New Approaches for Lab-in-a-Syringe Systems

by

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Statement of Co-authorship

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This paper constitutes Chapter One of the thesis. I. E. Mikhail (the candidate) was the first author of the review and co-wrote the paper with M. C. Breadmore, R. M. Guijt, A. A. Gooley, and M. Tehranirokh. I. E. Mikhail, M. C. Breadmore, R. M. Guijt, A. A. Gooley, and M. Tehranirokh contributed to the idea, structure, refinement, and presentation.

Paper 2. I.E. Mikhail, M. Tehranirokh, A.A. Gooley, R.M. Guijt, M.C. Breadmore, In-syringe electrokinetic ampholytes focusing coupled with electrospray ionization mass spectrometry, Analytical Chemistry 91 (2019) 8259-8266. doi:10.1021/acs.analchem.9b00942 [Research Article].

This paper constitutes Chapter Two of the thesis. The candidate was the first author and conducted all the experimental work and the data analysis. I. E. Mikhail, M. C. Breadmore, R. M. Guijt, A. A. Gooley, and M. Tehranirokh designed the experiments and co-wrote the paper. M. C. Breadmore, R. M. Guijt, A. A. Gooley, and M. Tehranirokh refined the paper.

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List of Publications and Presentations

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3. I.E. Mikhail, M. Tehranirokh, A.A. Gooley, R.M. Guijt, M.C. Breadmore, In-syringe electrokinetic ampholytes focusing coupled with electrospray ionization mass spectrometry, Analytical Chemistry 91 (2019) 8259-8266. doi:10.1021/acs.analchem.9b00942 [Research article].
4. I.E. Mikhail, M. Tehranirokh, A.A. Gooley, R.M. Guijt, M.C. Breadmore, Hyphenated sample preparation techniques for the direct analysis of biological fluids by electrospray and nano-electrospray ionization mass spectrometry [Review Article: in progress].
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6. I.E. Mikhail, M. Tehranirokh, A.A. Gooley, R.M. Guijt, M.C. Breadmore, In-syringe electrokinetic clean-up of biological samples for direct injection electrospray ionization mass spectrometry, 48th International Symposium

on High-Performance Liquid Phase Separations and Related Techniques, 16-20/06/ 2019, Milan, Italy [Oral presentation].

7. I.E. Mikhail, M. Tehranirokh, A.A. Gooley, R.M. Guijt, M.C. Breadmore, An electrokinetic lab-in-a-syringe – a new microfluidic platform for biological samples clean-up and interfacing with mass spectrometry, 10th Australia and NewZealand Nano and Microfluidics Symposium, 01-03/07/2019, University of Wollongong, Australia [Oral presentation: best oral presentation award (students' competition)/2nd place].
8. I.E. Mikhail, M. Tehranirokh, A.A. Gooley, R.M. Guijt, M.C. Breadmore, Lab in a syringe, ACROSS International Symposium on Advances in Separation Science, 30/11-02/12/2016, Hobart, Australia [Poster presentation: best poster presentation award/2nd place].
9. I.E. Mikhail, M. Tehranirokh, A.A. Gooley, R.M. Guijt, M.C. Breadmore, In-syringe electrokinetic clean-up of weakly acidic drugs in biological samples for direct injection electrospray ionization mass spectrometry, the 67th American Society for Mass Spectrometry (ASMS) Annual Conference, 02-06/06/2019, Atlanta, GA, USA [Poster presentation].

List of Abbreviations

APCI	Atmospheric pressure chemical ionization
BGE	Background electrolyte
BSA	Bovine serum albumin
CAs	Carrier ampholytes
CBS	Coated blade spray
CE	Capillary electrophoresis
CID	Collision induced dissociation
CIEF	Capillary isoelectric focusing
C_{max}	Peak plasma concentration
CNT	Carbon-nanotube
CSF	Cerebrospinal fluid
DART	Direct analysis in real time
DBS	Dried blood spot
DESI	Desorption electrospray ionization
DI-MS	Direct infusion mass spectrometry
DIMs	Direct ionization methods
DMF	Digital microfluidics
DMSPME	Dispersive magnetic solid phase micro-extraction
EIE	Extracted ion electropherogram
EkE	Electrokinetic extraction
EME	Electro membrane extraction
ESI	Electrospray ionisation
FIA	Flow injection analysis
FWHM	Peak's full width at half-maximum
GUI	Graphical user interface

HGB	Hemoglobin
HILIC	Hydrophilic interaction liquid chromatography
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HRAM-MS	High-resolution accurate mass-mass spectrometry
HRMS	High-resolution mass spectrometry
HSA	Human serum albumin
HV	High voltage
ID	Internal diameter
IEF	Isoelectric focusing
IM-MS	Ion-mobility mass spectrometry
IS	Internal standard
LC	Liquid chromatography
LESA	liquid extraction surface sampling
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LMJ-SSP	Liquid microjunction surface sampling probe
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass/charge
MALDI	Matrix-assisted laser desorption/ ionization
MESI	Membrane electrospray ionization
MEPS	Micro-extraction by packed sorbent
MNRB	Moving neutralization reaction boundary
MOI	Microfluidic open interface

MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Nano-ESI	Nanoelectrospray ionization
NRB	Neutralization reaction boundary
OD	Outer diameter
OPP	Open port probe
PAGE	Polyacrylamide gel electrophoresis
PE	Process efficiency
pI	Isoelectric point
PSI	Paper spray ionization
PTFE	Polytetrafluoroethylene
SCX	Strong cation exchange
SFME	Slug flow micro-extraction
SPE	Solid phase extraction
SPME	Solid phase micro-extraction
SSSP	Sealing surface sampling probe
TDM	Therapeutic drugs monitoring
TESI	Tip electrospray ionization
TLC	Thin layer chromatography
TOF-MS	Time of flight-mass spectrometry

Abstract

The work presented in this thesis describes an innovative approach for biological sample preparation in a micro-volume syringe based on electrokinetic separation. For the first time, the embedded stainless steel syringe needle and plunger served as electrodes and the syringe barrel served as a separation channel. The advantages of doing this in a syringe is such that all the preparation of biological samples can be done in the device, such as sample dilution with the required buffer, enrichment of the targeted analytes and/or interfering molecules removal, and finally infusion to the detector. Furthermore, the online coupling of the in-syringe sample preparation with the electrospray ionization-mass spectrometry (ESI-MS) opens up new opportunities for ultra-sensitive and selective analysis of complex biological samples in an automated manner.

Chapter 1 provides an insight into different sample clean-up and analyte enrichment strategies directly coupled with the ESI-MS and nano-ESI-MS for the analysis of biological fluids. The overview includes direct ionization methods (DIMs) along with the direct coupling of classical and innovative format of sample preparation techniques such as solid phase extraction (SPE), solid phase micro-extraction (SPME), liquid micro-extraction, and electrokinetic extraction (EkE) with ESI-MS and nano-ESI-MS.

Chapter 2 introduces the first electroseparation-in-a-syringe system where a 25 µL analytical glass syringe was utilized for isoelectric focusing (IEF) of amphoteric compounds using the stainless-steel needle and plunger as electrodes. The mechanism relied on the generation of hydronium and hydroxyl ion fluxes from water electrolysis to form a neutralization

reaction boundary (NRB) at which the amphoteric compounds can be focused within minutes. The focusing of different proteins such as hemoglobin, R-phycoerythrin, and bovine serum albumin was demonstrated by the developed IEF syringe. After optimization of the different experimental parameters affecting the IEF process and coupling of the IEF syringe with the ESI-MS, the IEF syringe-ESI-MS system was applied for the analysis of histidine in spiked urine samples as relevant for the diagnosis of histidinemia. Each 1 µL of urine was 10x diluted with the background electrolyte (BGE) *via* the IEF syringe itself and the final composition of the BGE was NH_4Ac, 1.0 mM, pH 4.0, in 70.0 % (v/v) acetonitrile. Voltages of −200 V (5 min) and −400 V were applied for the IEF step and the infusion step, respectively. The IEF syringe's contents and the sheath liquid (0.2% (v/v) formic acid) were infused into the Agilent triple tube ESI sprayer at a flow rate of 4.0 µL/min.

Furthermore, different systems for the clean-up of biological samples from the interfering proteins were developed. In Chapter 3, an EkE syringe is presented capable of the on-line removal of proteins from human serum samples within 320 seconds allowing the analysis of the weakly acidic compounds. The separation mechanism relies on the difference in the electrophoretic behavior of the serum proteins and the weakly acidic target analytes in a BGE composed of 50 mM formic acid (pH 2.5) in 30 % acetonitrile. 1 µL of spiked serum was 15x diluted with the BGE by the EkE syringe itself and the separation is accomplished by the application of a potential difference of 2000 V across the syringe for 320 seconds, utilizing the metallic syringe needle and plunger as electrodes, once the cationic proteins (pI > 2.5) are concentrated and precipitated close to the plunger, the syringe drive of a

syringe pump is actuated for infusion of the protein-depleted sample into the ESI-MS with a flow rate of 4.0 µL/min whilst applying 500 V. The syringe is interfaced with ESI-MS *via* a coaxial infusion of isopropyl alcohol 80.0 % (v/v) (flow rate, 10.0 µL/min) as a sheath liquid. The applicability of the EkE-ESI-MS method was demonstrated by the determination of naproxen and paracetamol in spiked serum using valproic acid as an internal standard (IS).

Chapter 4 exhibits the designing of a bubble free in-syringe EkE system relying on the dissolution of the water electrolysis gases by increasing the pressure inside the syringe barrel. A sealed system was designed to pressurize the gases into the solution using a push-pull valve or a 3-port selector valve at the needle side and a gas-tight plunger. The developed design was applied to the electrokinetic elimination of human serum albumin from the samples using different BGE systems (acidic and basic) and different syringe volumes.

Chapter 5 demonstrates the analysis of the weakly basic drugs in serum after the in-syringe EkE, the method is based on using NH_4OH (300 mM, pH 11.4) in 30 % acetonitrile as a BGE to negatively charge the serum proteins prior to their aggregation close to the plunger (the anode) by application of 800 V for 90 seconds on 10 µL of 5x diluted serum. Unlike the EkE system for acidic compounds analysis “Chapter 3”, a bubble-free EkE of the weakly basic compounds analysis was achieved by pressurizing the syringe as described in Chapter 4. After the EkE and valve switching, 8.0 µL of the syringe content were delivered to the ESI-MS at a flow rate of 5.0 µL/min. The sheath liquid was composed of methanol/water/formic acid, 75:24.5:0.5 and was infused with a flow rate of 5.0 µL/min. A reduced analysis time of 3.1 (1.5 min for the EkE and 1.6 min for the infusion) was achieved for the analysis of weakly basic drugs,

clomipramine, chlorphenamine, pindolol, and atenolol in spiked serum. Furthermore, a high sensitivity in ppb level was attained *via* coupling with ESI-MS/MS and spiking the serum with the deuterated isotopes as ISs.

In Chapter 6, a fully automated EkE-in-a-syringe platform is proposed, the system requirements, design, and workflow were recommended in details.

Finally, Chapter 7 of the thesis summarizes all the developed EkE syringe-ESI-MS systems and the different accomplished applications.

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Preface

Sample preparation is an essential research priority in bioanalysis. It is typically the most tedious part in the biochemical analysis because most of the effort and time are for sample preparation [1]. However, it is indispensable to enrich analytes of interest and/or to remove the interferences from complex matrices thus there are intense efforts to miniaturize, automate and integrate sample preparation with the final analysis [1].

At present, advanced mass spectrometers like high-resolution accurate-mass mass spectrometry (HRAM-MS) and ion-mobility MS (IM-MS) offer unprecedented sensitivity and selectivity [2], which has led to the resurgence of stand-alone MS technologies which depend solely on the MS power for the differentiation between the chemically related compounds. Many sample preparation techniques have been directly hyphenated with electrospray ionization mass spectrometry (ESI-MS) and nano-ESI-MS to boost the throughput of the bioanalysis by eliminating chromatographic separation. Nonetheless, the analysis reliability and sensitivity shouldn't be compromised so efficient sample preparation is even more critical given elimination of the chromatographic separation in the direct analysis techniques to remove the matrix interferences and/or to enrich the target analytes before the MS event.

This thesis describes an innovative electrokinetic extraction (EkE)-in-a-syringe approach for biological sample preparation and its coupling with ESI-MS. Owing to accurate liquid handling capability and being the most common element in many analytical procedures for the introduction of clean sample – both manually or through an autosampler - the preparation of complex biological samples in a syringe enables the combination of different analytical

steps in one platform and consequently offers a myriad of opportunities for the high throughput automated analysis.

Furthermore, the electrokinetic technique is implemented by exploiting the embedded stainless steel syringe needle and plunger as electrodes for the first time. This provides a potential alternative to conventional sample preparation techniques which suffer from many drawbacks, for instance, the difficulty in automation in protein precipitation [3] and liquid-liquid extraction [4] and the tedious method development and the high cost of solid phase extraction [3-5]. This thesis shows the potential and promise of an entirely new approach to sample preparation, based on the use of electric fields and the movement of ions, inside a simple liquid-handling device – the syringe – to completely overcome the issues associated with existing approaches to high throughput sample treatment.

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1. Hyphenated Sample Preparation Techniques for Direct Biofluid Analysis *via* ESI-MS

1.1 Introduction

Biological fluid analysis is fundamental in medical and biomedical applications, pharmacological studies, and biomarkers discovery. Biofluids such as serum, plasma, and urine, are much easier to obtain compared to tissue samples – the collection of urine is completely noninvasive and the collection of blood samples is a part of almost every routine physical investigation [1]. It is worth mentioning that blood is not a biofluid but it is referred to as a biofluid due to its accessibility and the similarity in processing to the other biofluids like the urine and cerebrospinal fluid (CSF) [2]. Further, biofluids such as serum can represent the body circulation profile and the performance of the entire body [3], thus, the analysis of biomarkers in biofluids, including whole blood, plasma, serum, saliva, urine, and CSF is critical in clinical diagnosis and prognosis [4]. Furthermore, therapeutic drug monitoring (TDM) and pharmacokinetics are usually based on the measurement of the different drugs and/or metabolites in biological fluids [5]. However, the concentration of the biomarkers in biofluids are usually lower than in tissues [1] so robust, selective, and sensitive detection methods are needed for analysis.

Mass spectrometry (MS) is a very powerful tool in analyzing complex samples due to its inimitable selectivity, sensitivity, and capability to provide molecular information regarding the target analytes [6]. The Nobel Prize-winning invention of the electrospray ionization (ESI) by John Fenn [7, 8] allowed for the gas phase ion production of fragile molecules from aqueous

solutions. Consequently, ESI has significantly broadened the scope of MS in biological macromolecule analysis [9] and enabled the coupling of MS with liquid chromatography for the quantitative analysis of biological samples in drug discovery, clinical studies, and biomedical research [10]. Furthermore, the version of ESI with a lower flow rate (10-20 nL/min) [11], nanoelectrospray ionization (nano-ESI), introduced by Matthias Mann and colaborators [12], has improved the efficiency of the ionization owing to uneven droplet fission which leads to the enrichment of the surface active analytes such as the peptides and proteins in the offspring droplets, in addition, the high surface/volume ratio facilitated access to analytes in the bulk of the droplets [13]. Nano-ESI allows the consumption of smaller volumes of samples, solvents, and buffers and shows high tolerance to non-volatile salts and buffers in more complex matrices [9]. Nonetheless, the major drawbacks of nano-ESI are the tedious adjustment, the poor stability, and the time-dependent performance, generally, the nano-ESI needles vary in the reliability and quality [13].

MS strategies can be classified into 3 main categories [14-16]: Chromatography-MS, stand-alone MS, and pure ambient MS. The analytical figures of merit and the throughput of these are shown in Figure 1-1.

Hyphenation of MS with different separation techniques especially, liquid chromatography (LC), has been the gold standard for the determination of different analytes in clinical samples [17]. Coupling with chromatography can provide the best analytical performance because of the minimum susceptibility to the matrix but it is labor intensive and time consuming, additionally, it still requires tedious sample pre-treatment [16, 17] so it has the lowest throughput among the strategies [14, 16]. Moreover, chromatographic alignment is very

challenging in LC-MS based metabolomics [18].

Elimination of the chromatographic separation and dependence only on the MS power for differentiation between the chemically related compounds is the main feature of the stand-alone MS strategy [14, 16], however, it still requires online or offline sample preparation to remove the matrix interferences, with liquid-liquid extraction (LLE) with immiscible solvents [16] being popular. Stand-alone MS has a more simplified workflow and a higher throughput compared to the LC-MS and it can offer satisfactory sensitivity for both qualitative and the quantitative analyses in some applications [14].

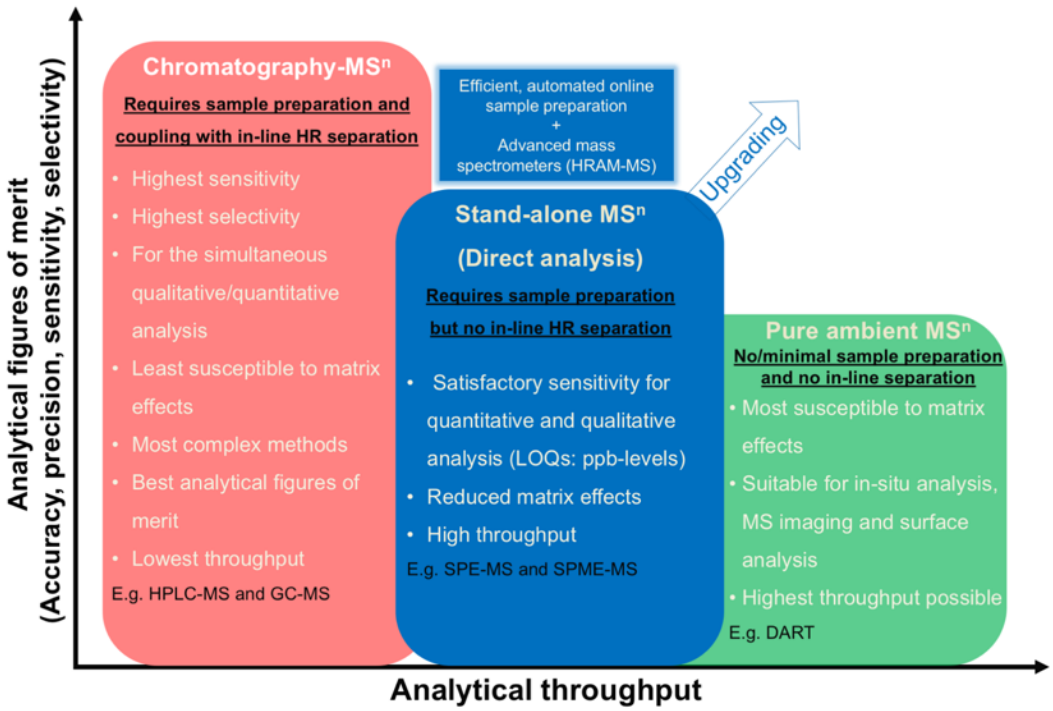

Figure 1-1. Comparison between the different MS strategies: chromatography MS, stand-alone MS, and pure ambient MS. Adapted from Ref. [16] with permission from Springer-Verlag GmbH.

The immediacy of sample processing differentiates pure ambient MS from other stand-alone MS strategies (direct analysis strategies) where sampling, processing, and ionization all take place in a simultaneous manner near the point of ion entry into the MS vacuum system, in another words, the sample is

available to the MS without any previous sample preparation in pure ambient methods, contrarily, direct analysis methods encompass sample preparation steps prior to the MS analysis [15]. Despite of the high analysis speed and the simplicity of pure ambient MS, it is highly susceptible to matrix effects [14] and most of the ambient methods such as direct analysis in real time (DART) and desorption electrospray ionization (DESI) are affected with intrinsic problems related to the geometry and surface of the specimen which makes the development of accurate and reliable quantitative analysis methods quite difficult [19].

Modern MS requires just a millisecond to obtain the signal of ions from the analytes [20] and the capabilities of the stand-alone MS were boosted by the virtue of the highly selective MS technologies such as the high-resolution accurate mass MS (HRAM-MS) and the ion-mobility MS (IM-MS) [16]. However, biological samples' analysis by MS usually requires extensive preparation steps, which is time consuming and represents a major source of error in every analytical workflow [21]. Therefore, the trending direction in sample treatment is miniaturization, integration into the final analysis, and full automation of the whole analysis [22]. Furthermore, there are many commercial systems for robotic automated sample preparation and coupling with ESI-MS such as Agilent RapidFire™ and TriVersa NanoMate LESA® both of which are now available in the market [23].

Thus, the coupling of the modern stand-alone MS with an efficient, online sample preparation can attain analytical figures of merits close to LC-MS technologies along with an outstanding throughput [14]. For instance, extraction times below 30 s can be achieved using the online SPE-ESI-MS technology,

Agilent RapidFire™ [24-29].

The scope of this review includes the directly coupled sample preparation-ESI-MS strategies for biological fluids analysis. These strategies depend only on the MS power for the differentiation between the chemically related compounds, i.e. not hyphenated with chromatographic separation, for instance, the strategies discussed here include online SPE-MS methods without gradient elution which work in load-elute mode or load-wash-elute mode for the removal of salts and interfering matrix components, but they don't chromatographically separate the chemically related molecules and depend solely on the MS for differentiation between the co-eluted compounds [30]. The sample preparation techniques included here are solid phase extraction (SPE), solid phase micro-extraction (SPME), slug flow microextraction (SFME), liquid extraction surface sampling (LESA), extraction electrospray, electrokinetic extraction (EkE), and extraction using digital microfluidics (DMF). This is the first comprehensive review for the direct coupling of the sample preparation techniques with the ESI-MS and nano-ESI-MS for biofluids analysis whether using the commercially standard ion sources or the open ion sources in the direct ionization methods (DIMs).

1.2 Direct Ionization Methods (DIMs) - Substrate Spray

Direct ionization methods (DIMs) are simple ambient ionization methods for analyzing both small organic compounds and large biomolecules [31]. Unlike many ambient ionization processes, DIMs based on ESI technique don't require special instrumentations for the ionization or the desorption but the electrospray is achieved directly from a substrate in front of the MS orifice [19]. The substrate-spray technologies depend on supplying elution/spray solvent to the

wicking materials, i.e. porous tips or paper strips, followed by the application of a high voltage to generate the gaseous ions from the sharp point of the substrate [14]. Unfortunately, instrumental simplicity comes at the expense of the compatibility with the required industrial safety requirements where the open ion-source with an exposed high voltage can be a major obstacle in commercialization but cartridge housings and fully automated systems can offer possible solutions for the safety concerns [19].

Accurate quantitative analysis is still a major weak point in the DIMs and no reliable results can be achieved without the incorporation of deuterated isotopes as internal standards (ISs) [19], hence, there is always a need to use a substrate which can work as an extracting phase to eliminate the interfering matrix components and to avoid the contamination of the instruments [14]. In return, more reliable quantification can be achieved by combining sample preparation with the substrate-spray methods [14]. This section highlights the DIMs such as the modified paper spray, the functionalized tip spray, and the coated blade spray (CBS) which allow for the sample clean-up and/or the analytes enrichment in addition to the sampling.

1.2.1 Paper spray ionization (PSI)

Direct ionization employing cellulose based material was first described by the Nobel Laureate, Fenn, in 1998 [32]. However, the work published by Cooks and Ouyang groups in 2010 is considered to be the actual start of the PSI-MS [33]. Paper spray is a three-step process with the first step being the application of the spray solvent onto the sample spot on the paper substrate such as the dried blood spot for analyte extraction followed by the transport of the analytes by solvent wicking to the sharp tip of the porous triangular material where a

high-voltage is applied using a metal clip connected to the paper triangle held in front of the mass spectrometer's orifice to perform the electrospray ionization [34]. PSI is considered the most popular DIM due to its simplicity, its reduced cost and the high analysis speed that can be achieved [19]. Also, PSI is suitable for the analysis of dried spots of blood [9, 33, 35] and urine [36, 37] owing to keeping salts and cell debris on the cellulose substrate [17]. However, PSI has a limited ability to clean-up the biological samples from the other matrix components [14] because the only treatment is accomplished *via* filtration during drying of the biofluid on the paper [15] so it is more susceptible to matrix effects and consequently has poor analytical figures of merit [14]. As such, additional sample treatment is required prior to PSI-MS for bioanalysis. Modified PSI is a rapidly developing field which incorporates coating of the paper substrate and/or designing paper-based microfluidics to minimize the matrix effects and to improve the sensitivity or to produce a more stable electrospray [17]. Here, the modified PSI methods for the analysis of biological fluids to achieve a more selective extraction prior to the spray step are summarized (Table 1-1).

Methacrylic acid based molecularly imprinted polymers were utilized to modify cellulose substrates for the SPME of cocaine from oral fluids [38] and dopamine from urine samples [39]. In both applications [38, 39], the triangular modified substrate was immersed in the sample for 5 min for target analyte extraction and the elution for PSI-MS analysis was accomplished using acidic methanol (0.1% formic acid (FA) in methanol) [38, 39] after substrate drying [38] or washing with deionized water (1 min) followed by drying [39]. A selective affinity extraction of codeine before the PSI-IM-MS analysis was achieved *via*

the immobilization of anticodeine aptamer on the cellulose paper [40].

