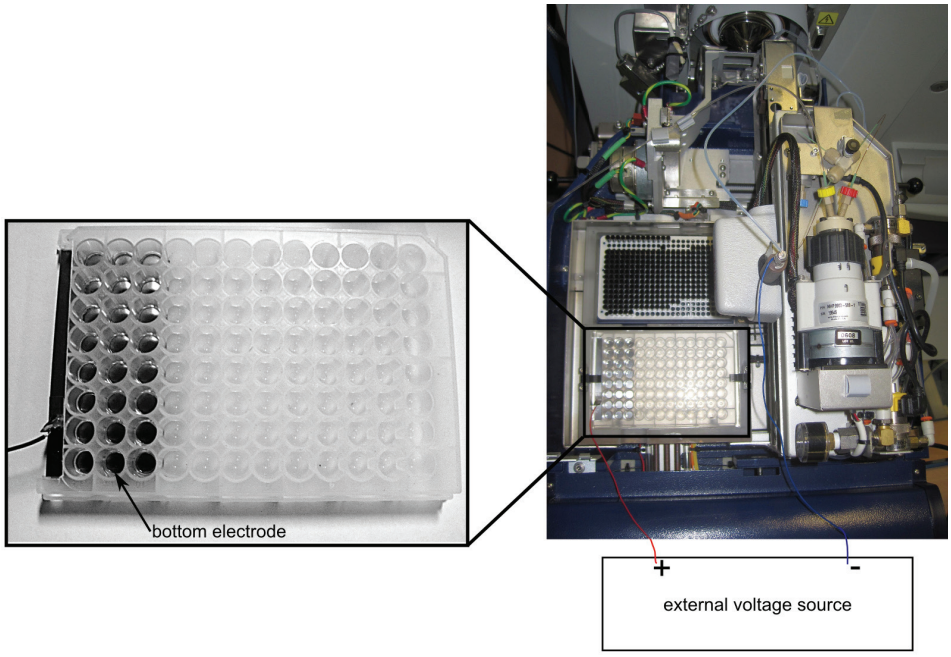
analyte	EF
carnitine	3.4
acetylcarnitine	5.1
propionylcarnitine	11.8
butyrylcarnitine	25.9
hexanoylcarnitine	70.5
octanoylcarnitine	77.3
decanoylcarnitine	81.9



Supporting Figure 1: Finite element simulation of the off-line 3-phase EE setup showing the electric field density distribution. As depicted, the electric field density is significantly lower at the top surface of the acceptor droplet, resulting in possible back-extraction processes imposed by K_2.



Supporting Figure 2: Averaged mass spectra of (A) BSA standard (500 µg/mL) (B) 3-phase EE of BSA standard (500 µg/mL) (C) Cyt C standard (500 µg/mL) (D) 3-phase EE of Cyt C standard (500 µg/mL)



Supporting Figure 3: Photo of a modified 96 polypropylene well plate with an electrode at the bottom of three well-rows integrated in the automated nanoESI robot.