Different PSI cartridges were designed to integrate sample processing with PSI-MS [41-43]. The concept of affinity extraction was achieved with different polyclonal antibody enrichment columns in a 3D printed cartridge for the detection of post-translational modifications of plasma proteins using paper modified with carbon-nanotube (CNT) and porous polyethylene as substrates [41]. The PSI cartridge allowed excess plasma components to be washed into a waste pad in the bottom cartridge and the MS detection of the retained proteins obtained after sliding the column to the elution position as indicated in Figure 1-2A. Online reduction of transthyretin post-translational modifications was accomplished by adding TECP-HCl to the spray solvent, the reduction reaction was enabled on the cartridge for 5 min prior to the ionization which was induced by applying a high voltage of 5 kV [41]. Another SPE column packed with a mixture of HLB particles and cellulose powder (to improve the wicking rate) was integrated into a PSI cartridge to extract a number of pharmaceuticals in plasma before the PSI-MS analysis. LODs were reduced 14-70 fold using the integrated SPE column compared to the direct PSI-MS analysis. Figure 1-2B shows a photograph of the coupling of the PSI cartridge with the mass spectrometer [42].

Commercially available membranes for blood fractionation were added to a PSI cartridge to allow for on-cartridge plasma separation from whole blood, the cartridge was applied for pharmaceuticals analysis in the whole blood drops *via* PSI-MS without any additional centrifugation step [43].

Coating of the paper with silica gel [44], ZrO_2 [45] or mesoporous graphene [46] and using silanized paper [34] have been also used for more efficient

separation prior to MS analysis but they are not covered in details in this review for the chromatographic separation free methods (extraction without baseline separation).

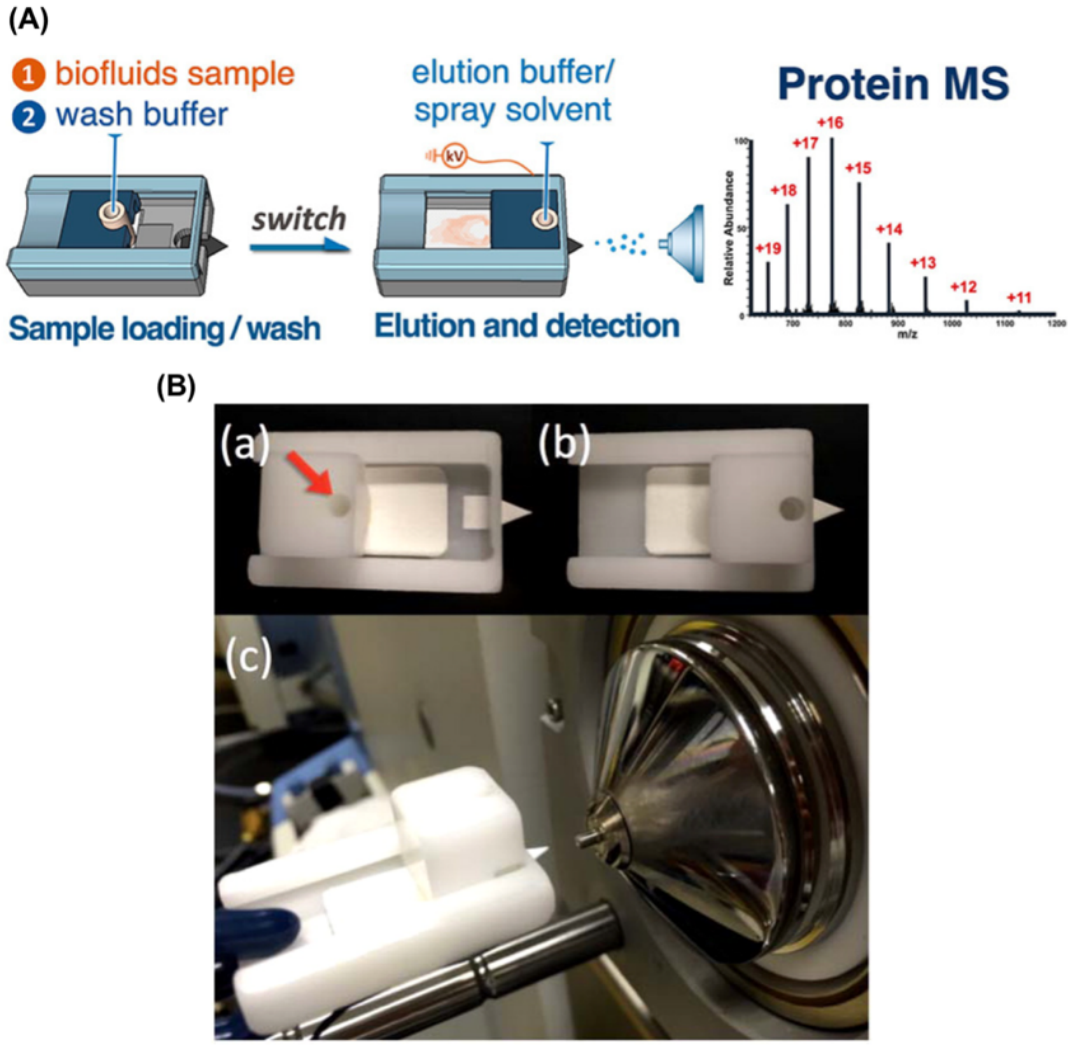

Figure 1-2. A, the workflow of the PSI-MS analysis using the PSI cartridge with an integrated antibody SPE column for proteins extraction and enrichment from plasma samples. B, photographs of a PSI cartridge with HLB-cellulose powder SPE column in loading position (a), elution position (b), and in front of MS-inlet (c) for the pharmaceuticals analysis in plasma samples. Figure 1-2A was reprinted from Ref. [41] and Figure 1-2B was reprinted from Ref. [42] with permission from American Chemical Society.

Table 1-1. Modified PSI-MS for the biofluids analysis.

Modification	Elution/spray solvent	Biofluid	Biofluid volume	Target analyte(s)	LOD	Extraction time	Ref.
Cellulose membrane modified with molecularly imprinted polymers	0.1% of FA in methanol	Oral fluid	Immersion in 1 mL of the sample	Cocaine	0.27 ng/mL	5 min	[38]
Coating of the paper with molecularly imprinted polymer	0.1% of FA in methanol	Urine	Immersion in 2 mL of the sample	Dopamine	0.24 ng/mL	5 min	[39]
Immobilization of anticodeine aptamer on the cellulose paper	Acetonitrile (ACN)/water, 90:10	Urine	Immersion in 15 mL of the sample	Codeine	3.7 ng/mL	30 min	[40]
Integrated of antibody enrichment column in a 3D printed PSI cartridge	Methanol/water with 2% acetic acid, 50:50	Plasma	20 µL spot	Plasma proteins, with posttranslational modifications: apolipoprotein c1, hemoglobin, glycated hemoglobin, and transthyretin	-	5 min (sample loading to the column)	[41]

SPE column (Mix of HLB and cellulose powders) integrated in a PSI cartridge	Methanol/water with 0.01% acetic acid, 95:5	Plasma	≥ 10 µL	Carbamazepine, atenolol, sulfamethazine, diazepam, and alprazolam	0.3, 2.2, 0.08, 6.1, and 1.3 ng/mL, respectively	Not given (-)	[42]
Addition of a plasma fractionation membrane in a PSI cartridge	Methanol/water/ acetic acid, 95:5:0.01	Whole blood	Uncontrolled drop	Detection of many pharmaceuticals and quantitation of atenolol and carbamazepine	0.04 and 0.03 µg/mL, respectively	15 min (for plasma separation)	[43]

1.2.2 Tip electrospray ionization (TESI)

Spray tips can provide a convenient sampling option for ESI-MS because it avoids the clogging problems found with the capillary-based ESI and the tip substrate can be functionalized to integrate the SPME in the ESI emitter [47]. Different tips were modified to enrich target analytes and remove the interfering matrix components before the TESI-MS [47-49]. One simple approach employed commercial pipette tips (10 µL ZipTips) with beds of 18-carbon modified silica (C18) for the purification and the enrichment of ketamine and norketamine in urine samples prior to the TESI-MS analysis [48]. Six aliquots of 10-µL urine were loaded onto the tip before washing with an aqueous acidic solution and elution with 1.0% FA in 80% methanol in front of the MS inlet [48].

Wooden tips were functionalized for the extraction of the perfluorinated compounds [49] and berberine [47] from different biological fluids. Enrichment by 100-500 fold for the perfluorinated compounds in whole blood was demonstrated using the functionalized wooden tip after silanization with n-octadecyldimethyl[3-(trimethoxysilyl)propyl] ammonium chloride [49], while the extraction of the cationic analyte, berberine, from urine samples was achieved using a sulphonic acid modified wooden tip [47].

1.2.3 Coated blade spray (CBS)

CBS is a technology merging SPME and substrate-spray ionization and was introduced by Pawliszyn's group [50] to integrate sampling and sample preparation in a single device. Typically, CBS utilizes a sword-like stainless steel blade partially coated with an extraction phase such as C18 [50] or hydrophilic-lipophilic balanced (HLB) particles attached to the substrate using polyacrylonitrile (PAN) as a binder [50-55].

Table 1-2 summarizes different biological fluids, target analytes and extraction conditions incorporated in the CBS-MS methods. The analytical protocol of the CBS-MS consists of three steps [14]: (1) analyte extraction which can be done either by application of the sample spot onto the CBS [51, 53, 55], or by immersion into the sample vessel [50, 52, 54, 55] usually under vortex agitation conditions to accelerate the extraction process [50, 54, 55]. Spot deposition has less experimental error due to the virtually exhaustive extraction conditions rather than the non-equilibrium conditions experienced in the large volume sample [55]; (2) rapid rinsing to remove the interfering matrix components; (3) elution and electrospray by applying a micro-volume of the elution solvent onto the coated area for 20 s before high voltage is applied to the uncoated stainless steel part of the blade to generate the electrospray from the blade's tip [14].

CBS-MS provides not only a more efficient sample extraction compared to the PSI-MS but also an increment in the sampling capacity which is limited to 100 µL (spot analysis only) in the ordinary PSI and a suitability in interfacing with the automated liquid handling tools (i.e. 96- and 384-well plates) for the high-throughput analysis [14]. Although using a cellulose substrate in PSI-MS offers a more economic technology compared to the CBS, CBS blades avoid the undesirable downsides of the cellulose material such as the poor electrical conductivity and the deformation tendency [14]. When compared to SPME-tips [53], improved LOQs can be achieved by CBS due to the higher surface-to-volume ratio.

An automated extraction could be performed using a holder carrying up to 96-CBS devices connected to a robotic arm moving among different 96-well

plates (Figure 1-3) [52, 54], however, the maximum speed of agitation could be achieved using the 96-autosampler is 1500 rpm (orbital agitation), therefore, a relatively long extraction time of ~15 min was required for every batch. Despite the short analysis time of 55 s per sample [54], the workflow was still segregated into two distinct steps: the automated sample preparation (blade conditioning, analyte extraction, and water rinsing) and the non-automated electrospray of the clean extract into the MS from each blade one by one, thus, the bottle-neck becomes the transition from the clean-up step to the MS event.

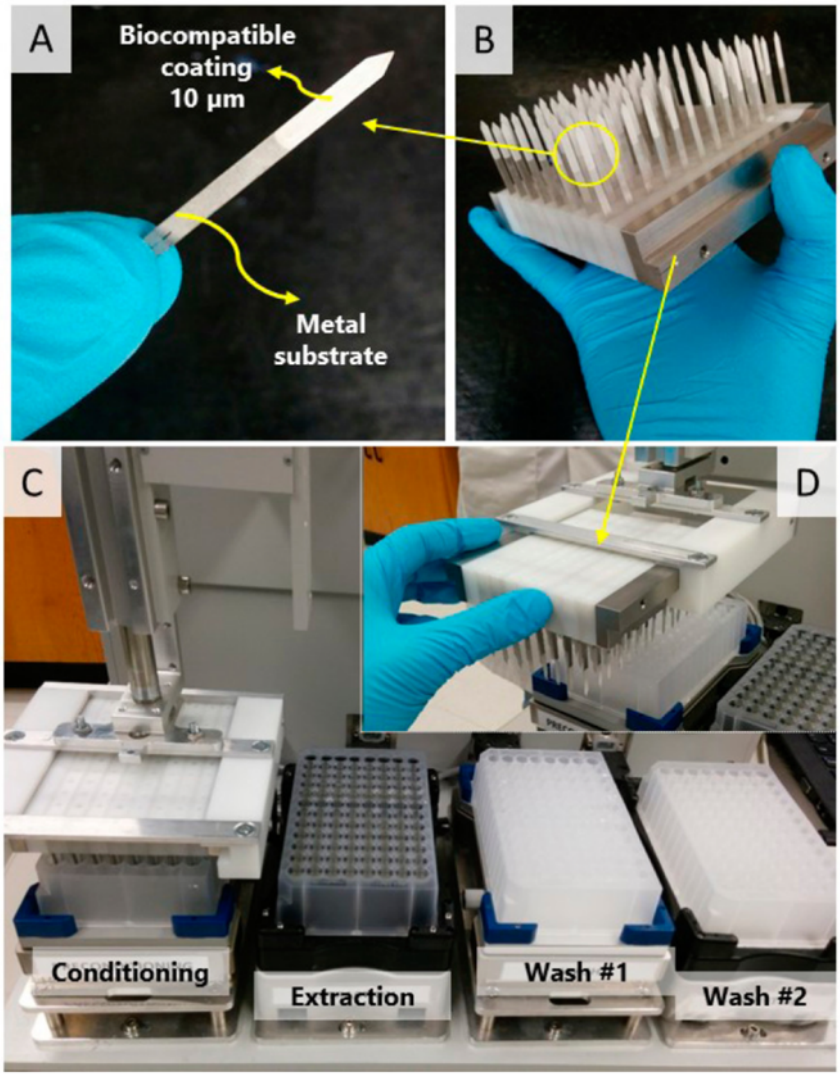

Figure 1-3. High throughput extraction of different analytes from urine and plasma using the CBS-brush which is compatible with the 96-well plates workflow. A, HLB-PAN coated blade, B, CBS-brush (96 blades), C, 96-autosampler, D, installation of the CBS-brush on the robotic arm. Reprinted from Ref. [54] with permission from American Chemical Society.

Table 1-2. CBS-MS methods for the analysis of biofluids.

Coating	*Elution/spray solvent	Biofluid	Biofluid volume	Target analyte(s)	LOQ	Analysis time	Ref.
C18-PAN	Methanol	Plasma	0.3-1.5 mL	Cocaine	0.5 pg/mL	3 min	[50]
				Diazepam	50 pg/mL		
		Urine		Cocaine	2 pg/mL		
HLB-PAN	0.1% FA in methanol/water, 95:5	Blood or plasma (biofluids modified with ACN or unmodified)	10 µL	Therapeutics and drugs of abuse	0.25 to 2.5 ng/mL in ACN modified blood spot	7 min	[51]
HLB-PAN	0.1% FA, and 10 mM NH_4Ac in methanol/ water, 95:5	Blood after proteins denaturation (0.1 M $ZnSO_4$ in water/ACN, 85:15)	150 µL	Tacrolimus, cyclosporine-A, sirolimus, and everolimus	1, 10, 2.5, 1 ng/mL, respectively	3 min/sample (Turn-around time 90 min)	[52]
HLB-PAN	0.1% FA in methanol/water, 95:5	Blood	5 µL	Amitriptyline	5 ng/mL	5 min	[53]
		Plasma	10 µL	Untargeted			

HLB-PAN	0.1% FA in methanol/water, 95:5	Urine and plasma	300 µL	Mixture of anabolics, diuretics, β-2 agonists, narcotics, stimulants, and β-blockers	0.1-10 ng/mL	2 min or 55 s/sample using the 96-well format	[54]
HLB-PAN	0.1% FA in methanol/water, 95:5	Plasma	300 µL (dynamic sampling)	Voriconazole	0.1 µg/mL	2 min	[55]
			10 µL (spot extraction)			3 min	

*Rinsing with water was included in all methods prior to the elution step.

1.2.4 Membrane electrospray ionization (MESI)

MESI added a unique feature to the substrate spray approach where compacting a dialysis membrane onto a pre-wetted filter paper acting as a waste pad allowed the clean-up of the sample - applied on the top of the dialysis membrane - in the vertical and horizontal dimensions, typically, a 2-µL sample was loaded on the dialysis membrane and in about 30 s, the flushing solvent (methanol/water, 6:4 v/v, 0.1% FA) along with the high voltage were applied onto the membrane for the spray generation [56]. The salts and small interfering molecules could pass through the membrane pores and absorbed by the filter paper which enabled the direct mass spectrometric detection of cytochrome C in human tears, angiotensin II in urine samples, progesterone in human saliva, and Met-Arg-Phe-Ala peptide in human serum [56].

The horizontal separation on the membrane is not as efficient as the cellulose-based substrates especially for the interfering molecules with high masses which could not be filtered in the vertical direction by the membrane due to the hydrophobic nature of the membrane [56]. Thus, the membrane was modified to allow for a sensitive determination of cytochrome C in human urine (LOQ,10 ng/mL), the membrane was coated with anti-cytochrome C prior to dipping into the sample for 15 min to allow the binding of cytochrome C with the antibody, this binding can be resolved after the washing step by adding urea to the membrane [56].

1.3 Solid Phase Extraction (SPE)

SPE has been considered for many years as a very efficient technique for biological samples' clean-up and the selective preconcentration of different analytes [57]. SPE depends on the interaction between the analytes and the

sorbent as in liquid chromatography but the size of the sorbent particles is generally greater in SPE where the particle size can reach up to 30 µm [58]. Many sorbents and embodiments are now available and the online coupling with different techniques has been routine for years now, online SPE is able to provide better precision than the off-line procedures because of the constant flow of the entire process and the complete transfer of the analytes [57]. A very rapid analysis of complex samples can be achieved by direct coupling of SPE with ESI-MS, with almost real-time analysis of 7 s/sample [28]. Although this approach is very cost-effective, there are some restrictions that should be considered to develop a robust method. Since the analytical column is absent, a much higher efficiency is required for the sample preparation step by the SPE procedures to avoid the co-elution of the endogenous interfering compounds which subsequently can result in a significant ion suppression and poor precision and accuracy [57].

1.3.1 SPE columns

The column format of SPE is very popular due to the feasibility of its online coupling with ESI-MS *via* valve-switching techniques [59-66], or high-speed robotics [24-29]. Classically, automated online-SPE systems are composed of an autosampler, a cartridge switching device (optional), one or more switching valves, and solvents dispensers [57].

Table 1-3 is summarizing the published methods for the online clean-up of biofluids by the SPE columns prior to the ESI-MS analysis. Methods that involved the incorporation of SPE columns in the PSI cartridges are discussed in “1.2.1 Paper spray ionization” section. Different SPE sorbents have been coupled with ESI-MS using this approach such as C18 particles [59-62] and

hydrophilic lipophilic balance (HLB) copolymers (divinylbenzene-co-*N*-vinylpyrrolidone) [63, 64]. C18 traps were efficient for the clean-up of urine samples [59-62] removing creatinine (polar compound) to avoid creatinine-adduct formation during ESI-MS analysis [57]. HLB particles allow for the proteins exclusion [63] making it an efficient sorbent for plasma [64] and serum [63] clean-up. Additionally, polymeric sorbent performs over the full pH range to allow the retention/elution of target analytes at very low and very high pH values [58]. One of the drawbacks of classical online SPE-ESI-MS is the long time required for sample analysis ($\geq$ 15 min for many methods [59-62]) to load, wash and elute the analytes. Levi *et al.* [65] managed to accomplish the whole analysis within 4.5 min by using 30 µm particles to allow the use of high flow rates without the high back pressure issue [65]. Some methods used a flow splitter [59, 63, 64] to attain the optimum flow rate for the ESI-MS which resulted in a loss of analytes and consequently a compromised sensitivity.

Unlike classical online SPE-ESI-MS, the commercially available system Agilent RapidFire™ integrates high-speed robotics and fast-switching valves to allow for ultra-fast online SPE-MS [29]. The microfluidic interface is available in 96- or 384-well formats and the system has an ultra-low dead volume autosampler and an SPE cartridge with few µLs bed volume [28]. The system can handle up to 63 plates and each SPE cartridge is capable of enduring more than 3000 injections before replacement [23]. The RapidFire system was coupled with ESI-IM-MS for untargeted analyses of human urine and plasma using different SPE cartridges [29]. Despite, the high throughput of the online SPE-ESI-MS process itself, the workflow includes other offline sample pre-treatment by protein precipitation [24, 26, 29] or LLE [27]. Also, the clean-up of

the microfluidic lines is required to avoid the lines clogging and the sensitivity suppression [23].

Online SPE-ESI-MS suffers from some drawbacks. First, it is not suitable for point-of-care analysis as it still requires the bench-top high performance pumps to flush the SPE columns with the washing and elution solvents [24-29, 59-66]. Also, conventional SPE (column format) coupled with ESI-MS require a relatively large volume of samples as shown in Table 1-3 where the required biofluid volume for the conventional SPE ranged from 50 µL to 500 µL, i.e. $\geq$ 10 times higher than the sample volume for the spot analysis by the CBS-MS [53].

Table 1-3. Methods for the analysis of biofluids by online SPE-ESI-MS.

Sorbent	Washing solution	Elution solution	Biofluid (offline pre-treatment)	Biofluid volume	Target analyte(s)	Sensitivity*	Analysis time	Ref.
C18	Water	Methanol	Urine (filtration)	400 µL	S-phenylmercapturic acid (SPMA)	0.15 ng/mL (Deuterated isotope addition)	15 min	[59]
						0.35 ng/mL	17 min	[60]
C18	SPE trap washing: water	Loop and SPE trap elution: methanol	Urine (filtration)	400 µL	Urinary benzene exposure biomarkers: trans,trans-muconic acid (ttMA) and SPMA	7.43 and 0.23 ng/mL, respectively	15 min	[61]
						1.27 and 0.042 µg/g creatinine, respectively		[62]
HLB	5 mM ammonium formate, pH 4.0	ACN/5 mM ammonium formate, pH 4.0, 95:5	Serum	200 µL	Melatonin	0.1 ng/mL	9 min	[63]

HLB	Water	Methanol/water, 95/5	Plasma	500 µL	Docetaxel	LOQ: 1 ng/mL	7 min	[64]
Oasis MAX (water-wettable, mixed-mode polymeric sorbent for acidic compounds)	Water followed by tetrahydrofuran	Tetrahydrofura/water/FA, 95:5:5	Plasma and urine	50 µL	Valsartan and candesartan	LLOQs: 5 and 1 ng/mL, respectively	4.5 min	[65]
Polymeric divinylbenzene resin	0.1% FA in 10% methanol	1% FA and 2% acetic acid in 40% ACN	Serum	500 µL	Cathelicidin	2.5 ng/mL	12 min	[66]
C18	10 mM NH_4Ac, 0.9% FA, and 100 µL/L trifluoroacetic acid (TFA) in water	50% methanol	Whole blood (hemolyzation and protein precipitation)	100 µL	Cyclosporine A, Tacrolimus, Everolimus, and Sirolimus	LLOQs 15.5, 1.0, 1.3, and 1.0 ng/mL, respectively	13 s	[24]

Silica modified with 4 carbon (C4)	10 mM NH_4Ac and 0.1% FA in water	0.1% FA in methanol/water, 50:50	Urine (hydrolysis with NaOH, dilution, and centrifugation)	50 µL	Synthetic cannabinoids	1 ng/mL	9 s	[25]
		0.09% FA and 0.01% TFA in water/acetone/ ACN, 50:25:25			Synthetic cathinones	5 ng/mL	12.6 s	
C4	5 mmol/L ammonium formate in water with 5% ACN	ACN with 5% of the washing solvent	Plasma (protein precipitation with methanol)	50 µL	Imatinib, nilotinib, and lapatinib	LLOQs: 50, 50, 100 ng/mL, respectively	29 s	[26]
C18	10 mmol/L of NH_4Ac, 0.1% FA, and 0.009% TFA in water	50% methanol	Plasma (LLE with n-butyl chloride)	100 µL	Busulfan	6.25 pg/mL	20 s	[27]

Graphitic carbon	0.1% TFA	0.1% TFA in 80% methanol	10% of human serum was added to the enzymatic reaction mixture (protein precipitation by trichloroacetic acid (TCA))	-	Cystathionine	LOQ: 80 nM	7 s	[28]
C18	0.1% FA	FA/isopropanol (IPA)/methanol, 0.1:49.95:49.95	Plasma (protein precipitation with ACN) and urine (urease pre-treatment and methanol extraction)	50 µL	Endogenous metabolites and xenobiotics	≤ 10 nM	10 s	[29]
Mixed mode hydrophobic Imtakt scherzo sm-C18	0.1% FA							
Graphitic carbon	0.1% FA	FA/ACN/acetone/water, 0.1:25:25:49.9						
hydrophilic interaction liquid chromatography (HILIC) cartridge	FA/ IPA/hexane, 0.1:10:89.9	FA/IPA /methanol, 0.1:49.95:49.95						

*Sensitivity is expressed by the LOD unless otherwise mentioned.

1.3.2 Miniaturized SPE

Miniaturization of the SPE column results in lower consumption of sample and solvents, lower cost, enhanced performance, and higher throughput, however, the ability of the miniaturized system to efficiently handle complex samples on the same microfluidic device is essential for final successful applications [67].

On-chip SPE devices for the clean-up of urine samples were designed and built by *in situ* UV light-initiated polymerization of alkylacrylate based monolith as an SPE column in a Zeonor microchip [67, 68]. One of the chips was coupled with the ESI-MS using a pneumatically assisted micro ion sprayer connected to the on-chip SPE column *via* a fused silica capillary [68]. The micro ion sprayer [68, 69] incorporated a self-contained liquid junction to enable the infusion of the make-up liquid. The other microchip included a side channel for the infusion of the make-up liquid and it was interfaced with the MS *via* an integrated on-chip electrosprayer tip [67]. Furthermore, each chip included more than one integrated SPE column prepared at the same time to improve the extraction reproducibility, for instance, an array of the 8 SPE columns allowed the determination of imipramine in human urine with a good reproducibility (RSD 3.8%) and a LLOQ of 0.025 µg/mL [68]. On the other hand, this approach suffered from elongated analysis time using a load-wash-elute protocol, in one of the methods, 20 µL of the spiked urine sample was loaded onto a preconditioned monolithic column, washed for 10 min and eluted to MS in with a total analysis time of ~15 min [67].

Microextraction by packed sorbent (MEPS) was described in Abdel-Rehim's work [70] which incorporated the sorbent material into a liquid handling device

(syringe) to accomplish the sample pretreatment and injection into the analytical instrument by a single device. Candish *et al.* [71] have hyphenated MEPS directly to the ESI-MS to screen opiates and codeine metabolites in urine within 5 min. A two-way valve was incorporated in the barrel of a hand-held digital analytical syringe (eVol®) to manipulate the liquid flow direction, by sliding the needle within the valve body, the negative pressure could force the eluent through the side port to be applied from the top of the adsorbent minimizing the sample dilution and the carryover between runs [71]. A reduction in the carryover from 65% to only 1% was reported for this controlled flow approach compared to the conventional MEPS [71].

1.4 Solid Phase Microextraction (SPME)

SPME is a non-exhaustive extraction technique. Unlike SPE, it is driven by diffusion and equilibrium between phases and the extractant/sample ratio is significantly lower than the exhaustive approach used in SPE. [72].

Many published works included the direct coupling of SPME with ESI-MS. Methods which involved the direct electrospray or nano-electrospray from the extractant itself as in CBS-MS are discussed in “1.2 Direct Ionization Methods” section. In this section, approaches based on the direct elution of the adsorbed analytes from the SPME devices by their mounting onto the sprayer tubes or by using the different desorption units were indicated.

1.4.1 SPME fibers

Different applications in the field of biofluid analysis were accomplished *via* the direct coupling of SPME fibers with ESI-MS as summarized in Table 1-4. Ghiasikhou *et al.* developed a fully automated system for the online coupling of SPME with ESI-MS [73]. The system had a robotic arm to control the movement

of the C18 coated stainless steel extraction tool. Extraction was performed by dipping the SPME device in the sample followed by automatic placement in a chamber for eluent infusion toward the spray capillary *via* a microfluidic platform [73]. The system was called a “gap sampler” because of the micro-sized gap between the elution solvent capillary and the spray capillary, this design allowed a nL-liquid bridge for the adsorbed analyte elution with a minimal dead volume as illustrated in Figure 1-4A [73]. The gap sampler was utilized for the extraction of benzodiazepines from human plasma and the reversed phase coating was reported to be reusable for up to 30 extractions, however, 30 min of loading time was required to achieve the equilibrium between the sample and the extractant [73].

Other systems [74-80] were developed for the online elution of the adsorbed analytes from the SPME fibers to the electrosprayer but the sampling workflow was not fully automated and it was segregated into two main steps; manual sample loading and rinsing and online desorption with the elution solvent to the ESI-MS sprayer. Gomez-Rios *et al.* [74] introduced the SPME fiber directly into the nano-ESI emitter prefilled with the desorption solution, they used a biocompatible mixed mode SPME fiber (C18-strong cation exchange (SCX) coating) for the extraction of different drugs in blood and urine within 2 min by agitation at 3200 rpm before the rinsing and the online desorption steps. The advantage of using the biocompatible coating is the prevention of proteins adsorption when exposed to complex biological samples to avoid the clogging of the nano-ESI-MS emitter [74]. Likewise, other methods [75-77] were developed based on mounting the SPME fiber onto the nano-ESI emitter for the determination of ketamine in urine [75], phosphopeptide I in serum [76], and

hydroxylated polycyclic aromatic hydrocarbons in urine samples after the enzymatic hydrolysis by glucuronidase/arylsulphatase [77]. Figure 1-4B illustrates the coupling of the SPME fiber with the nano-ESI-MS *via* a glass-capillary emitter.

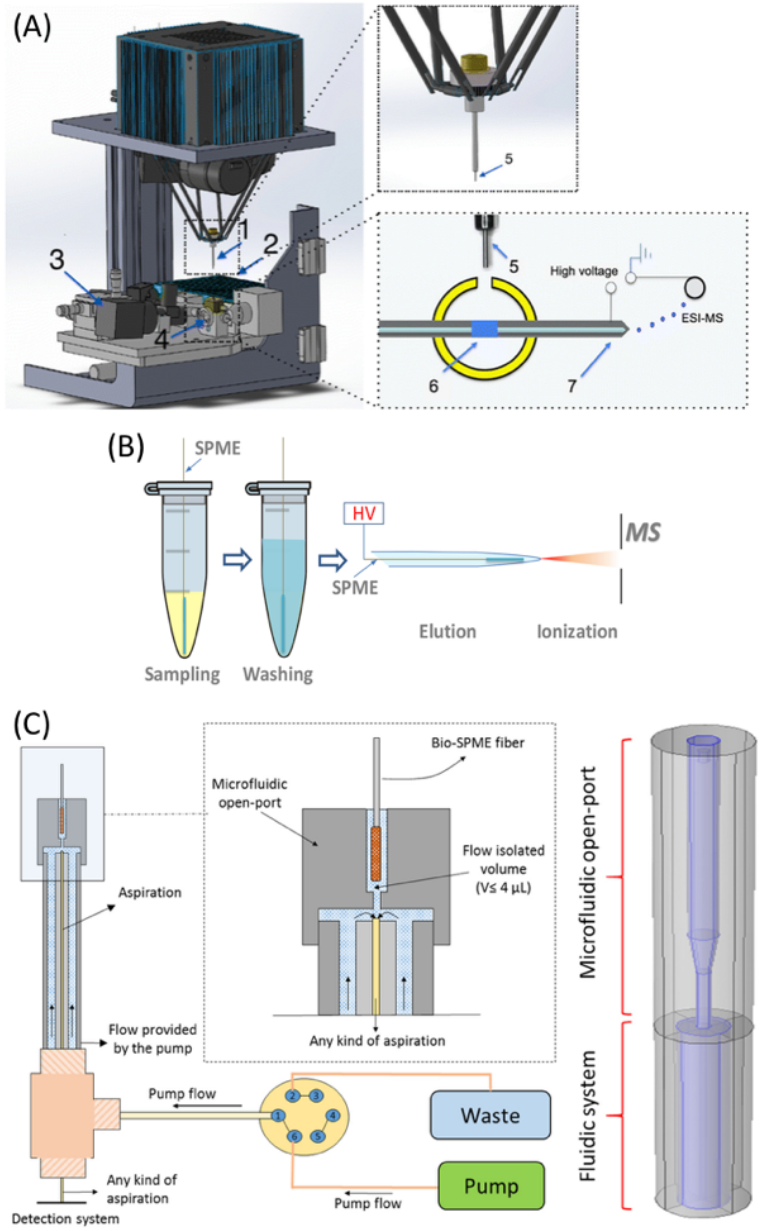

Figure 1-4. Different platforms for the coupling of SPME fibers with ESI-MS. A, the capillary gap sampler: extraction tool held by delta robot arms (1), microwell plate (2), camera (3), pressure chamber (4), solid stainless steel pin (SPME tool) (5), liquid bridge for the desorption (6), and stainless steel ESI capillary (7). B, coupling of the SPME with the nano-ESI-MS by mounting the SPME fiber into a glass capillary emitter. C, schematics of the microfluidic open interface for the coupling of the SPME fibers with the ESI-MS. Figure 1-4A was reprinted from Ref. [73] with permission from Springer-Verlag GmbH, Figure 1-4B was reprinted from Ref. [77] with permission from Elsevier, and Figure 1-4C was reprinted from Ref. [80] with permission from American Chemical Society.

In a different approach, a desorption chamber was designed and placed in the loop of a two-position six-port valve, where the elution solvent can fill the loop and desorb the analytes [78]. A polypyrrole (sol-gel) coated syringe needle was used for the headspace extraction of venlafaxine in urine and protein precipitated plasma, then the needle was dried and placed in the V-shape junction of the desorption chamber to ensure the sealing, finally, venlafaxine was eluted by methanol to the electrospray needle of ESI-IM-MS [78].

Gomez-Rios and collaborators [79] utilized an open port probe (OPP) that was designed by Van Berkel *et al.* [81] as a sampling interface for atmospheric pressure chemical ionization (APCI)-MS to couple SPME fibers with the ESI-MS. The probe used a co-axial vertically aligned tubes arrangement for the delivery of the solvent to the sampling end through the tubing annulus [79, 81]. The dome-shaped sampling surface increased the contact area with the SPME fiber and it could allow continuous sampling by setting the delivery rate to the interface to be higher than the aspiration rate to the ion source which depends on the nebulizing gas flow rate [79]. The OPP is more suitable for automation compared to the nano-ESI emitter [74-77] due to the less ionization interruption with dry-fibers insertion and the continuous flow resulted in a low carry-over [79].

For enhanced sensitivity, the same group further developed a microfluidic open interface (MOI) interface [80], indicated in Figure 1-4C, with chamber dimensions for a micro-volume of desorption solvent (≤ 7 µL) without sacrificing the fiber length which was 2.5 times higher than the fiber length in the OPP interface [79]. Compared to the OPP interface [79], the MOI [80] with a longer SPME fiber and a minimal sample dilution allowed the determination of

tacrolimus, sirolimus, everolimus, and cyclosporine A with an enhanced sensitivity of 3-, 30-, 15-, and 7- fold, respectively, in blood samples treated with ACN/0.1M $ZnSO_4$/water, 30:60:10 for plasma proteins denaturation, however, an elongated extraction time of 90 min was required [80]. Unlike the nano-ESI emitters for the SPME fibers elution [74-77], the desorption *via* the MOI can be tuned from few seconds to minutes where the rapid evaporation of the solvent was prevented by the enclosed chamber [80].

Table 1-4. Coupling of the SPME fibers with ESI-MS and nano-ESI-MS for the biofluids analysis.

Interface with MS	Fiber coating	*Elution/spray solvent	Biofluid	Biofluid volume	Target analyte (s)	Sensitivity**	Extraction time	Ref.
Desorption chamber (the gab sampler)	C18	0.1% FA in ACN/water, 80:20	Plasma	≤ 40 µL	Benzodiazepines	0.3 µg/mL	30 min (equilibrium)	[73]
Nano-ESI-MS emitter	C18-SCX	0.1% FA in methanol	Urine	10 µL - 1.5 mL	Salbutamol, codeine, methadone, and oxycodone	1.1, 2.1, 0.1 and 1.4 ng/mL, respectively	2 min	[74]
			Whole blood		Amitriptyline and imatinib	1.6 and 2.3 ng/mL, respectively		
Nano-ESI-MS emitter	C18-benzenesulfonic acid (mixed mode)	0.1% FA and 12 mM NH_4Ac in 95% methanol	Urine	300 µL	Ketamine	0.027 ng/mL	10 min	[75]
Nano-ESI-MS emitter	TiO_2 modified silica	2% NH_3-H_2O in 50% ACN	Serum	-	Phosphopeptide I	0.39 µg/mL	2 min	[76]

Nano-ESI-MS emitter	C18	0.05% NH_4Ac in acetone	Urine	500 µL	Hydroxylated polycyclic aromatic hydrocarbons	0.05 ng/mL	2 min	[77]
Desorption chamber	Polypyrrole (PPy)	Methanol	Urine	-	Venlafaxine	0.5 ng/mL	30 min (headspace extraction)	[78]
			Protein precipitated plasma (using TCA)	1 mL		11 ng/mL		
Open port probe	C18-SCX	0.1% FA in methanol	Urine	300 µL	Clenbuterol, fentanyl, and buprenorphine	0.03, 0.05, and 0.25 ng/mL, respectively	5 min	[79]
Microfluidic open interface	HLB	0.1% FA and 12 mM NH_4Ac in methanol	Blood treated with ACN/ 0.1 M $ZnSO_4$/water, 30:60:10	100 µL	Tacrolimus, sirolimus, everolimus, and cyclosporine A,	LOQs: 1, 0.3, 0.3, 0.6 ng/mL, respectively	90 min	[80]

*Washing with water was included in all methods prior to the elution step except Ref. [76] which encompassed washing with 1% TFA in 50% ACN and Ref. [78] which included drying step instead of rinsing with water.

**Sensitivity is expressed by the LOD unless otherwise mentioned.

1.4.2 Dispersive magnetic solid phase micro-extraction (DMSPME)

The high surface area and the versatility that can be offered by dispersive magnetic solid phase microextraction (DMSPME) encouraged some research groups to directly couple the DMSPME with ESI-MS for the biological fluid analysis [82, 83]. Nanocomposites of polypyrrole-coated Fe_3O_4 magnetite [82] and magnetic nanoparticles of Fe_2O_3 functionalized with C18 [83] were utilized for the extraction of 1-hydroxypyrene, a polycyclic aromatic hydrocarbon exposure biomarker [82] and the extraction of cocaine, methadone, fentanyl, sertraline, and propranolol [83], respectively, from urine samples. For the analysis of 1-hydroxypyrene [82], 1 mg of nanocomposite was mixed with 10 mL of undiluted urine for 1 min under vortex mixing conditions, the suspension mixture was loaded into a syringe with an external magnet to gather the nanocomposites during the urine discharge to the waste, washing with deionized water, and elution with acetone/benzene/acetic acid, 90:10:1. The elution fluid was pumped to the ESI-MS using the same syringe through a capillary with a magnet positioned outside to prevent the magnetic particles from reaching the spray nozzle.

A microfluidic open interface (MOI) was designed for coupling of DMSPME with ESI-MS for the determination of different drugs in urine samples [83]. A 3D-printed holder containing an embedded magnet was used for the collection of the magnetic particles from the dispersive extraction and the washing steps then the holder was set on top of the MOI for the desorption step. The MOI comprised two concentric tubes and the gap between them was filled with solvent to form a droplet for the magnetic nanoparticles re-extraction. After 5 s of re-extraction, the flow was switched to allow for rapid aspiration of the droplet

through the inner tube to the MS due to the self-aspiration property of the ESI-source [83].

Despite of the short analysis time provided by the internal extractive-ESI-MS method [82] and the MOI [83], 4 min and 1 min, respectively, the process was not fully automated and the sample loading and rinsing were accomplished offline before the online desorption to ESI-MS.

1.5 Slug Flow Microextraction (SFME)

Avoiding the drawbacks of conventional liquid-liquid extraction such as usage of large amounts of the organic solvents and being time-consuming [84], Ren *et al.* [85] introduced a new technique entitled “slug flow microextraction (SFME)” in 2014 for the online clean-up of biofluids using just few microliters of the sample and organic solvent. A simple analysis of organic compounds in blood and urine samples was achieved by coupling SFME with nano-ESI-MS. Five µL adjacent plugs of the immiscible organic solvent and sample were sequentially injected into the capillary then the slug flow was induced by capillary tilting or by applying a push-and-pull force using air pressure through a pipette to increase the interfacing area and the extraction efficiency, after analyte extraction into the organic solvent, the organic solvent plug was pushed to the tip of the capillary and nano-ESI generated by voltage application using a stainless-steel wire inserted in the organic solvent plug [85]. Ethyl acetate was the organic solvent of choice for the extraction of methamphetamine, nicotine, and benzoylecgonine in urine and amitriptyline, verapamil, and methamphetamine in diluted blood samples. Samples in the capillary were tilted five times to reach equilibrium and a LOD of 0.05 ng/mL was reported for verapamil [85]. One of the main advantages of the SFME is the ability to probe

the biological and chemical properties only present in wet samples, for instance, protein enzymatic functions are quenched in DBS so this approach has been applied successfully for monitoring of cholinesterase activity after adding acetylthiocholine iodide as a substrate in blood samples [85]. Furthermore, an online chemical derivatization of anabolic steroids was achieved to improve their ionization by injecting an aqueous solution of hydroxylamine between the organic solvent and the urine sample plug [85].

Additionally, SFME was adapted for the analysis of the hydrophilic compounds such as amino acids by designing a three phase system, in such case, the immiscible organic solvent (hexane) formed a bridge between the biofluid and the polar extractant (methanol/water) [86]. In the ordinary SFME experiment, most of the salts, proteins, and cell debris can be blocked by the non-polar organic solvent, however, the non-polar solvent can only extract a tiny amount of the polar target compounds in each SFME cycle so the system was modified to let the extracted compounds be transferred into a third polar extractant plug exploiting the efficient mass transfer due to the slug flow [86]. Using a hydrophobic Teflon tubing instead of the glass tube was necessary to keep the three-phase system during the SFME and 300 cycles were required to reach the equilibrium during the analysis of amino acids in the urine matrix by the SFME [86]. Figure 1-5 illustrates the difference between the normal SFME and the three phase SFME.

SFME was also coupled with PSI for the determination of trace macrolide in whole blood where the organic layer was spotted onto a paper triangle after the extraction and dried out for the PSI-MS analysis [84].

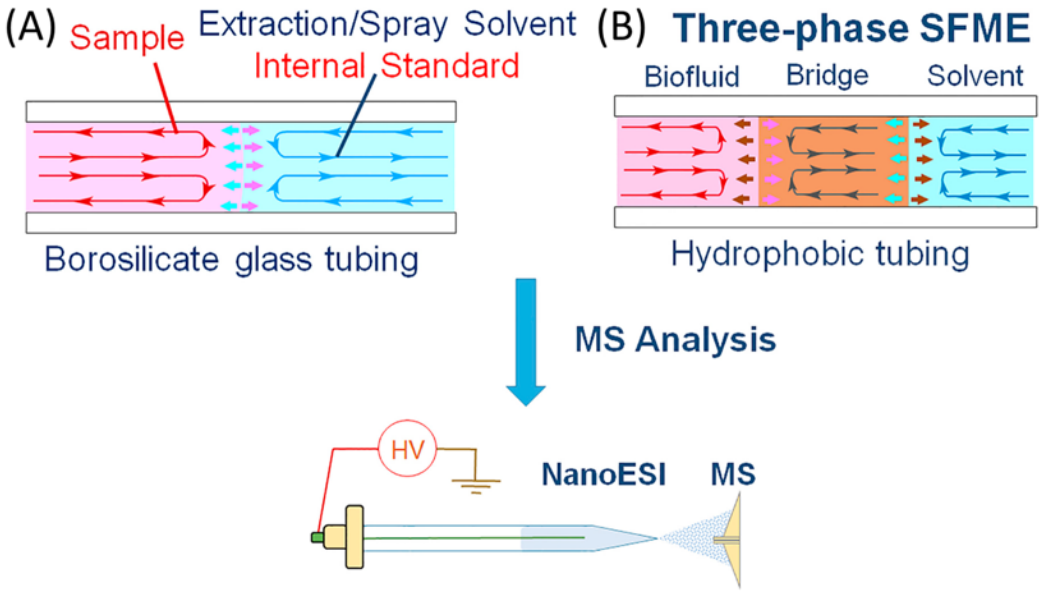

Figure 1-5. A, normal SFME, B, three-phase SFME for the hydrophilic compounds extraction, followed by nano-ESI-MS analysis. Reprinted from Ref. [86] with permission from Elsevier.

1.6 Liquid Extraction Surface Analysis (LESA)

LESA uses a liquid conduit probe for the direct extraction of analytes from different surfaces. The probe contacts the substrate surface with a confined liquid stream or a droplet of the extracting solvent then the solution after extraction is transferred to the ionization source *via* the same probe [87]. There are two kinds of LESA probes, namely, sealing surface sampling probe (SSSP) [87] and liquid microjunction surface sampling probe (LMJ-SSP) [88]. SSSP allows the extraction from the porous material like paper by sealing the probe to the surface using the probe's integrated knife edges but it didn't allow the extraction from the hard and nonflexible material [87]. LMJ-SSP is characterized by having a wall-less liquid microjunction between the probe's end and the surface of the substrate, it is suitable for the extraction from nonporous materials such as glass and metal but it suffers from poor repetitive sampling when applied to the extraction from porous surfaces such as the

normal phase TLC plates and most paper substrates [88]. This is due to irregular solvent spreading where the liquid was conducted out into the porous wider surface instead of being aspiration back into the probe [88]. Thus, the two probes complement each other.

SSSP was used for the extraction of acetaminophen from a DBS on paper substrates prior to the ESI-MS analysis [87]. Figure 1-6A shows the coupling of a stainless steel SSSP with the ESI-MS (linear ion trap mass spectrometer) *via* a switching valve and an LC system to pump the extraction solvent (methanol) to the probe inlet [87]. The downsides of this system are leaking in case of a clogged frit and the need for extracting a blank spot for 60 s between the sample spots to eliminate the carryover [87].

A fully automated system (TriVersa NanoMate, Advion, USA) was developed to couple LMJ-SSP probes with MS through a nano-spray microchip for the analysis of the dried biological fluid spots [88-92]. A robotic arm was controlled to move the conductive tip to a certain sample spot for surface extraction and to engage the tip with the back of the nano-ESI chip which contains microfabricated nozzles for nano-spray generation. The system was applied for the quantitation of sitamaquine in DBS with an accuracy within 15% at a concentration level of 100 ng/mL [89]. The power of coupling with a high resolution Orbitrap mass spectrometer was explored for the identification of haemoglobin (Hb) variants (homozygous and heterozygous HbS, HbC, and HbD variants) after the direct surface sampling of the DBSs with the NanoMate system [90]. This method allowed for the repeated analysis of the same spot [90]. Also, the coupling of a high field asymmetric waveform ion mobility spectrometry with the NanoMate robot enabled a gas phase separation of lipids

(phosphatidylcholine, and sphingomyelin species) and haemoglobins α- and β-subunits, 1% FA in methanol/water, 1:1 was used as a solution for the surface extraction in this multiplexed clinical assay [91]. The LMJ-SSP probe was used to provide chemical profiles of fingerprints, oral fluid, and urine along and the detection of illicit drugs and their metabolites in the studied matrices [92]. Only 0.2 µL drops of the urine or the oral fluid were deposited onto a glass slide and stored until extraction and MS analysis [92].

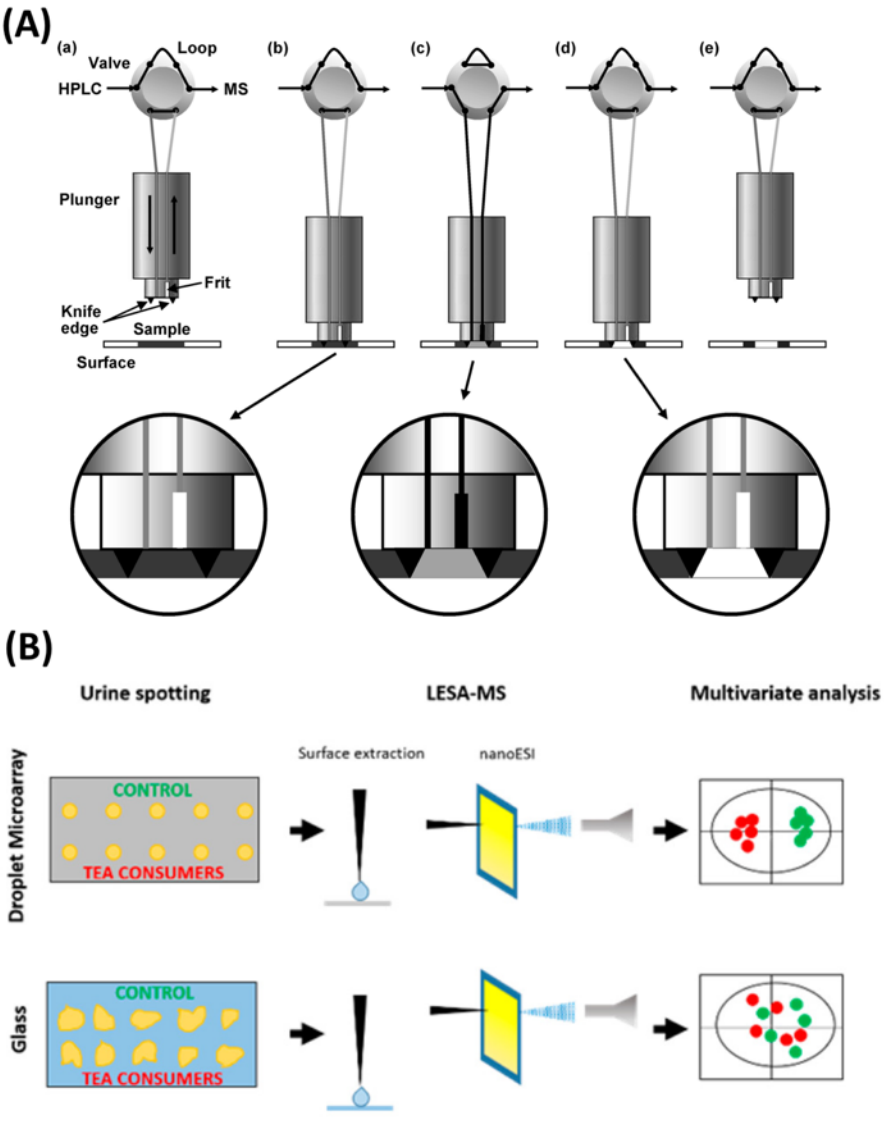

Figure 1-6. A, individual steps of the surface sampling to ESI-MS using the SSSP with expanded views of the sealed probe. B, LMJ-SSP-nano-ESI-MS analysis utilizing an array of superhydrophilic spots surrounded by a superhydrophobic material *versus* the extraction from a standard glass substrate. Figure 1-6A was reprinted from Ref. [87] and Figure 1-6B was reprinted from Ref. [88] with permission from American Chemical Society. Further permissions related to the material excerpted should be directed to the ACS.

To sort out the problem of the relatively poor reproducibility of this technique due to the extracting solvent spreading beyond the limits of the dried sample, a droplet microarray was designed by bordering the superhydrophilic material for the sample spots with a superhydrophobic material as illustrated in Figure 1-6B [88]. This pattern was achieved by chemical modification of the nanoporous 2-hydroxyethyl methacrylate (HEMA)-ethylene dimethacrylate (EDMA) polymer with alkyne groups to confine both the sample and the extraction solvent in a certain area [88]. The relative standard deviation was reduced by at least 3 fold owing to this approach in a metabolomic study of urine samples before and after tea consumption [88].

Although the sampling speed of the SSSP and LMJ-SSP were comparable, 2.5 min/sample and 2 min/sample, respectively, an additional washing step (1 min) was required for SSSP to clean the probe face and tubes (between the probe, the valve, and the ion source) to eliminate the carryover between the different samples [89]. Cleaning was not required in the NanoMate LMJ-SSP due to the high speed of the robotic system and each nozzle and pipette tip being single use [89].

1.7 Extraction Electrospray

Extraction electrospray is a direct MS analysis approach where the dried sample on a paper substrate is placed in the ESI tube for the fast extraction of target analytes with an organic solvent and the ions produced by the applied high voltage for the spray generation [93] as shown in Figure 1-7. The graph (Figure 1-7) also indicates that the water soluble molecules including blood cells can be washed off from the paper strips if there is an aqueous component in the solvent mixture so methanol and other pure organic solvents are

recommended as extraction solvents to avoid clogging of the spray tube [93]. Borosilicate glass tubes with pulled tips are utilized for the extraction process and nano-electrospray generation [93, 94]. Extraction Electrospray can require sample volumes as low as 0.2 µL [93] and it was coupled with a miniaturized mass spectrometer (25 Kg) to enable on-site determination of synthetic cannabinoids in blood and urine with LOQs ranging from 10 to 20 ng/mL [94].

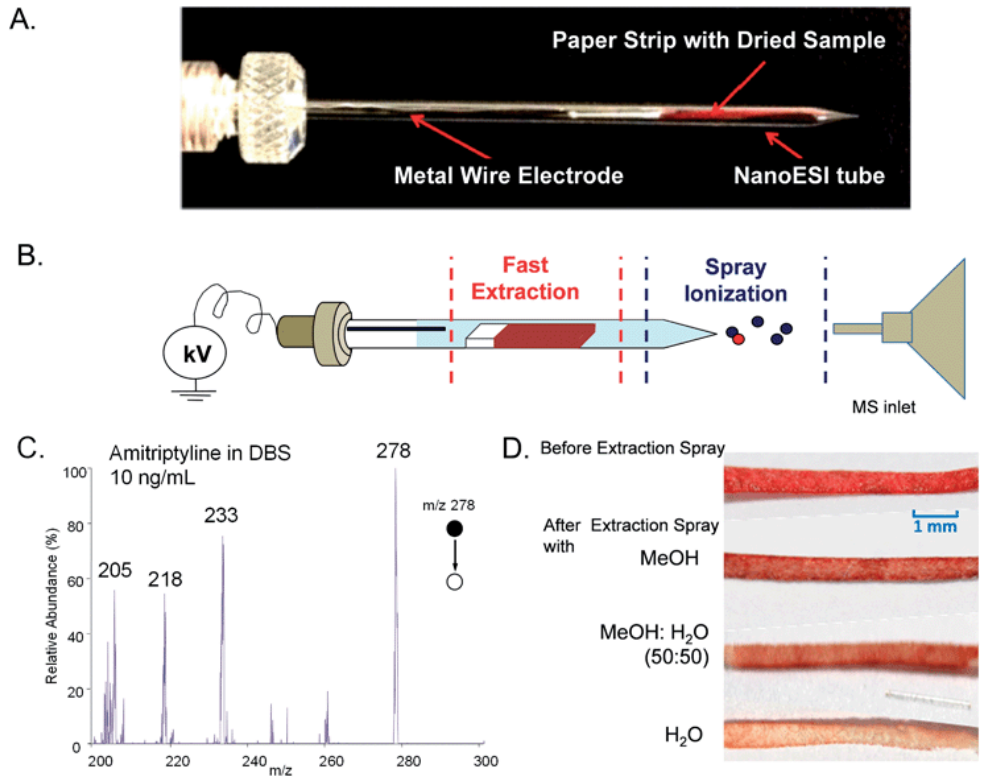

Figure 1-7. A, photo of an extraction nano-electrospray ionization source for MS. B, schematic of extraction nano-electrospray. C, MS/MS spectrum of 10 ng/mL amitriptyline in dried blood sample using extraction nano-electrospray. D, photos of dried blood strips before and after the extraction using different solvents. Figure 1-7 was reprinted from Ref. [93] with a permission from Royal Society of Chemistry.

1.8 Electrokinetic Extraction (EkE)

Electrokinetic sample preparation is based on the isolation and/or the concentration of the target analytes through an electric field application [21]. Electrokinetics represents a very attractive approach for designing online sample preparation systems due to the simple hardware requirement and the compatibility with the miniaturization, detector integration, and automation [21].

Electro membrane extraction (EME) was integrated into a microfluidic chip for the extraction of basic drugs in urine prior to the online UV or ESI-MS detection (Figure 1-8A) [95]. A porous hydrophobic polypropylene membrane separated the acceptor reservoir and sample channels and a DC potential applied to transport basic analytes to the membrane at one side and out into the acceptor at the other side of the membrane [95]. The aqueous media was acidic (100 mM FA) on both sides of the membrane to protonate the basic drugs, this design allowed the selective extraction of the relatively non-polar basic compounds while the inorganic salts along with the acidic/neutral/polar components in the matrix can be discriminated by the membrane [95]. The design of the system enabled the dynamic flow in both channels and a nanospray interface (in-house built) was used for accomplishing the online analysis by the ESI-MS [95]. Methadone was determined in urine after the clean-up with a LOD of 40 ng/mL and an enrichment factor of 75 fold in 12 min operation [95].

Another configuration is based on using a liquid membrane to separate the sample and acceptor phase in a three-phase electroextraction system. A drop of acceptor phase (2 µL of 5% formic acid in 33% methanol) was submerged using a conductive pipette tip into the organic solvent layer (ethyl acetate:methyl acetate, 3:2) where the immiscible solvent layer acted as a filter to prevent salt and protein transfer from the sample to the acceptor phase during the extraction of acylcarnitines from spiked diluted human plasma [96]. In the second step of this protocol, a high voltage applied to the conductive tip to generate a protein-free nano-electrospray for the mass spectrometric detection with concentration factors $\leq$ 7 [96].

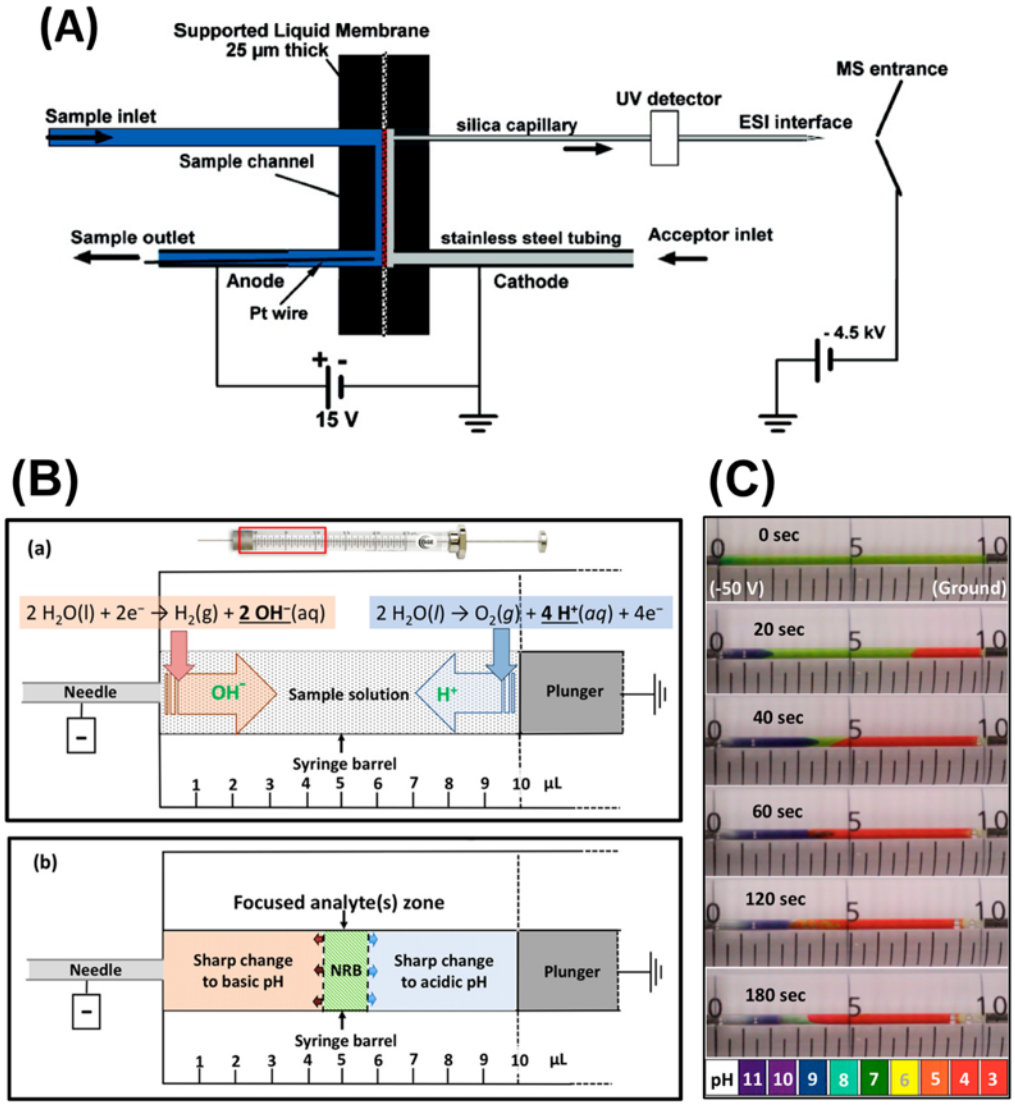

Figure 1-8. A, illustration of the on-chip electro membrane extraction (EME) system coupled with ESI-MS interface. B, formation of the neutralization reaction boundary (NRB) inside the CAF-IEF syringe due to water electrolysis where (a) represents the application of electric field on sample solution using the needle and the plunger as electrodes and generation of fluxes of OH^- and H^+ ions and (b) indicates the formation of the NRB. C, demonstration of the pH step gradient inside the CAF-IEF syringe using a 10 µL of 5.0 mM NH_4Ac (pH 7) containing a universal pH indicator (pH 3−11). Figure 1-8A was reprinted from Ref. [95] and Figure 1-8B and Figure 1-8C were reprinted from Ref. [97] with permission from American Chemical Society.

Recently, our group introduced a novel approach for electrokinetic sample clean-up and target analyte preconcentration in a micro-volume syringe. The in-built metallic needle and plunger were used as electrodes to apply the voltage and the syringe barrel served as a separation channel [97]. A 25 µL glass syringe (SGE analytical syringe, Trajan Scientific and Medical, VIC, Australia) was used for carrier ampholytes free-isoelectric focusing (CAF-IEF)

of amphoteric compounds, with proteins (*pIs* 3-11) such as hemoglobin and albumin, focused in the middle of the syringe within minutes [97]. Figure 1-8B depicts the mechanism of the CAF-IEF and Figure 1-8C demonstrates the pH step generated inside the syringe. The CAF-IEF syringe was coupled directly with the ESI-MS through an interface with a triple tube sprayer for the co-axial sheath liquid infusion and the IEF syringe-ESI-MS system was applied for the determination of histidine in urine samples with an enhancement factor of 8.5 fold [97]. 5 min was required for the CAF-IEF step and the use of the syringe allowed for the sample dilution (1 µL of urine:9 µL of the BGE, 1.1 NH_4Ac in 77.8% ACN), preparation by CAF-IEF, and infusion to ESI-MS using a single device without the need of any modification in the standard ionization source [97]. Further, the electrokinetic approach didn't require excessive use of toxic solvents compared to the LLE, only 7 µL of ACN was required for every CAF-IEF cycle. It has much simpler method development and a less sample consumption than the SPE – only 1 µL of urine was required for the EkE while 100 µL of urine was required for the SPE [97].

Johannesson *et al.* [98, 99] developed two methods for urine sample desalting and target analyte preconcentration using a C18 derivatized monolith in fused silica column (sol-gel columns) to trap the target analytes. High voltage application was used during the elution step for analyte focusing. In the first work [98], urine samples spiked with hydrophobic peptides (neurotensin, angiotensin II, oxytocin, leucine-Enkephaline, and luteinizing hormone-releasing hormone) were desalted by loading onto the sol-gel columns for 5 min under slight pressure (1 bar), followed by washing with 5 mM NH_4Ac for 15 min, then the column inlet was placed into the running buffer vial containing 5 mM

NH_4Ac in 60% ACN and a voltage of 20 kV was applied, all the peptides were eluted as a sharp band (about 70 times preconcentration) in less than 20 min except for leucine-Enkephaline which penetrated more in the porous monolith owing to its smaller size and it was eluted as a broad peak at ~20 min. The other work applied the same concept on the determination of escitalopram in urine samples with a LOD of 10 pg/mL [99].

1.9 Digital Microfluidics (DMF)

DMF is a technique for fluid-handling by manipulating discrete droplets of reagents and samples on a microfluidic open surface using an array of insulated electrodes to apply a series of electrical potentials [100]. The actuation of droplets is driven by the generated electromechanical forces on the droplets dipoles in case of the dielectric liquids and the droplets meniscus free charges in case of the conductive liquids [100]. DMF is suitable for DBS analysis because there are no channels to be clogged by any particulates present in the sample or the substrate, additionally, DMF is compatible with the mesoscale extraction, i.e. extraction of the analytes from the filter paper punches in millimeter diameter [101].

Jebrail *et al.* [100] introduced online extraction, derivatization, and analysis of the DBSs by nano-ESI-MS/MS utilizing DMF. They developed a hybrid microchip with a nano-ESI-MS emitter at a corner of the device where the nano-spray was generated by applying a high voltage to the top plate electrode of the DMF device [100]. The developed method was applied for the quantification of amino acids in DBS after online processing for one hour which included extraction with methanol containing the deuterated amino acids (ISs) and derivatization to the corresponding butyl esters to give characteristic

fragmentation patterns [100]. Another DMF device was developed for the quantification of succinylacetone in DBSs as a marker for hepatorenal tyrosinemia by nano-ESI-MS [101]. The system, indicated in Figure 1-9, was characterized by a pulled glass capillary emitter sandwiched between the two DMF device substrates, with a feedback control system introduced to enable the high-fidelity manipulation of the droplet across the DBS samples without any manual intervention through an array of twenty-two actuation electrodes [101]. Extraction efficiency of 85.2% was achieved and the LOD was 4.95 μM after the online derivatization of succinylacetone with hydrazine to the hydrazone derivative which can be quantified with tandem MS [101].

DMF devices with four independent extraction modules were developed [102, 103]. The first device was applied for the multiplexed analysis of pharmaceuticals, namely, sitamaquine, proguanil, benzethonium chloride, acetaminophen, simvastatin, and ibuprofen in DBSs [102]. The device design allowed the extraction of four DBSs in the 4 modules in parallel and each module interfaced with a nano-ESI emitter [102]. Each nano-ESI-MS analysis was obtained by serially positioning each emitter in front of the MS inlet [102]. The other device [103] was modified by exposing the indium-doped tin oxide coated glass substrates to form four hydrophilic sample anchors on the device top plate, the device was integrated with a portable mass spectrometer (25 kg) and a linear ion trap lab-scale mass spectrometer and applied for the quantitative analysis of drugs of abuse, namely, cocaine, benzoylecgonine, and codeine in dried urine spots. The LOQ of cocaine using this miniaturized system was 40 ng/mL which was found to be compatible with the required performance criteria (United Nations Office on Drugs and Crime) and the total analysis time

was less than 15 min for the four urine samples [103].

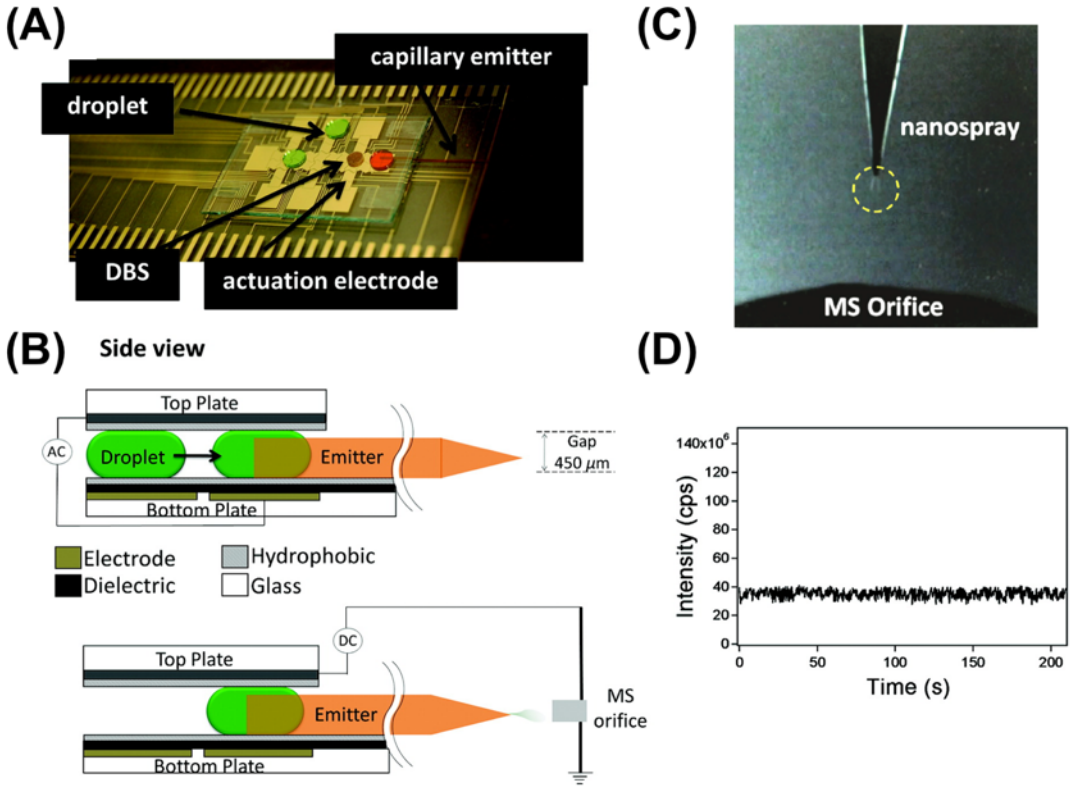

Figure 1-9. DMF-nano-ESI-MS interface. A, image of the DMF device coupled with a capillary emitter. B, schematics of the AC electric potentials applied to actuate the droplets (top) and the DC electric potentials applied to generate the nanoelectrospray (bottom). C, the generated spray. D, plot of total ion count versus time for a 15-μL droplet of tyrosine, with a RSD of 7.3% in 200 s. Reprinted from Ref. [101] with permission from American Chemical Society.

1.10 Summary and Future Perspective

At the present time, mass spectrometers offer better sensitivity and selectivity than ever, and this is anticipated to continue to improve which will increase the demand for stand-alone MS analysis. Different sample preparation techniques have been directly coupled with ESI-MS and nano-ESI-MS to augment the throughput of the analysis and eliminate the high resolution chromatographic separation without compromising the analysis reliability and sensitivity.

For the direct analysis of biofluids by ESI-MS, every hyphenated sample preparation strategy has its pros and cons as summarized in Table 1-5. In terms

of the extraction speed, online SPE using the RapidFire system required less than 7 s but many offline sample pre-treatments preceded the SPE step such as offline protein precipitation, thus not presenting fully automated sample-in/answer-out capability. The other strategies have minimum extraction times between 1 min and 5 min. Only few microliters of the sample can be analyzed by all the techniques except the conventional online SPE which required at least 50 µL of the sample. Some, such as EkE *via* the IEF syringe, extraction electrospray, and LESA can extract $\leq$ 1 µL of the crude biofluid. The variety of the coating and packing sorbents, extracting liquids and the electrokinetic system could provide more selective extraction compared to the dried samples spot extraction by LESA, extraction electrospray, and DMF approaches. In addition to the analyte extraction, online derivatization could be accomplished by the PSI cartridges, SFME, and DMF to detect proteins post-translational modifications, enhance the ionization efficiency, or monitor the enzymatic activity. At the moment, fully automated MS methods were already developed based on the SPE, LESA, EkE, and DMF techniques however the transition from the sample extraction to the MS is still the main challenge in the CBS, modified PSI, and SPME (only one SPME method is fully automated). All the techniques were adapted for the quantitative analysis of different analytes in biofluids except for LESA which is more suitable for surface imaging and was applied mostly in the biofluids qualitative analysis. Although the advanced robotics in LESA and the high performance pumps in the online SPE systems boosted the throughput of the analysis, these bulky instrumentations made both of the techniques not suitable for the on-site analysis. Furthermore, the disposable extraction devices in DIMs, SFME, extraction electrospray, and

LESA minimized the carryover between the different samples, on the other hand, the reusable devices in SPE, SPME, EkE, and DMF can reduce the operating costs but additional steps should be added for the devices washing and conditioning before every run.

Challenges including contamination, carryover, ion suppression, and device fouling can limit the sensitivity of the standalone MS technologies integrated with sample preparation. However, advancement in instrumentations such as ion mobility MS which provides an orthogonal mode of separation of isobaric compounds and future developments in microfluidics can alleviate these challenges.

The other future aspect is the development of comprehensive POC-MS systems for different clinical applications *via* coupling of the portable sample preparation tools with the miniaturized MS. Miniaturized MS instruments that are capable of direct biofluids analysis were already reported and coupled with the sample processing methods as seen in the extraction electrospray-miniature MS system. In the next few years, we expect to see more integrated portable miniature MS systems with improved performances and smaller sizes.

The electrokinetic in-syringe sample preparation approach presented in this thesis offers many advantages over the current hyphenated approaches and the widespread use of syringes makes the novel approach attractive for sample treatment in bioanalysis. Given the accuracy of the syringe in liquid handling and the ease of its integration with autosamplers, the in-syringe approach can avoid the segregation between the sample preparation event and the MS event presenting a high potential for a totally automated bioanalysis. Moreover, the ability to deal with liquid samples can allow for the monitoring of enzymatic

functions in biofluids. Additionally, the instrumental simplicity of the in-syringe systems is ideal for POC analysis. Furthermore, minute amounts of samples and organic solvents are consumed by the in-syringe approach relying on the electrokinetic techniques.

Table 1-5. Comparison between the different hyphenated techniques of biological fluids preparation for the stand-alone ESI-MS and nano-ESI-MS.

	DIMs (modified PSI and CBS)	**SPE**	**SPME**	**SFME**	**LESA**	**Extraction electrospray**	**EkE**	**DMF**
Extraction time*	2 min	7 s	2 min	Dozens of seconds	2 min	< 1 min	5 min	5 min
Biofluid volume**	5 µL	50 µL	10 µL	5 µL	0.2 µL	0.2 µL	1 µL	5 µL
Extraction selectivity	Selective	Selective	Selective	Selective	Non selective	Non selective	Selective	Non selective
Analytes derivatization	Applied	Not applied	Not applied	Applied	Not applied	Not applied	Not applied	Applied
Automation of the analysis***	Not allowed	Allowed	Allowed	Not allowed	Allowed	Not allowed	Allowed	Allowed
Suitability for the quantitative analysis	Suitable	Suitable	Suitable	Suitable	Less suitable	Suitable	Suitable	Suitable

Suitability for on-site analysis	Suitable	Not Suitable	Suitable	Suitable	Not suitable	Suitable	Suitable	Suitable
Reusability of the extraction device	Disposable	Reusable	Reusable	Disposable	Disposable****	Disposable	Reusable	Reusable

*The minimum extraction time could be achieved by one of the methods under each approach.

**The minimum biofluid volume required by one of the methods under each approach.

***The automation of the analysis is considered allowed if one of the method is fully automated.

****The pipette tips and spray nozzle tips were disposable only in the LMJ-SSP (NanoMate systems).

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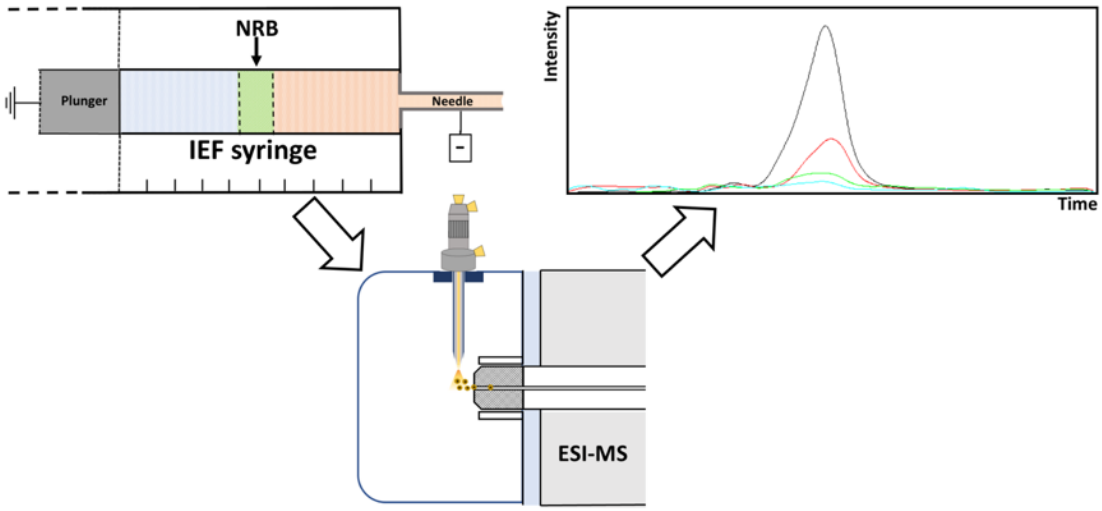
2. In-Syringe Electrokinetic Ampholytes Focusing for ESI-MS

This chapter has been published in Analytical Chemistry. 2019, Volume 91, Pages 8259-8266 as a research article. Minor changes e.g. numbering, font size, layout, and style were introduced in order to match the formatting style of the thesis, keeping the original features of the article as much as possible.

2.1 Abstract



A 25 µL analytical glass syringe has been used for isoelectric focusing (IEF) utilizing the stainless-steel needle and plunger as electrodes. The generation of protons and hydroxyl ions at the electrodes facilitated a neutralization reaction boundary (NRB) mechanism to focus different amphoteric compounds, such as hemoglobin, bovine serum albumin, R-phycoerythrin, and histidine, within minutes. After optimization of different

experimental parameters affecting the IEF process and the coupling of the IEF syringe with electrospray ionization mass spectrometry (ESI-MS), a background electrolyte (BGE) composed of NH_4Ac, 1.0 mM, pH 4.0, in 70.0% (v/v) acetonitrile was used for the IEF of histidine. A voltage of −200 V was applied for 5.0 min to accomplish the IEF and increased to −400 V during the infusion to ESI-MS at a flow rate of 4.0 μL/min. The coaxial sheath liquid consisting of 0.2% (v/v) formic acid was added at 4.0 μL/min. The detection limit was found to be 2.2 μg/mL and a nonlinear quadratic fit calibration curve was constructed for histidine over the range of 4.0−64.0 μg/mL with a correlation coefficient (r) = 0.9998. For the analysis of urine samples, only 1 μL of urine was diluted 10 times with the BGE *via* the syringe itself prior to the in-syringe IEF. The determination of histidine in spiked urine samples as relevant for the diagnosis of histidinemia was demonstrated by the IEF syringe-ESI-MS system with accuracy ranged from 88.25% to 102.16% and a relative standard deviation less than 11%.

2.2 Introduction

Despite the adoption of advanced and efficient detection technologies, such as tandem mass spectrometry (MS/MS), appropriate sample preparation is still pivotal to successful chemical analysis. The common ionization techniques for MS analysis, such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), necessitate minimization of agents that adversely affect the ionization [1]. This requires suitable sample preparation, which, despite the significant advances in automation over the last few decades, is still a major bottleneck in many analytical processes. High-throughput automated workflows are essential to be able to process the number of samples required

for large studies and for the commercial viability of an analytical laboratory. Moreover, automated sample preparation offers higher assay efficiency, better quality of data, and potential cost-savings [2]. However, accurate liquid handling and transfer between different processing steps remains a challenging aspect that needs to be overcome.

The analytical glass syringe is a common element in many analytical procedures and often the way in which a processed sample is introduced into the analytical instrument whether manually or through an autosampler. The ability to implement sample treatment within the syringe for direct coupling with a powerful detection technique, such as MS, could potentially simplify the workflow and lead to significant gains in analytical performance. Over the past few years, in-syringe systems were proposed for sample preconcentration, which could be coupled with various analytical techniques, such as electrothermal atomic absorption spectrometry [3, 4], inductively coupled plasma mass spectrometry [5], and gas chromatography−mass spectrometry [6], as well as a stand-alone system with built-in spectrophotometric detection [7]. Also, gold nanoparticle-based systems were developed for in-syringe immunosensing of biomarkers [8] and colorimetric discrimination of alanine enantiomers [9]. As a platform, the syringe provides a broad range of opportunities for new/alternative approaches for automated and integrated sample treatment.

Electrokinetic focusing techniques, such as isoelectric focusing (IEF), have long been recognized as a powerful concentration and separation approach for amphoteric molecules. The IEF concept was initiated by Alexander Kolin in 1954, who described the separation of hemoglobin and cytochrome C mixture

in a solution of varying pH stabilized by sucrose or glycerol using a cell with U-tube arms [10]. Kolin referred to such separation according to the isoelectric points (pIs) as an “isoelectric spectrum”, and the separated protein lines were sharper and more stable than the bands separated according to the electrophoretic mobility [11, 12].

IEF is typically assisted by carrier ampholytes (CAs) in gels or solutions to enforce a pH gradient and aid in resolving different targets. CAs are expensive [13] and complicate MS analysis, such that a special interface or tedious procedures are often required to remove CAs before injection of the focused targets into the MS [14]. Many scientists have explored the possibilities of instating a pH gradient generation without the use of CAs using so-called carrier ampholyte free (CAF) systems. One of the CAF isoelectric focusing (CAF-IEF) strategies is based on the formation of a pH gradient by controlling the electrolysis-driven production and movement of H^+ and OH^- from water [13, 15, 16]. Brod *et al*. designed an in situ water splitting system utilizing bipolar membranes with electrical control of the electrolytic generation of H^+ and OH^- ions along the separation channel [17]. Using this microfluidic CAF system, protein and peptide separations comparable to those obtained using conventional IEF with CAs were realized. Also, Pospićhal *et al.* [18] described a method for the concentration and separation of amphoteric molecules from their matrix using controlled flows of the H^+ and OH^- from electrode chambers at opposite sides of the separation column toward the center of the column forming an neutralization reaction boundary (NRB) between the two zones with a sharp change of pH. Amphoteric molecules with *pI*s between the pHs of either

side of the column are focused on this NRB. The NRB is reasonably stable with time and over time depleted from the nonamphoteric matrix components. Electrophoretic behavior known from isotachophoresis was observed, with adjacent sharp boundary zones of constant composition increasing in length with increasing concentration [19]. Important features of this NRB, such as its position, length, direction, velocity, and pH gap, are influenced by the current used for electrolytic generation of the solvolytic ions, sample nature and concentration, background electrolyte concentration, separation time, and additives [18-20]. Furthermore, Cao's group has developed a series of simulations accompanied by experimental data to study the moving NRB (MNRB) and to select the appropriate systems for the IEF [21-24]. One of their works included formation of a MNRB in agarose gel using HCl as an anolyte and NaOH as a catholyte, demonstrating the observed MNRB velocities at different conditions were consistent with the theoretical computations [22]. The same group used the MNRB concept to design a tunable nonimmobilized pH gradient isoelectric focusing (non-IPG-IEF) via the concept of the MNRB by selecting the appropriate pairs of catholyte and anolyte then the non-IPG-IEF was combined with polyacrylamide gel electrophoresis (PAGE) for the analysis of colon cancer cell lysates [24]. Additionally, the focusing dynamics using a pH boundary was investigated via high-resolution computer simulation studying the stacking of weak bases [25]. The formation of the NRB was utilized for the injection of focused proteins in capillary electrophoresis [26, 27], as well as being applied for preconcentration and purification of proteins prior to offline analysis by MALDI-TOF-MS [28, 29].

Here, we present the first implementation of an electrokinetic technique

within a syringe, the electroseparation syringe, exploiting the embedded stainless steel syringe needle and plunger as electrodes. Following the rich history of CAF-IEF, the electrolysis of water at the needle and plunger was used to induce a NRB to focus amphoteric molecules, such as proteins, peptides, and amino acids. In-syringe CAF-IEF was coupled with ESI-MS for the determination of clinically relevant levels of histidine in urine samples.

2.3 Experimental Section

2.3.1 Chemicals and solvents

Ammonium acetate (NH_4Ac) (assay ≥98%), glacial acetic acid, ammonium hydroxide (28−30% aqueous solution), formic acid (≥98%), L-histidine (≥99%), N,N-dimethylformamide anhydrous (DMF) (99.8%), (hydroxylpropyl) methyl cellulose (HPMC) (viscosity 2600−5600 cP, 2% in H_2O), Chromeo™ P465, hemoglobin porcine (HGB) (lyophilized powder), bovine serum albumin (BSA) (lyophilized powder, ≥96%), and R-phycoerythrin (RPE) were purchased from Sigma-Aldrich (St. Louis, USA). Chromeo™ 488 NHS-ester was purchased from Santa Cruz Biotechnology (Texas, USA), acetonitrile (LC/MS grade) was obtained from Honeywell−Burdick and Jackson (Muskegon, USA), and universal indicator UL3000 (pH 3− 11) was purchased from Chem-Supply Pty Ltd. (SA, Australia). Deionized water was produced by a Milli-Q reagent water system (MA, USA).

2.3.2 Instruments

ESI Mass spectrometry was carried out using an Agilent 6330 Ion Trap mass spectrometer (Santa Clara, CA, USA) with 6300 series TrapControl software, version 6.1, for method development and acquisition monitoring. Acquired mass spectral data were analyzed by Bruker's DataAnalysis software,

version 3.4 (Bremen, Germany). IEF experiments were conducted in a 25.0 µL SGE analytical syringe (P/N 003050) (Trajan Scientific and Medical, Australia), comprising of a borosilicate syringe barrel with 0.73 mm internal diameter (ID), stainless steel plunger, and removable needle with 0.20 mm ID, which was cut to 30.0 mm in length. The contents of the IEF syringe and sheath liquid were delivered to Agilent triple tube ESI sprayer through fused silica capillaries (ID, 50.0 µm) at accurate rates via two KDS 100 syringe pumps (Holliston, USA). Voltage was applied using an in-house built DC voltage USB power supply (power = 0.5 W, output voltage = 0 to ±3000 V, and maximum output current= 0.17 mA; University of Tasmania, Hobart, Australia). Focusing of different fluorescent labeled compounds was investigated by Dino-Lite Edge digital fluorescence microscope (model number AM4115T-GFBW) (Taiwan).

2.3.3 IEF experiments

The IEF experiments were implemented in a 25.0 µL SGE analytical syringe (Trajan Scientific and Medical, Australia). Voltage was applied using an in-house built high voltage USB power supply connected to the stainless-steel needle and plunger through alligator clips. The needle served as the cathode and the plunger served as the ground. NH_4Ac was selected as a background electrolyte inside the IEF syringe because of its volatility and MS compatibility. Under optimized conditions, NH_4Ac, 1.0 mM, pH 4.0, in 70.0% (v/v) acetonitrile was used for the IEF of histidine.

2.3.4 Labeling of BSA and histidine with Chromeo dyes

Labeling of BSA and histidine with Chromeo™ 488 NHS-ester and of BSA with Chromeo™ P465 were performed according to the vendor protocols [30, 31]. Chromeo™ 488 labeled compounds have absorption and emission maxima

(λ_{ex} and λ_{em}) of 488 and 517 nm, respectively [30], and the λ_{ex} and λ_{em} of the Chromeo^{TM} P465 conjugated proteins are 465 and 630 nm, respectively [31].

2.3.5 System optimization and coupling with ESI-MS for histidine determination

The parameters studied in the system optimization and the interface with ESI-MS are described in the "2.8 Supporting Information".

2.3.6 Construction of the calibration curve

The standard solution of histidine (1.0 mg/mL) was prepared in Milli-Q water and sonicated for 5.0 min. The standard solution was kept in a refrigerator at 4 °C and found to be stable for 7 days under these conditions. Working solutions were prepared by transferring specific volumes of standard solution quantitatively into a set of glass vials to obtain final concentrations of 4.0−64.0 µg/mL. To each vial, 100.0 µL of 10.0 mM NH_4Ac (pH 4) was added, followed by 700.0 µL of acetonitrile, and the final volume was completed to 1000.0 µL by adding Milli-Q water. Also, a blank solution was prepared by following the same procedures except the addition of histidine standard solution. Aliquots (10.0 µL) of the working solutions and the blank solution were aspirated by the IEF syringe and −200 V was applied for 5.0 min. Following the CAF-IEF process, the solutions were injected to ESI sprayer with a flow rate of 4.0 µL/min, while keeping a −400 V applied. The interface included a coaxial flow of the sheath liquid (SL) (0.2% (v/v) formic acid) with a flow rate of 4.0 µL/min. A quadratic fit calibration graph was constructed by plotting the average peak heights (n= 3) from the extracted ion electropherograms (EIE) (*m/z* 156.0 ± 0.1) (migration time = 2.1 ± 0.1) versus the corresponding drug

concentration in micrograms per milliliter.

2.3.7 Analytical performance for histidine determination

The developed method accuracy, precision, limit of quantitation (LOQ), and limit of detection (LOD) were assessed according to the ICH Q2 (R1) recommendations [32]. The accuracy and precision were investigated by analyzing histidine standard solutions at 4.0, 8.0, 16.0, 32.0, and 64.0 µg/mL. The accuracy was determined as the percentage recovery of each nominal concentration using the mean (n= 3) of experimentally obtained concentrations from calibration curve.

The precision of the method, that is, repeatability and intermediate precision, was expressed by the relative standard deviation (RSD) using the standard deviation and the mean of the assayed triplicates of each concentration [33].

LOD and LOQ were calculated based on the standard deviation of the blank:

$LOD = x_B + 3s_B$ [33]

$LOQ = x_B + 10s_B$ [33]

where x_B is the analyte concentration giving a signal equal to the blank signal and s_B is the standard deviation of the blank.

2.3.8 Application to the analysis of spiked urine samples

Urine samples obtained from a healthy male volunteer were spiked by adding 4.0, 8.0, and 16.0 µL of histidine stock solution (10.0 mg/mL) to polypropylene tubes, and for each tube, urine was added to 1.0 mL (to give samples with added histidine concentrations of 40, 80, and 160 µg/mL). The unspiked and spiked urine samples were double filtered using a 0.45 µm filter and stored at 4 °C.

Each urine sample was diluted 10 times by first aspirating 1.0 µL of the BGE (1.1 mM NH_4Ac, pH 4 in 77.8% (v/v) acetonitrile) followed by 1.0 µL of urine sample then 8.0 µL of the BGE using the IEF syringe. This effectively resulted in a 10× dilution of the urine in 70.0% (v/v) acetonitrile containing 1.0 mM NH_4Ac as a BGE. The procedures and conditions described in the "2.3.6 Construction of the calibration curve" section were followed; the obtained peak heights (n = 3) from the EIEs (*m/z* 156.0 ± 0.1) were plotted versus the final concentrations of histidine (µg/mL) to give a quadratic fit calibration graph, and the corresponding regression equation was derived. The accuracy of the developed approach for histidine determination in the spiked urine samples was determined by the ratio of the found and the added concentrations and both of repeatability and intermediate precision were expressed by the relative standard deviation (RSD). The accuracy, repeatability, and intermediate precision, were determined by using 9 of spiked urine samples diluted with the BGE to give 3 different levels of added histidine concentrations: 4.0, 8.0, and 16.0 µg/mL.

2.4 Results and Discussion

2.4.1 Mechanism of NRB formation inside the IEF syringe

The stainless steel needle and plunger of the IEF syringe were utilized as electrodes to apply voltage across the glass barrel filled with a NH_4Ac based background electrolyte (BGE). Despite the traditional tendency to use of Pt as inert electrode material for application of the separation potential in electrophoresis, stainless steel has been used as a cost-effective and available material for the high voltage application in the electrophoretic separations [34-36]. Its suitability as HV electrode material was demonstrated by its use to apply

a voltage of 25 kV (90 s) for 240 consecutive separations without compromising separation performance [34]. In another work, stainless steel electrodes were used in an automated, online system used for the monitoring of cell cultures for four consecutive days, again without any indication of electrode deterioration or changes in separation performance [35].

In this work, the syringe needle was used as a cathode and the stainless-steel plunger was used as ground. When the voltage is applied, the reduction of water at cathode results in the production of OH^- ions and simultaneously, generation of H^+ ions at ground electrode due to water oxidation [13]. Thus, there is a flux of OH^- ions from the cathode and a flux of H^+ ions from the ground toward the center of the syringe barrel. When and where these two fluxes meet, a NRB is formed between acidic and basic solutions in the syringe barrel [18-20, 26-29], allowing amphoteric molecules with *pI* values within the NRB pH gap to be focused as indicated in Figure 2-1.

The span of the pH gap could be reduced by changing the flux of the solvolytic ions to allow the separation of a mixture of proteins [18], but this would require a more sophisticated system/platform than what is presented here. The formation of the NRB was confirmed experimentally by applying the voltage across 10.0 µL of a solution containing 80% (v/v) universal indicator and 5.0 mM NH_4Ac, pH 7 inside the syringe. The syringe barrel was coated with 1.0% (w/v) HPMC prior to filling the barrel with the universal indicator in BGE to limit the electroosmotic flow. Figure 2-2 shows a gradual increase of the red color originating from the ground side (plunger) after the application of −50 V indicating the generation of a H^+ flux. Likewise, a purple color developed

originating from cathode (needle) due to the OH^- flux. Flow instabilities were observed at the interface between the two fluxes for about 1 min prior to the formation of a relatively stable NRB.

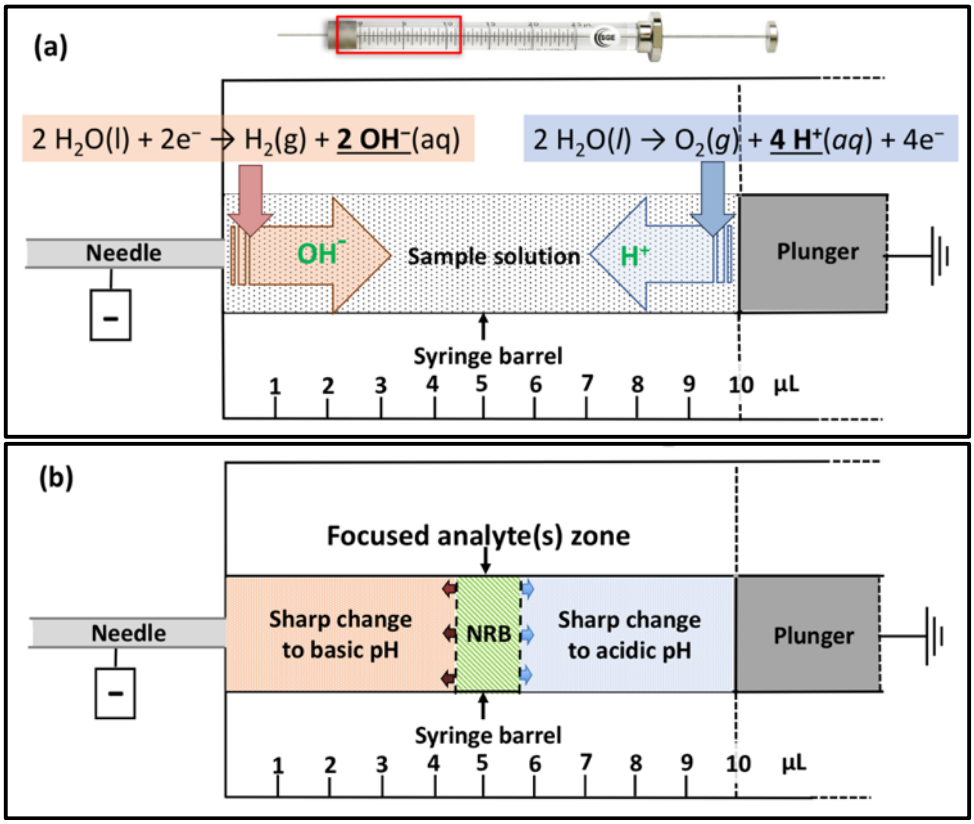


Figure 2-1. Mechanism of NRB formation inside the IEF syringe due to the electrolysis of water. a, application of electric field on sample solution utilizing the stainless-steel needle and plunger as electrodes in a 25.0 µL glass syringe and generation of OH^- and H^+ fluxes from the cathode and ground, respectively. b, formation of the NRB and focusing of the amphoteric compounds having *pI* values within the NRB pH range.

These instabilities may be caused by differences in ionic conductivity where the H^+ and OH^- fluxes meet. Convective instabilities accompanying the electrokinetic phenomena have been reported to occur in the presence of electrical conductivity gradients [37, 38]. At the interface of the conductivity gradients, charge accumulation leads to electric body forces, which drive the flow into unstable dynamics [37, 38]. The stable NRB was formed within 3 min under the conditions described, and a video recording of the process is provided (Movie S-1).

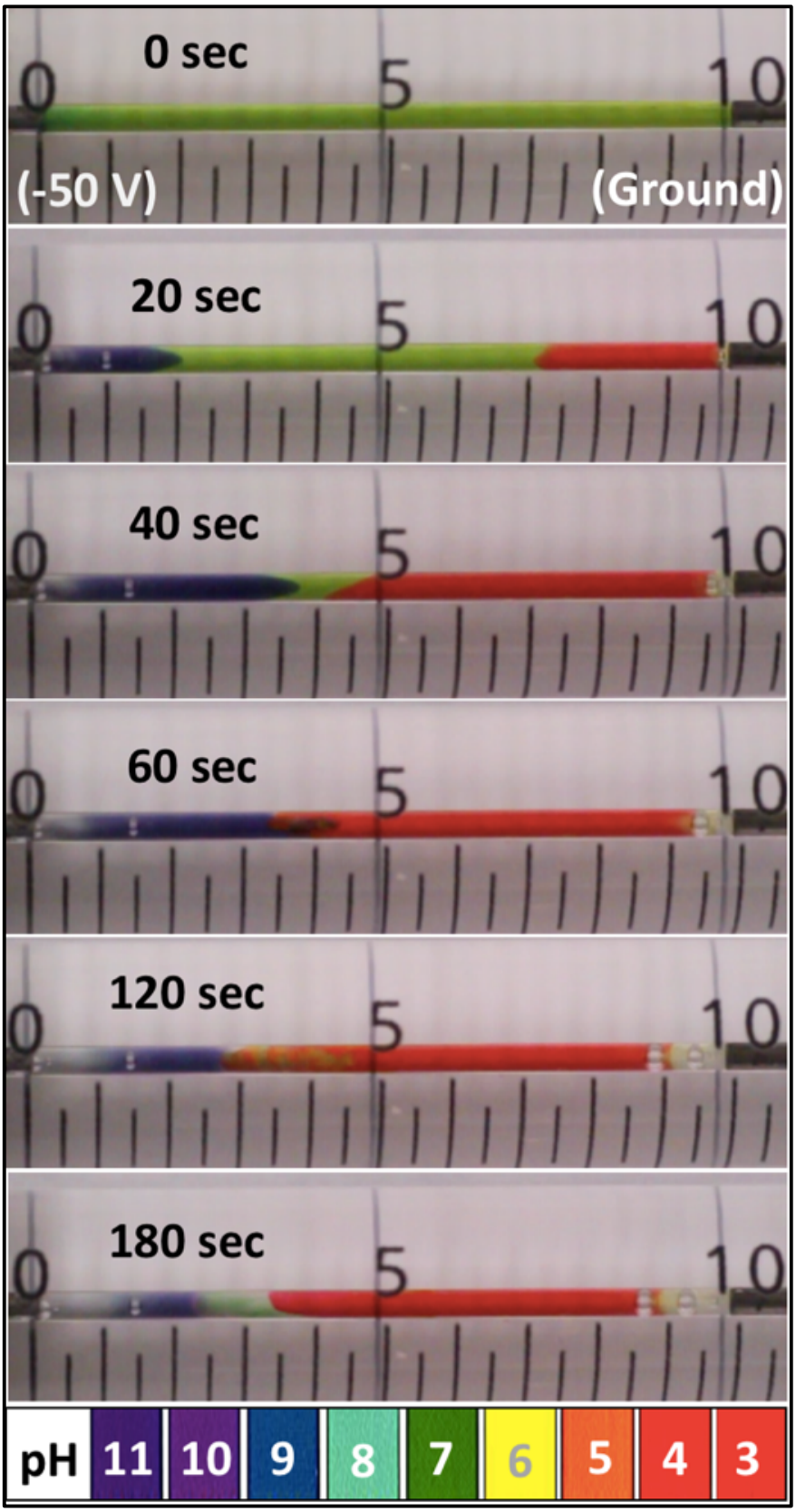


Figure 2-2. Demonstration of the pH step gradient for CAF-IEF using a color indicator. The color pattern at different time intervals during the application of a potential difference of 50 V across the stainless-steel needle and plunger of a 25 µL syringe containing 10 µL of a solution of 80% (v/v) universal indicator (pH 3−11) and 5.0 mM NH_4Ac (pH 7) shows the changing pH along the barrel. The syringe was coated with 1.0% (w/v) HPMC before use.

As illustrated in Figure 2-2, the pH gradient is a step gradient from approximately pH 3 to pH 11 positioned approximately 3−4 mm from the needle. The formation of a sharp boundary agrees with other reports [18-22,26,28,29], and its formation was found to be repeatable under the same conditions [21,22]. Its position is determined by the electrophoretic mobility ratio of H^+ and OH^- ions [13], and the NRB will be stagnant only if the fluxes of the solvolytic ions (H^+ and OH^-) are balanced [18, 29]. The syringe based method was used to concentrate proteins with *pIs* within the NRB pH gap, including Chromeo™ labeled BSA (*pI* 4.7), R-phycoerythrin (*pI* 4.2), and hemoglobin (*pI* 6.9).

Figure 2-3 illustrates the CAF-IEF of Chromeo™ P465 labeled BSA at 60 s intervals over 300 s and Figure S-1 shows the focused bands of R-phycoerythrin and hemoglobin, as well as the bands for a sample containing a mixture of Chromeo™ 488 labeled BSA and hemoglobin. While baseline separation was not achieved, the higher *pI* value of hemoglobin leads to its focusing closer to the cathode (needle), while BSA with lower *pI* focuses closer to the ground (plunger). Movies S-2, S-3, Movie S-4, and S-5 display the IEF of Chromeo™ 488 labeled BSA, R-phycoerythrin, hemoglobin, and a mixture of Chromeo™ 488 labeled BSA and hemoglobin, respectively.

The control of bubble formation in the miniaturized system is challenging owing to the generation of H_2 and O_2 during the electrolysis of water. This can lead to the formation of gas bubbles, which can distort the focused analyte band or the electric field. In the described system, the electrolysis of water is required to form the NRB and the separation channel (the syringe barrel) is not isolated from the electrodes (the needle and the plunger). To control bubble generation,

a simple strategy was used by adding acetonitrile because this decreases the amount of water available for electrolysis and minimizes the bubble size by decreasing the surface tension of the solution [39].

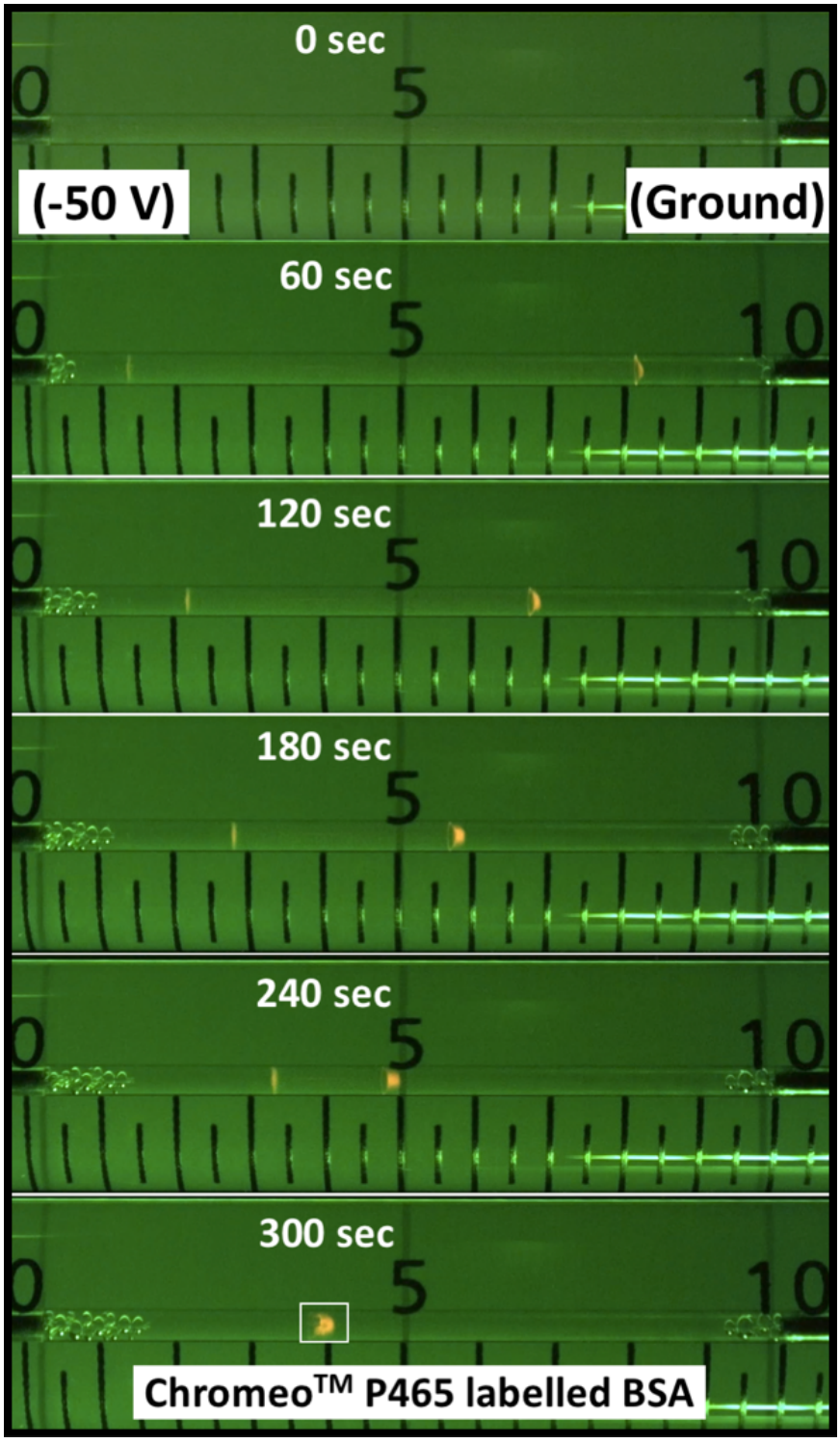


Figure 2-3. CAF-IEF of BSA (*pI* 4.7) (100.0 µg/mL), labeled with Chromeo™ P465, at different time intervals using applied voltage of −50 V to the needle, 5.0 mM NH_4Ac (pH8) as a background electrolyte and HPMC (1.0% (w/v)) coated IEF syringe.

2.4.2 System optimization and coupling with ESI-MS for histidine determination

The potential of CAF-IEF in a syringe for sample preparation is demonstrated with the analysis of the amino acid histidine (*pI* 7.47) in urine samples by ESI-MS. Histidinemia is an autosomal recessive disorder resulting from a histidase enzyme deficiency that can be detected by elevated histidine levels in body fluids [40]. The reference histidine range in healthy adult urine is 130−2100 μM (20−326 μg/mL) [41].

Previously reported methods relied on dilution, in addition to purification by centrifugation [42], ultrafiltration [43]' or perchloric acid precipitation [44]. Here, the syringe was used to aspirate 1.0 μL of filtered urine and a total of 9.0 μL of BGE, Movie S-6 indicates the homogeneous dispersion of 1% cochineal red dye diluted by the same pattern of the urine sample with the BGE inside the IEF syringe's barrel. Upon application of a potential difference across the plunger and needle of the same syringe, CAF-IEF was used to focus the histidine, followed by actuation of the plunger for infusion of the focused band into the ESI-MS. Selected ion monitoring at *m/z* 156.0 allowed for the selective detection of histidine without the need for additional purification steps other than a simple filtration procedure.

The experiments were performed using an uncoated 25 μL syringe as HPMC adversely affects the precision of MS detection. An electrolyte containing 1.0 mM NH_4Ac in 70% acetonitrile was used as a volatile BGE to be compatible with ESI-MS. A molar strength of 1.0 mM was enough to render the required conductivity without excessive gas generation at the electrodes. Gas generation was further minimized though the use of 70.0% (v/v) acetonitrile,

decreasing the amount of water available for electrolysis (Figure S-2). The position of the NRB moved closer to the cathode (needle) with decreasing starting pH of the BGE, as evidenced by the position of the focused band shown in Figure S-3. To minimize the diffusion during infusion into the MS, close proximity of the focused band to the needle was preferred and a BGE pH of 4.0 was selected for further experiments.

When coupling capillary isoelectric focusing (CIEF) to MS, a sheath-flow is often required [45], employing a dedicated interface to mix the sheath liquid with CIEF effluent to allow for stable electrophoretic separations and optimum ionization. The sheath liquid may contain an organic solvent or a volatile electrolyte, such as formic acid [45]. To interface the IEF syringe with the ESI-MS, a coaxial sheath flow of sheath of 0.2% (v/v) formic acid in water was used (Figure S-4). A flow rate of 4.0 µL/min provided a good compromise between sensitivity and precision, decreasing the sheath liquid flow rate gave a higher sensitivity at the expense of precision as shown in Figure S-5. The infusion conditions were established by infusing histidine using a syringe without application of a voltage for CAF-IEF.

Employing in-syringe CAF-IEF, the influence of the applied voltage during both the IEF and infusion steps on the efficiency was studied by ESI-MS (EIEs, *m/z* 156.0 ± 0.1), determining the peak's full width at half-maximum (FWHM) as a measure of efficiency. To delineate between CAF-IEF and infusion, two sets of experiments were done: first the CAF-IEF voltage was varied with the infusion voltage set at 200 V (Figure 2-4, red trace), and then, the voltage during infusion was varied with the CAF-IEF voltage set at 200 V (Figure 2-4, green trace). Although the FWHM at voltages of −250 and −300 V was slightly smaller

than the FWHM at −200 V, −200 V was selected for further experiments as a decrease in sensitivity was noted for higher CAF-IEF voltages. This decrease in sensitivity may be due to increased Joule heating, which may result in the inner cyclization of histidine [46]. The decrease in FWHM with increasing voltage applied during infusion, see Figure 2-4, shows the importance of applying a voltage (−400 V) during infusion to keep the band focused.

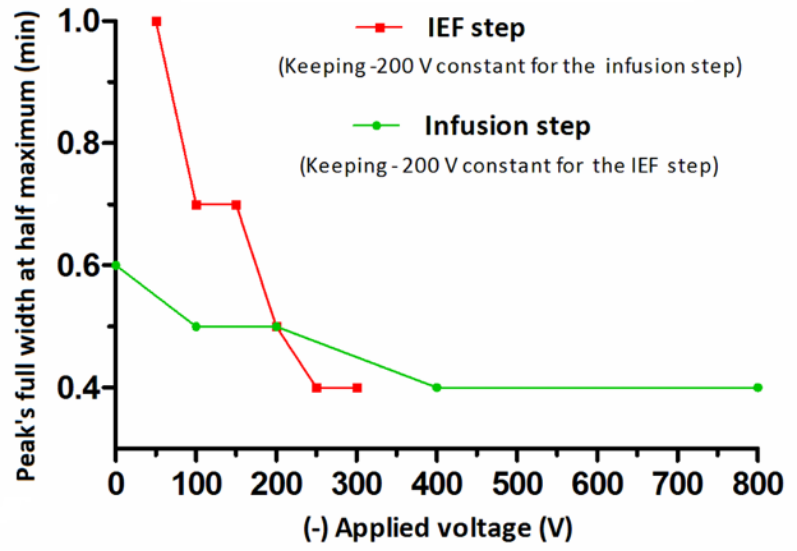


Figure 2-4. Effect of the applied voltage on the peak width at half height, determined by monitoring signal intensity by ESI-MS (EIEs, *m/z* 156.0 ± 0.1). CAF-IEF of histidine (30.0 µg/mL) for 5.0 min before infusion into ESI at a flow rate of 4.0 µL/min.

The CAF-IEF syringe coupled with the ESI-MS was successfully applied for the quantitative determination of histidine (*m/z* 156.0) from standards. Compared with direct infusion ESI-MS, the signal intensity increased 6.5-fold using CAF-IEF, as shown in Figure S-6. Moreover, an enhancement factor of 8.5 was achieved using the CAF-IEF syringe for the analysis of histidine in urine (Figure 2-5). The mass spectra recorded at 2.2 min of infusion with and without the use if the CAF-IEF step (Figure 2-5b and c, respectively) showcase the increased signal intensity when using CAF-IEF. The two dominant ions are creatinine (*m/z* 114.1; *pI* 11.19) and histidine (*m/z* 156.1; *pI* 7.47), both showing an increase in intensity when using CAF-IEF.

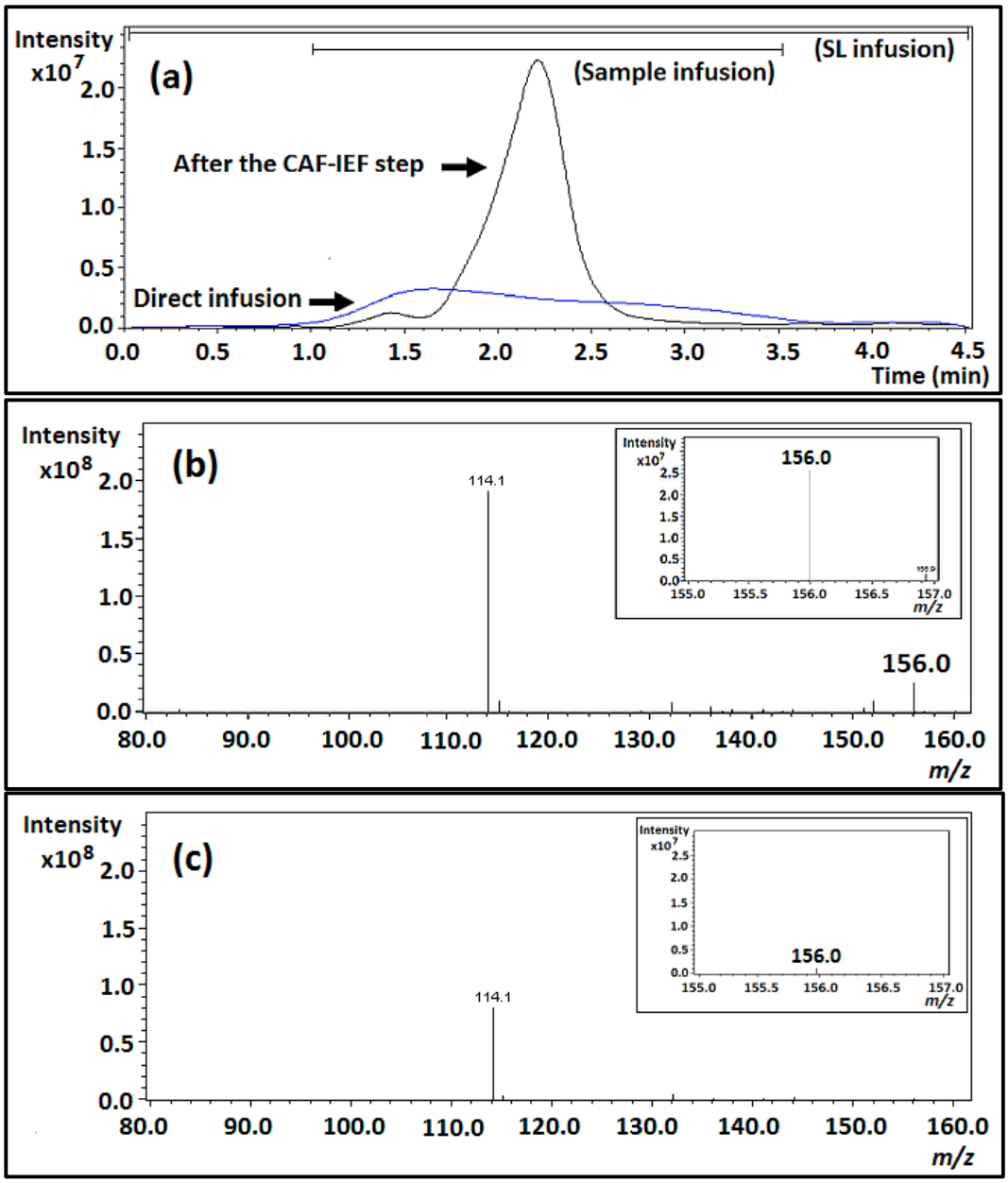


Figure 2-5. a, EIEs (*m/z* 156.0 ± 0.1) of urine samples spiked with 16.0 µg/mL histidine (concentration after 10× dilution in the syringe) using direct infusion (blue) and CAF-IEF (black). b, mass spectrum of urine sample spiked with 16.0 µg/mL histidine, infusing for 2.2 min after CAF-IEF step. The inset represents a magnified view of the ion region *m/z* 155−157. c, mass spectrum after 2.2 min infusion without application of the CAF-IEF step (direct infusion). The inset represents a magnified view of the ion region *m/z* 155−157.

2.4.3 Analytical performance for histidine determination

In the absence of a real biological blank, a calibration curve and a range of analytical attributes including accuracy, precision, range, LOD, and LOQ were determined using histidine standard solutions. A plot of peak height (EIEs, *m/z* 156.0 ± 0.1) versus concentration was fit with a nonlinear quadratic

equation ($y = -1922000 + 1450000x + 51918x^2$) with a range of 4.0−64.0 µg/mL and correlation coefficient (r) = 0.9998. The method accuracy and precision data are summarized in Table 2-1. Considering the 10-fold dilution of urine in BGE in the syringe, the LOD and the LOQ of histidine were found to be 22 µg/mL and 36 µg/mL, respectively, the calculations of the method's LOD and LOQ are indicated in Table S-1. Clinically, histidine levels in urine are considered normal below 2100 µM (326 µg/mL) [41], indicating that the sensitivity and dynamic range of the developed method allow for clinical decision making regarding histidinemia when using the in-syringe 10-fold dilution in BGE, with no further sample preparation than a simple filtration step.

Additionally, another calibration graph for the spiked urine samples was constructed mainly to evaluate the accuracy and the precision of the spiked histidine determination in the presence of the endogenous histidine, urine samples obtained from a healthy volunteer were spiked with histidine (0.0, 40.0, 80.0, and 160.0 µg/mL) prior to the 10× dilution with the BGE using the IEF syringe. The EIEs (*m/z* 156.0 ± 0.1) are presented in Figure S-7. The correlation between the peak height and concentration can be represented by a polynomial equation ($y = 1\ 343\ 000 + 58\ 975x + 80\ 831x^2$) with a regression correlation coefficient (r) of 0.9997. The calibration curves have been shown in Figure S-8. The quadratic fit regression has been used many times before with the ESI-MS technique even after coupling with the separation techniques and the addition of internal standards [47, 48]. Generally, the spray droplets saturation with the analyte molecules and the more relaxation of the ions internal energy with the higher analyte concentration (in case of molecular ion monitoring) are the common reasons for the nonlinear calibration graphs with ESI-MS [49]. The

accuracy ranged from 88.25% to 102.16% and the precision data were determined at 4.0, 8.0, and 16.0 µg/mL (n = 3) and found to be satisfactory with RSD less than 11% (Table 2-1).

Table 2-1. Accuracy and precision for the determination of histidine by CAF-IEF syringe-ESI-MS

Matrix	Concentration (µg/mL)	% Recovery	Repeatability *RSD (%) (n=3)	Intermediate precision *RSD (%) (n=3)
Standard solutions	4.0	94.72	3.54	7.58
	8.0	91.86	8.37	4.72
	16.0	92.98	7.14	8.74
	32.0	102.16	4.33	6.10
	64.0	99.89	0.69	9.90
Spiked Urine samples	**Concentration (µg/ml)**	**% Found**	**Repeatability *RSD (%) (n=3)**	**Intermediate precision *RSD (%) (n=3)**
	40.0	88.25	9.86	10.66
	80.0	102.16	3.14	9.97
	160.0	99.91	4.82	9.24

*relative standard deviation, RSD = 100 S/ $\bar{x}$

The required time for the analysis of 1.0 µL urine using the CAF-IEF syringe-ESI-MS system is 7.5 min comprising 5.0 min for the CAF-IEF step and 2.5 for the infusion step. Only 7 µL of acetonitrile is required for the CAF-IEF step, a significant reduction in comparison with protocols used for solid-phase extraction (SPE) prior to HPLC-MS/MS analysis, where for example 100 µL of urine and 15 mL of methanol were used conditioning, washing, and eluting in the SPE protocol for the analysis of amino acids in urine [50].

Considering no CAs are used, the CAF-IEF syringe provides a simple and economic system for the preconcentration of the amphoteric compounds (*pI* 3−11) within minutes. Performing the focusing step within a syringe facilitates accurate dilution of the sample in BGE, as well as compatibility with online coupling with the ESI-MS utilizing a syringe pump. Without further control

on the generation of solvolytic ions, the current approach is limited to the use of a step gradient in pH and, therefore, is not able to resolve proteins based on *pI*. Also, the generation of gas bubbles due to water electrolysis increases experimental variability, but through the use of ACN in the BGE, the RSD for the determination of histidine in urine by CAF-IEF syringe-ESI-MS system was < 11%, which is acceptable. Future research, however, will be required to further suppress or prevent bubble generation during the IEF step.

2.5 Concluding Remarks

This study describes the first use of a syringe for electrokinetic separations, as demonstrated by isoelectric focusing of amphoteric molecules by CAF-IEF. The use of a volatile BGE without carrier ampholytes make this method fully compatible with ESI-MS. The proposed method was effective in reducing ion suppression due to matrix effects for amphoteric analytes, a significant challenge in the analysis of biological samples by ESI-MS. The use of a syringe, an established liquid handling device, allows for sample dilution, electrokinetic focusing of the targeted compounds and infusion to ESI-MS using a single platform. With liquid handling driven by a syringe pump, this approach opens up new opportunities for integrated and automated workflows for complex samples, for example, online coupling with ESI-MS.

2.6 Acknowledgments

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2.8 Supporting Information

System optimization and coupling with ESI-MS for histidine determination

Chromeo™ 488 labelled histidine was used for the optimization of experimental parameters affecting the isoelectric focusing (IEF) step including NH_4Ac molar strength, starting pH, and fraction of acetonitrile. Unlabelled histidine was used to optimize both the IEF step and infusion of the focused band into the MS.

The infusion of the concentrated histidine band from the IEF syringe to triple tube ESI sprayer (mounted in front of the Ion Trap MS inlet) was achieved through fused silica capillary (250 mm length, 360.0 µm OD, 50.0 µm ID, and 0.5 µL volume) connected to the IEF syringe needle by MicroTight® fitting. This sprayer allowed co-axial flow of sheath liquid (SL) consisting of 0.2% (v/v) formic acid. Two separate syringe pumps were utilized to infuse the focused histidine zone and the sheath liquid at a flow rate of 4.0 µL/min while the voltage along the IEF syringe was kept at -400 V.

The ESI-MS was performed in a positive mode of ionization with the following source settings: nebulizer gas pressure, 5.0 psi; dry gas temperature, 365°C; dry gas flow rate, 4.0 L/min; electrospray voltage of the ion source, -4500 V; skimmer, 24.9 V; capillary exit, 87.5 V; target mass, *m/z* 156; scan range, *m/z* 50−200; with the use of ion charge control; smart target, 200000; maximum accumulation time, 100 ms. DataAnalysis software was used to integrate mass spectra and to produce the corresponding extracted ion electropherograms (EIE) (*m/z* 156.0±0.1). EIEs were smoothed using the Gauss smoothing algorithm at 2.003s.

Table S-1 Calculations of the limit of detection and limit of quantitation of histidine by the developed CAF-IEF syringe-ESI-MS method

Blank solution	Height of the signal at migration time 2.1 min	x_B* (µg/ml)	Mean (µg/ml)	Standard deviation (s_B)	LOD** (µg/ml)	LOQ*** (µg/ml)
Replicate 1	727891	1.721	1.675	0.189	2.2	3.6
Replicate 2	915697	1.836				
Replicate 3	317335	1.467				

*x_B is the analyte concentration giving a signal equal to the blank signal, obtained from the calibration equation:
$y=-1922000+1450000*x+51918*x^2$, where y is the response (the signal height) and x is the corresponding analyte concentration.
** LOD = x_B+3S_B [1]
***LOQ= x_B+10S_B [1]

Supplementary Videos

https://cloudstor.aarnet.edu.au/plus/s/cKtxPHCVJAdyhxG
Movie S-1. Formation of the neutralization reaction boundary (NRB) within the isoelectric focusing (IEF) syringe, coated with (1.0% (w/v) HPMC by application of -50 V using the stainless-steel needle and plunger on 10 µL solution containing 80 % (v/v) universal indicator (pH 3-11) and 5.0 mM NH_4Ac (pH 7).

https://cloudstor.aarnet.edu.au/plus/s/4xJl07SIm68ByfX
Movie S-2. Focusing of Chromeo™ 488 labelled BSA (*pI* 4.7, 100 µg/mL) by the IEF syringe coated with (1.0% (w/v) HPMC using a BGE composed of 5.0 mM NH_4Ac (pH 8) and applied voltage of -50 V.

https://cloudstor.aarnet.edu.au/plus/s/oJV1RDV7uXspXA2
Movie S-3. IEF of R-phycoerythrin (*pI* 4.2, 40.0 µg/mL) using 5.0 mM NH_4Ac (pH 8) as a BGE.

https://cloudstor.aarnet.edu.au/plus/s/2WA4QnMtaeYH9Xc
Movie S-4. IEF of haemoglobin, (*pI* 6.9, 350.0 µg/mL) using 5.0 mM NH_4Ac (pH 8) as a BGE.

https://cloudstor.aarnet.edu.au/plus/s/6Z3y15MFT06GU7f
Movie S-5. IEF of a mixture of Chromeo™ 488 labelled BSA (100 µg/mL) and haemoglobin (350.0 µg/mL) using 5.0 mM NH_4Ac (pH 8) as a BGE.

https://cloudstor.aarnet.edu.au/plus/s/QW5VDsPFrimqdPg
Movie S-6. Aspirating 1 µL of cochineal red dye between 1 µL and 8 µL of the BGE, respectively, using the IEF syringe.

Supplementary Figures

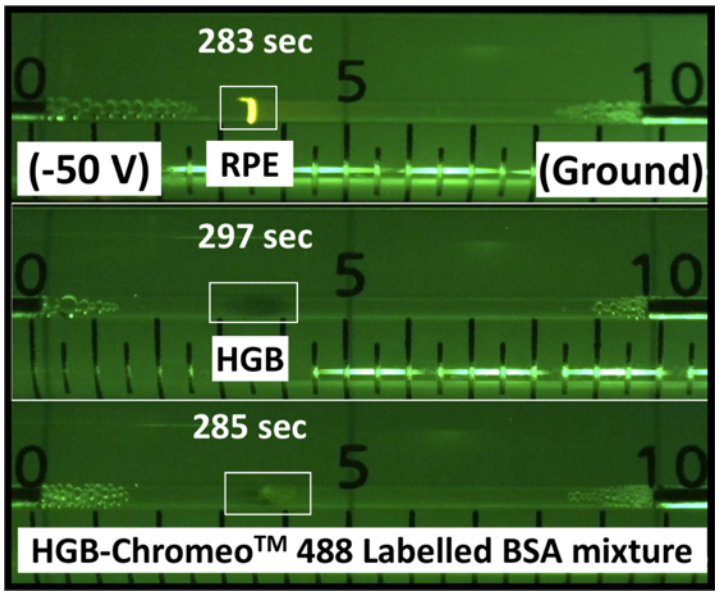
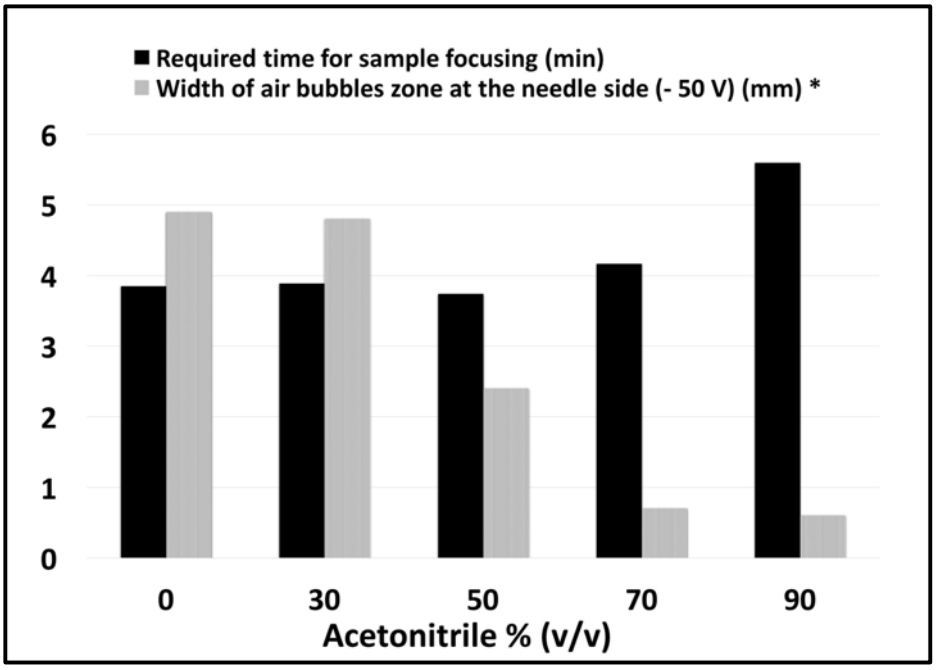
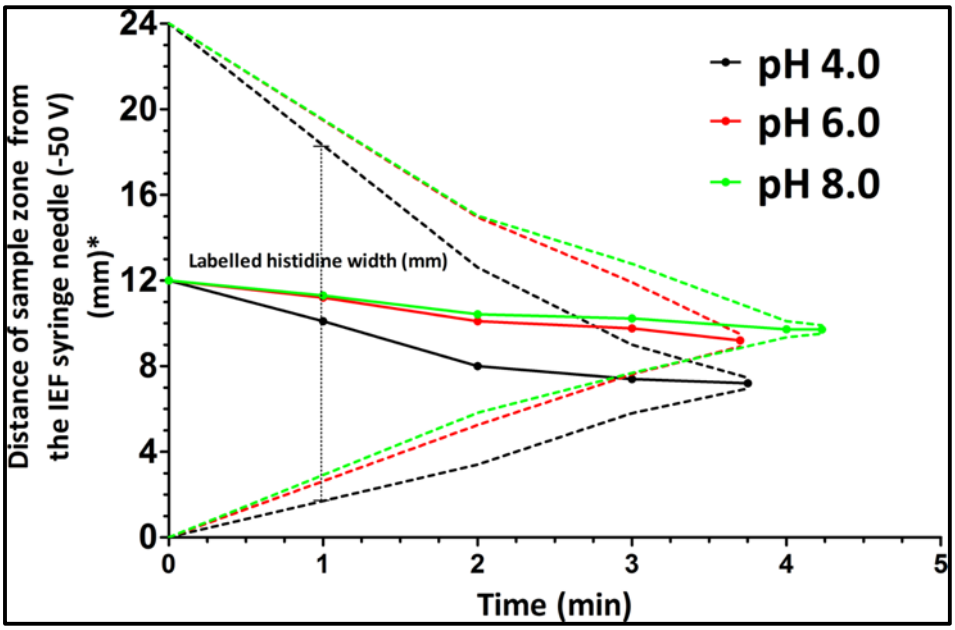


Figure S-1. Different proteins after the IEF process: RPE (R-phycoerythrin, *pI* 4.2, 40.0 µg/mL); HGB (haemoglobin, *pI* 6.9, 350.0 µg/mL); and mixture of Chromeo™ 488 labelled BSA (*pI* 4.7, 100 µg/mL) and HGB (350.0 µg/mL) with the IEF syringe (coated with 1.0% (w/v) HPMC) using 5.0 mM NH_4Ac (pH 8) as a background electrolyte.



*Width was measured after the IEF process with ImageJ 1.50i software.

Figure S-2. Effect of acetonitrile on the gas generation at the needle side and the time required for the IEF of Chromeo™ 488 labelled histidine (150.0 µg/mL).



*The distance was measured from the midpoint of the Chromeo™ 488 labelled histidine band to the needle.

Figure S-3. Effect of the starting pH on the time required for the IEF of Chromeo™ 488 labelled histidine (150.0 µg/mL) and the position of the focused histidine band inside the syringe barrel.

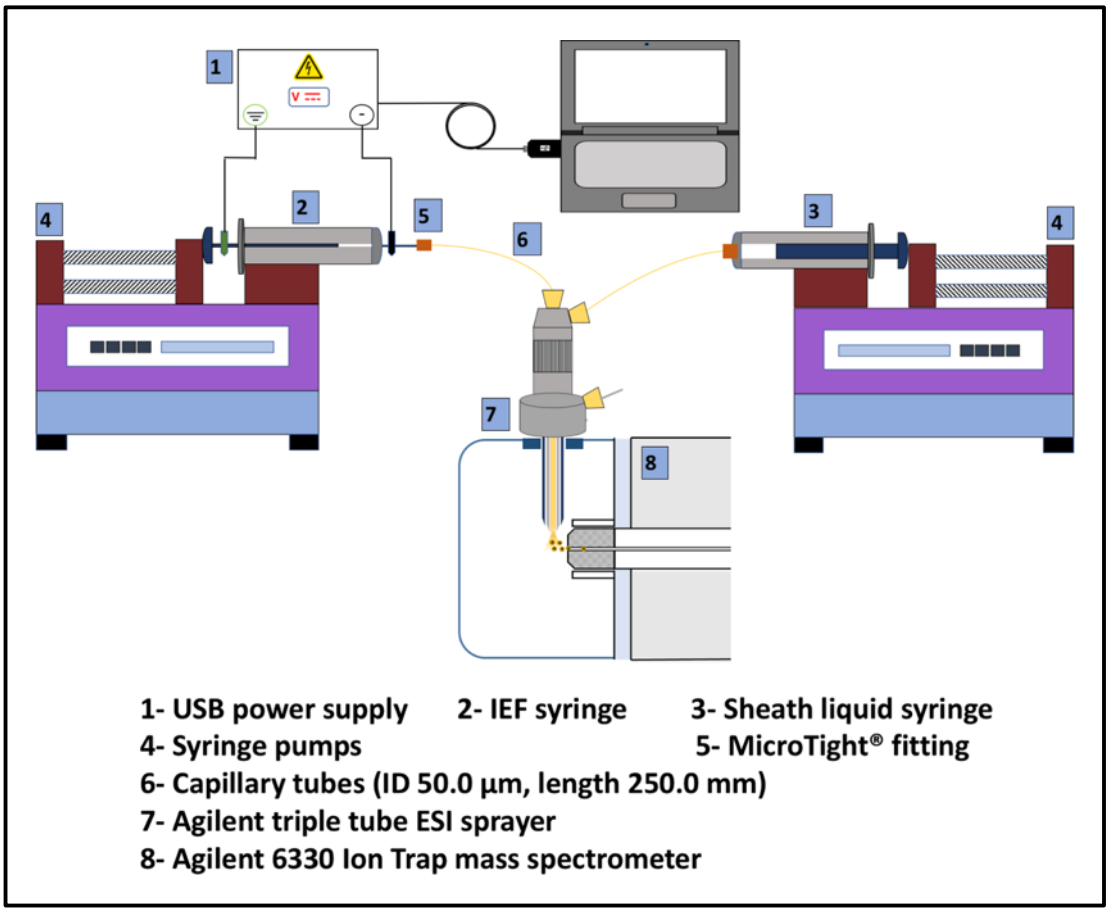


Figure S-4. Coupling of IEF syringe with ESI-MS for histidine determination (IEF syringe-ESI-MS interface).

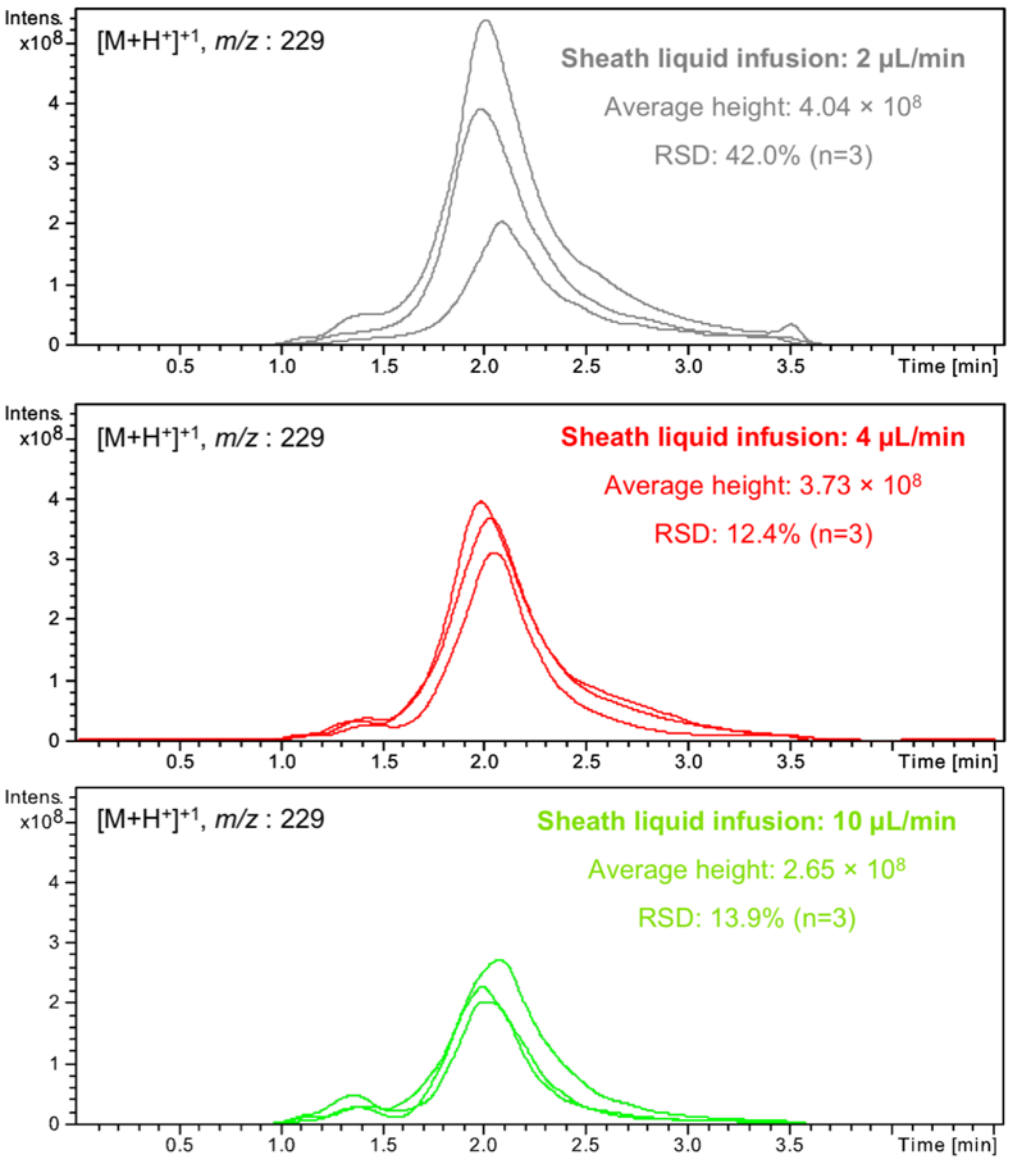


Figure S-5. Effect of the flow rate of the sheath liquid on the sensitivity and repeatability of the analysis. EIEs (*m/z* 156.0±0.1) of histidine solutions (30.0 µg/mL) infused with a flow rate of 4.0 µL/min after application of the IEF step using different flow rates of the sheath liquid, 0.2 % formic acid.

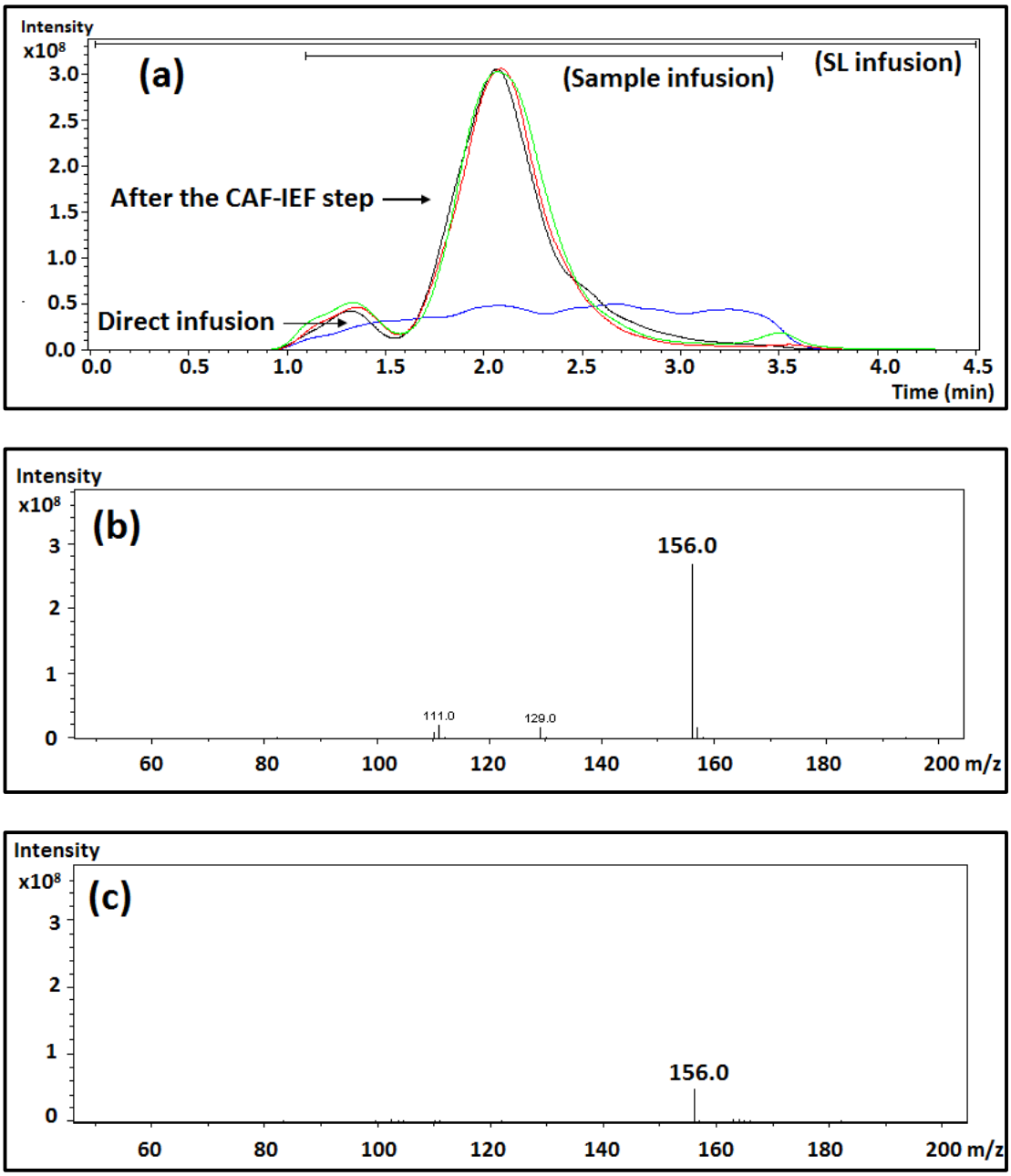


Figure S-6. a, EIEs (*m/z* 156.0±0.1) of standard histidine solutions (64.0 µg/mL) infused with a flow rate 4.0 µL/min with a co-axial flow of the sheath liquid (4.0 µL/min); after application of the IEF step (three replicates) and without application of the IEF step. b, mass spectrum of histidine (64.0 µg/mL) at migration time 2.1 min after application of the IEF step. c, Mass spectrum of histidine (64.0 µg/mL) at migration time 2.1 min without application of the IEF step (direct infusion).

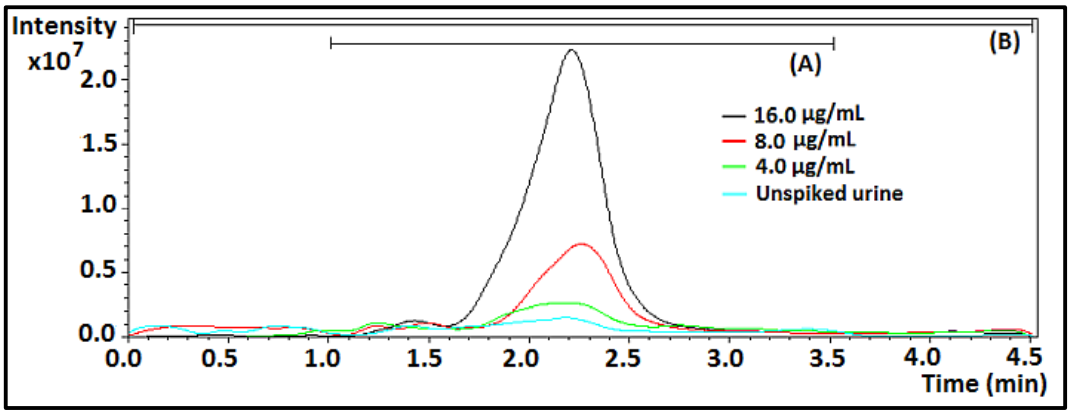


Figure S-7. EIEs (*m/z* 156.0±0.1) of CAF-IEF of untreated and spiked urine samples. Urine samples spiked with 4.0, 8.0, and 16.0 µg/mL histidine (concentration after the 10x dilution in the syringe); IEF syringe infusion with a flow rate 4.0 µL/min (A); a co-axial flow of sheath liquid (0.2 % (v/v) formic acid) with a flow rate of 4.0 µL/min (B). The applied voltages were -200 V and -400 V for the IEF step and infusion step, respectively.

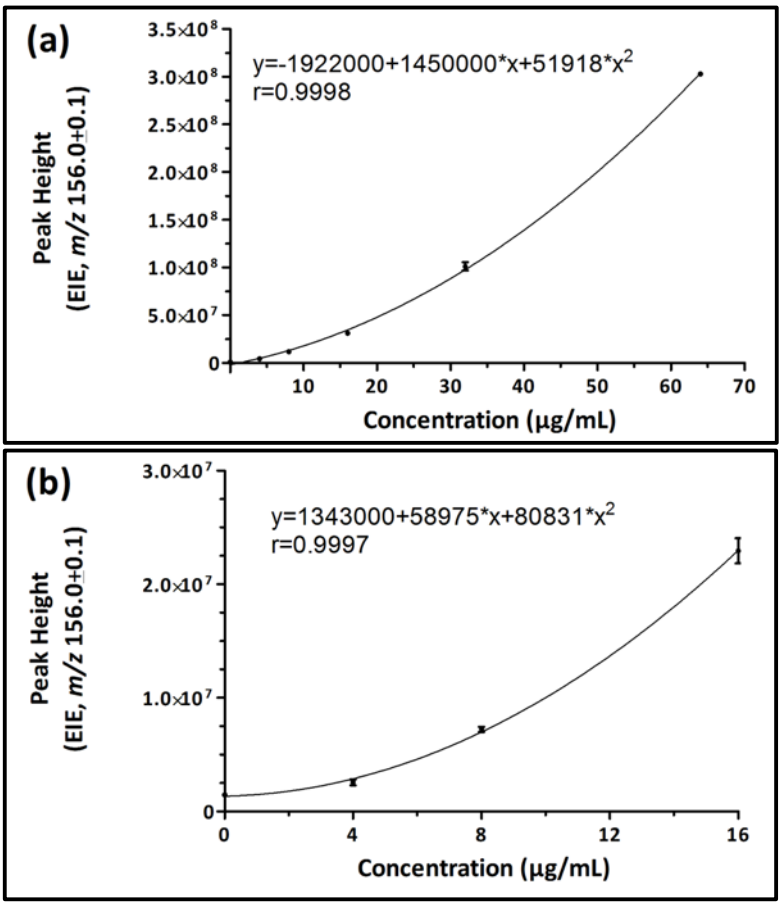


Figure S-8. a, calibration graph for histidine determination in standard solutions. b, calibration graph for histidine in spiked urine samples.

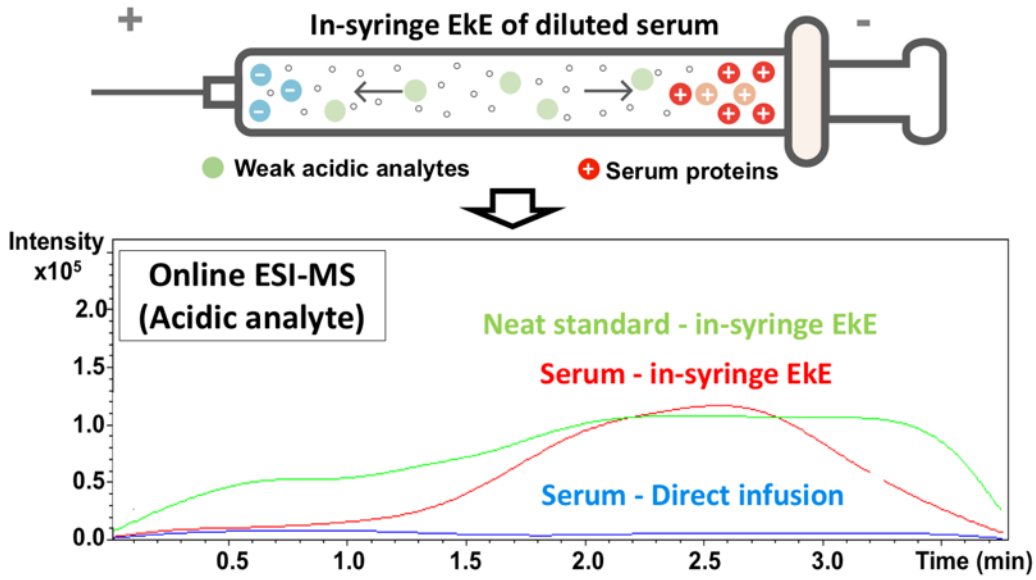
References

[1] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 6th ed., Pearson Education Limited, UK, 2010.

3. In-Syringe Electrokinetic Preparation of Serum Samples for Analysis of Weak Acidic Compounds *via* ESI-MS

This chapter has been included in an accepted research article in Angewandte Chemie Int. Ed. [https://doi.org/10.1002/anie.202006481].

3.1 Abstract



Matrix effects can compromise ionization and hence reliability of electrospray ionisation mass spectrometry (ESI-MS), making sample clean-up including protein removal critical in bioanalysis. An electrokinetic extraction (EkE) syringe is presented here allowing for on-line electrokinetic removal of serum proteins before ESI-MS utilizing the metallic syringe needle and plunger as electrodes. Under acidic conditions, most proteins are cationic facilitating their accumulation at a cathodic plunger. Actuation of the plunger enables infusion of the deproteinated sample remaining in the barrel into the ESI-MS for detection of acidic molecules such as naproxen and paracetamol. Signal

enhancements of 7.7 and 10.8 fold were achieved compared to direct infusion of diluted serum for naproxen and paracetamol, respectively. An EkE-ESI-MS method was developed for the analysis of naproxen and paracetamol in serum using valproic acid as internal standard. Every 1 µL of spiked serum sample was 15x diluted with the background electrolyte (BGE) using the EkE syringe where the final BGE composition was 50 mM formic acid in 30 % (v/v) acetonitrile. -2000 V was applied using the needle and plunger as electrodes for 320 s to achieve the in-syringe EkE. The first 14 µL of the syringe content were Infused to ESI-MS sprayer (flow rate = 4.0 µL/min) after the EkE while keeping -500 V applied. A coaxial flow of isopropyl alcohol 80.0 % (v/v) with a flow rate of 10.0 µL/min was utilized as a sheath flow to optimize the ionization. The LOQs were 3.1 µg/mL and 2.9 µg/mL for naproxen and paracetamol, respectively. The % found of the studied drugs using the EkE-ESI-MS method ranged from 81 % to 116 % with a precision $\leq$ 19. The corresponding regression equations were y=0.0279+0.0328*x for naproxen and y=-0.0075+0.0099*x for paracetamol with a regression correlation coefficient (r) $\geq$ 0.998 for both drugs.

3.2 Introduction

In bioanalysis, sample preparation typically is the most labor-intensive and time-consuming part of the workflow, additionally, the use of off-line processes and manual handling make the sample preparation prone to errors and a source of variability [1, 2]. However, sample preparation is indispensable to prevent matrix effects impacting on the reproducibility and efficiency of the analysis [3]. The development of microfluidic-based, online, and automated sample preparation systems offer many merits to the routine bioanalytical protocols such as low consumption of samples and solvents, high extraction efficiency,

enhanced robustness, and high analysis speed [3], therefore, there are intense efforts to automate and integrate the sample preparation with the final detection step [4].

Currently, mass spectrometry is considered amongst the most important techniques for selective and sensitive bioanalysis [5]. Driven by a demand for increased throughput, direct infusion mass spectrometry (DI-MS) has rapidly gained popularity in metabolomics, because the time required for the high resolution separation in the hyphenated techniques can be eliminated [6]. Moreover, peak alignment and retention times variation are very challenging in the chromatography-MS based metabolomics compared to the DI-MS approach [7]. However, the retention times still can represent an additional feature for the metabolites identification along with the *m/z* ratio. Recently, the capabilities of the stand-alone MS have been boosted by the virtue of highly selective and sensitive MS technologies such as high-resolution MS (HRMS) and ion-mobility MS (IM-MS) [6].

Still, for the analysis of small molecule pharmaceuticals and metabolites from plasma and serum samples, a sample preparation step for protein removal is highly recommended [2]. For example, in non-quantitative metabolomics by direct-infusion high-resolution MS (DI-HRMS), protein removal was realised by centrifugation following the addition of deuterated standards in methanol [7].

Protein precipitation, liquid-liquid extraction (LLE), solid phase extraction (SPE), and ultrafiltration are common methods used for biological sample preparation [8]. Protein precipitation is the simplest sample preparation approach for protein removal where proteins are denatured using heat, organic solvents, or acids [8] but it suffers from the variation in the recovery based on

the precipitating agent [3]. Also, many issues are related to protein removal *via* LLE such as having inferior selectivity, low recovery, emulsion formation, and low efficiency for extracting highly hydrophilic compounds, in addition to the difficulty in automation [9, 10]. Although SPE can provide advantages over other methods including the higher selectivity of extraction and the ease of automation [11], it is affected by many limitations as tedious method development, high cost, poor inter-batch reproducibility, and co-elution of the endogenous contaminants from the SPE cartridges such as polyethylene glycols and phthalates [2, 9]. For ultrafiltration methods, a relatively large volume of sample (> 25 µL) is required [12] and in many instances, filtration was not efficient in removing the interfering compounds specially with high-molecular weight target analytes [13].

Electrokinetic techniques provide an attractive alternative for the clean-up of biological samples owing to the high-speed, and selectivity that can potentially be realized [14, 15]. One of the demonstrations for the coupling of the electrokinetic extraction (EkE) with ESI-MS was the utilization of a conductive tip for the electro-extraction of acylcarnitines from plasma in 3-9 min using a three-phase system where a 10-fold enhancement in the detection limit and a protein-free nano-ESI signal were attained [16]. Furthermore, electro membrane extraction (EME) was incorporated in a micro-chip for the extraction of basic drugs in the complex matrices like urine prior to the online ESI-MS detection [17].

A syringe is the most common device for sample introduction into different analytical instruments, making sample preparation within a syringe highly attractive. In-syringe sample preparation can simplify the workflow and

enhance the analytical performance by combining different liquid handling steps within one system which creates new opportunities for the integrated/automated analysis. Over the last few years, in-syringe systems were coupled with various analytical techniques such as electrothermal atomic absorption spectrometry [18], gas chromatography-mass spectrometry [19], and inductively coupled plasma spectrometry [20]. Recently, we electrokinetically created a pH boundary inside a syringe for the concentration and purification of amphoteric molecules using the stainless steel needle and plunger as the electrodes, the developed electroseparation syringe was applied for the analysis of histidine in urine for the diagnosis of histidinemia [21].

In this chapter, the electrokinetic extraction (EkE)-in-a-syringe was applied for the clean-up of serum samples prior to the analysis of the weakly acidic compounds by ESI-MS. An acidic background electrolyte (BGE) (formic acid, 50 mM, pH 2.5) with a pH below the serum proteins' isoelectric points (*pIs)* directs protein electromigration towards the plunger (cathode) leaving a protein-depleted region in the syringe while neutral weakly acidic compounds will not move and remain dispersed across the syringe at this low pH. Subsequent actuation of the plunger infuses neutral molecules into the ESI interface, allowing for their MS detection with no influence from the proteins. Naproxen and paracetamol were used as model weakly acidic drugs, and were measured in spiked serum using valproic acid as an internal standard with the EkE syringe-ESI-MS system.

A very simplified workflow is presented by the EkE syringe compared to the other conventional protein precipitation protocol along with a significant

reduction in the needed sample volume, the organic solvent consumption, and the analysis time as highlighted in Figure 3-1.

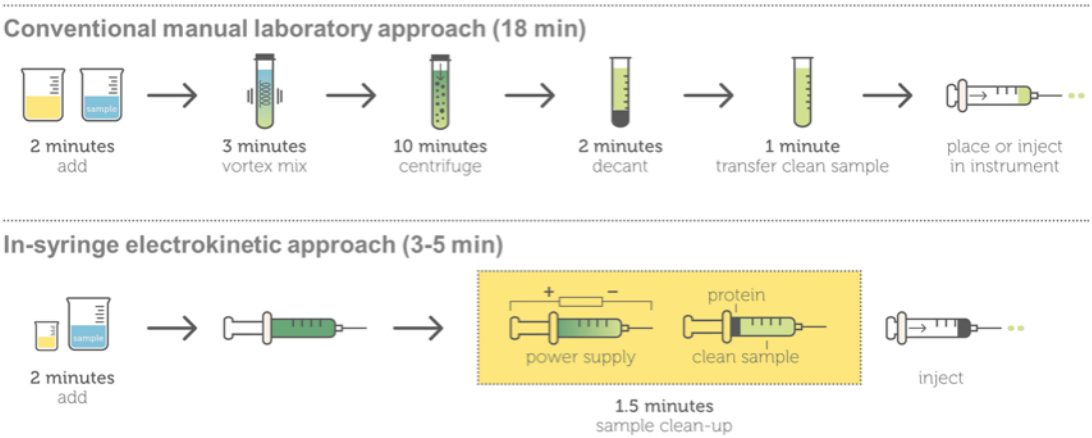


Figure 3-1. Comparison between the conventional protocol for the proteins crushing and the in-syringe electrokinetic proteins removal.

3.3 Experimental Section

3.3.1 Chemicals and reagents

Albumin from human serum (HSA) (≥99 %, fatty acid free, globulin free), formic acid (≥98 %), 4-acetamidophenol (paracetamol) (98 %), naproxen (≥98.5 %), eosin B (99.8 %), and methanol (HPLC grade, ≥99.9 %), were purchased from Sigma Aldrich (St. Louis, USA). Isopropyl alcohol (LC/MS grade) and acetonitrile (LC/MS grade) were bought from Honeywell-Burdick & Jackson (Muskegon, USA). Deionized water was produced by a Milli-Q reagent water system (MA, USA). Chromeo™ 488 NHS-ester was purchased from Santa Cruz Biotechnology (Texas, USA) and the fluorescent labelling of HSA by Chromeo™ 488 NHS-ester was accomplished according to the vendor protocol [22].

3.3.2 Instruments

The ESI-MS analysis was performed using an Agilent 6330 Ion Trap mass spectrometer (Santa Clara, USA) and 6300 series TrapControl software, version 6.1 was used for the method development and monitoring of the

acquisition. Mass spectral data were inspected by DataAnalysis software, version 3.4, Bruker (Bremen, Germany). The contents of the electrokinetic extraction (EkE) syringe were infused to the ESI sprayer accurately *via* a KDS 100 syringe pump (Holliston, USA). Focusing of the fluorescent labelled HSA was visualized with a Dino-Lite Edge fluorescence microscope (AM4115T-GFBW, Taiwan).

3.3.3 System optimization and coupling of the EkE syringe with ESI-MS

The EkE was performed in 25 µL SGE analytical syringes (P/N 003050, Trajan Scientific and Medical, Victoria, Australia). Each syringe has a stainless steel plunger, removable needle (ID 0.2 mm) and a syringe barrel's ID of 0.73 mm. The metallic needle was shortened to 35 mm to reduce the needle's dead volume to 1 µL. An in-house built USB DC voltage power supply (high voltage component: UltraVolt® 3V5-N0.5-EI-W, Colorado, USA) was connected to the stainless-steel needle and plunger using alligator clips to apply high voltage, allowing for the plunger and the needle to serve as the cathode and ground, respectively. The connection between the EkE syringe and the Agilent triple tube ESI sprayer was accomplished by a fused silica capillary (250 mm length, 50 µm ID, 360 µm OD, and 0.5 µL volume) connected to the needle by a MicroTight® fitting. The ESI-MS analysis was performed in the negative mode of ionization with the following source settings: nebulizer gas pressure, 7.5 psi; dry gas temperature, 225°C; dry gas flow rate, 5.0 L/min; electrospray voltage of the ion source, 4000 V; with the use of ion charge control; smart target, 200000; maximum accumulation time, 20 milliseconds.

To minimize the carryover between runs, the aggregated proteins were removed from the EkE syringe by N_2 gas after removing the needle and the

plunger. The EkE syringe was then reassembled and washed with Milli-Q water, acetonitrile 50 % (v/v), and the BGE, respectively. Also, the capillary to the ESI-MS was flushed with Milli-Q water, acetonitrile 50 % (v/v), and the BGE, 25 µL each, after every run.

An Agilent triple tube ESI sprayer rendered a co-axial flow of the sheath liquid and two separate syringe pumps were used to infuse the sample and the sheath liquid to the ESI sprayer. The sheath liquid encompassed isopropyl alcohol 80.0 % (v/v) (10 µL/min).

The composition of the background electrolyte (BGE) was optimized by ESI-MS experiments and monitoring of the Chromeo^{TM} 488 labeled HSA focusing using the fluorescence microscope.

3.3.4 Investigation of the ion suppression by serum proteins and the significance of the EkE

To affirm the results obtained from the fluorescence microscope and to determine the rate of albumin elimination from the sample, a mixture of naproxen (8.0 µg/mL) as a weak acidic drug and HSA (3.0 mg/mL) as the most abundant protein in serum was performed by ESI-MS without the EkE step (t=0) and after -2000 V application for different time intervals (t=20, 40, 80, 160, 320, and 640 sec) using 50 mM formic acid in 30 % (v/v) acetonitrile as a BGE. The first 14 µL of the syringe were infused to the ESI after each time interval with a flow rate of 4.0 µL/min while keeping -500 V applied, and the sheath liquid was isopropyl alcohol, 80 % (v/v) infused at a flow rate of 10.0 µL/min. Naproxen, EIE was for the molecular ion $[M-1]^{-1}$ (*m/z* 229.1) using the source conditions mentioned in “Section 3.3.3”. For HSA, the method of Babic *et al*. [23] was used applying source-induced fragmentation by raising the declustering potential to

350 V where adjusting the voltage difference between the capillary exit and the skimmer to 335 V resulted in the same fragmentation pattern of the first 30 residues from the N terminus of HSA. The resulting b_{24}^{4+} fragment ion (most abundant fragment, *m/z* 685.1) was detected utilizing the positive mode of ionization and selected for the HSA monitoring. Finally, the mass spectra of the runs at each time interval (one in negative mode for naproxen and one in positive mode for the b_{24}^{4+} fragment of HSA) were integrated and the intensity enhancement/reduction (ratio of the average intensity after each time interval and the average intensity at time=0) of naproxen and HSA were plotted *versus* the time.

3.3.5 Determination of the weakly acidic drugs in spiked serum samples

Standard solutions of naproxen (1.00 mg/mL), paracetamol (1.00 mg/mL), and valproic acid (IS) (4.000 mg/mL) were prepared in methanol by transferring 25.0 mg of naproxen and paracetamol and 100.0 mg of valproic acid into separate 25.0 mL volumetric flasks, 20 mL of methanol was added to each flask, the solutions were sonicated for 5.0 min and finally methanol was added to each flask till the mark. The working solutions of naproxen and paracetamol were prepared by appropriate dilution of the standard solution with methanol. Both the standard solutions and the working solutions were kept in the refrigerator at 4 °C and found to be stable for at least 14 days.

The collection and preparation of the human blood was done under the ethics approval (approval number H0016575) by The Tasmanian Health and Medical Human Research Ethics Committee. Fresh human blood was collected by pinprick of a fingertip of a healthy adult. The blood was allowed to clot by leaving it undisturbed at room temperature for 30 min followed by centrifugation

at 6400 RPM for 10 min to remove the clot and the liquid component (serum) was transferred into clean Eppendorf tubes. All serum samples were kept at -20 °C until analysis. Spiked serum samples were prepared by adding specific volumes of the working solutions of naproxen and paracetamol to the serum samples to obtain final concentrations of 4.00, 8.00, 16.0, 32.0, and 64.0 µg/mL of naproxen, and 3.00, 6.00, 12.0, 24.0, and 48.0 µg/mL of paracetamol, respectively. Each serum sample was spiked with 160.0 µg/mL of the IS and finally vortex-mixed for 30 s.

The BGE was prepared separately from the spiked serum samples and it was composed of 53.3 mM formic acid in 32.0 % (v/v) acetonitrile. Every spiked serum sample was 15-fold diluted with the BGE by aspiration of 1 µL of the spiked serum between 3 and 11 µL of the BGE, respectively, to give a final BGE composition of 50 mM formic acid in 30 % (v/v) acetonitrile.

The clean-up step was achieved with the EkE syringe by application of -2000 V using the plunger as a cathode for 320 s then 14 µL of the EkE syringe content (to avoid the needle clogging with the precipitated protein close to the plunger) was Infused to ESI-MS sprayer with a flow rate of 4.0 µL/min while keeping -500 V applied along with a coaxial flow of a sheath liquid composed of isopropyl alcohol 80.0 % (v/v) (flow rate, 10.0 µL/min).

The calibration graphs were constructed by plotting the average intensity ratio between the molecular ion of each drug and the molecular ion of the IS (n=3) from the integrated mass spectra of the whole run (0-3.75 minutes) *versus* the corresponding drug concentration in µg/mL and the corresponding regression equations were derived.

3.3.6 Method validation

The developed method was validated according to the ICH Q2 (R1) guidelines [24]. The accuracy and the precision of the simultaneous determination of naproxen and paracetamol in the spiked serum samples were evaluated at different concentration levels; 4.00, 16.0, and 64.0 μg/mL for naproxen and 3.00, 12.0, and 48.0 μg/mL for paracetamol. The precision of the method (repeatability and intermediate precision) was expressed by the relative standard deviation (RSD) using the standard deviation and the mean of the assayed triplicates of each studied concentration level [25].

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated based on the standard deviation of the response and the slope of the calibration curve [24]:

LOD=3.3 σ/b [24]

LOQ=10 σ/b [24]

where σ is the standard deviation of the blank samples response and b is the slope of the calibration curve.

3.3.7 Process efficiency

Four samples sets were prepared to evaluate the significance of the developed EkE approach for the samples clean-up; set A included neat standard solutions diluted by the BGE and infused to the ESI-MS, set B enclosed neat standard solutions like set A but after the application of EkE using the experimental conditions mentioned in Section “3.3.5 Determination of weakly acidic drugs in spiked serum samples”, set C involved pre-spiked serum samples after the EkE application, and set D encompassed pre-spiked serum samples diluted by the BGE and injected without the EkE. Every set was

prepared at three different concentration levels covering the whole range of the calibration graph of each drug.

Process efficiency (% PE) was assessed as recommended by Matuszewski *et al.* [26] by comparing the average intensities between set C and set A.

PE (%) = C/A × 100 [26]

Furthermore, the ion suppression due to the serum matrix was evaluated as follows:

Ion suppression = 100 – (D/A × 100) [27].

3.4 Results and Discussion

The depletion of proteins in serum samples is based on the difference in charge between the dominant serum proteins and the target analytes due to the pH of the BGE. Serum proteins include mainly albumins (58 %), globulins (38 %), and fibrinogen (4 %) [28] which have *pI*s of 4.7-4.9 [29], 5.3-7.3 [29], and 5.5-6.3 [30], respectively. Using of an acidic BGE at pH 2.5 (50 mM formic acid) ensures the serum proteins are positively charged and upon voltage application using the plunger as a cathode and the needle as a ground, the cationic serum proteins migrate, focus and aggregate close to the plunger while weakly acidic analytes remain unfocused as indicated in Figure 3-2a. The EkE mechanism was experimentally verified in Figure 3-2b and Figure 3-2c which show the focusing of Chromeo™ 488 labelled human serum albumin (HSA) and no focusing of eosin B (a weak acidic dye with pKa values of 2.2 and 3.7), respectively, using an electrolyte composed of 50 mM formic acid in 30 % (v/v) acetonitrile and an applied voltage of -2000 V.

The structures, pKa values, and plasma protein binding levels of the studied weakly acidic analytes are summarized in Figure 3-3.

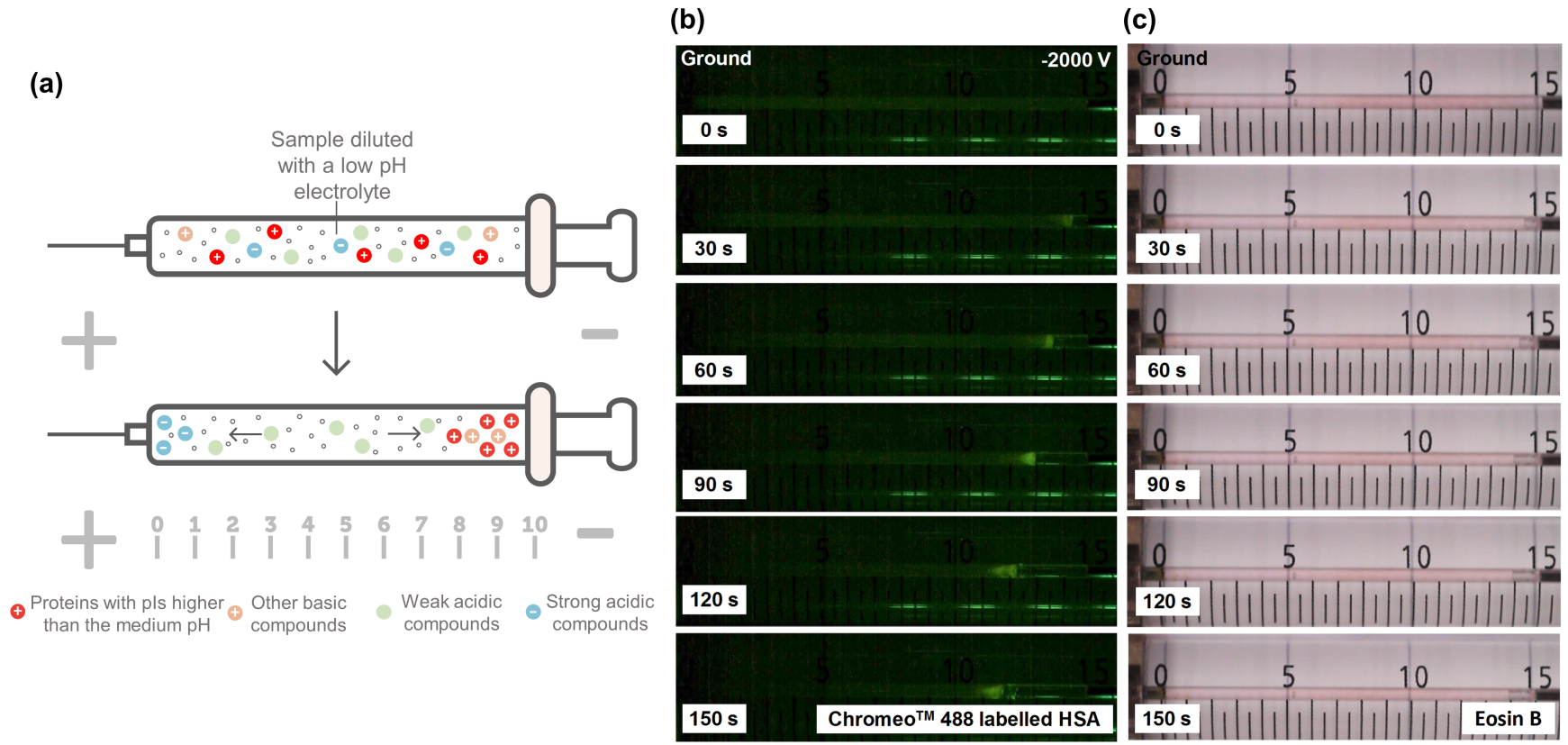
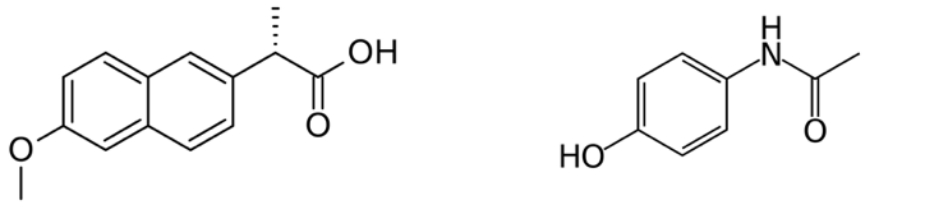


Figure 3-2. Electrokinetic clean-up mechanism for the analysis of weakly acidic compounds. a, a schematic presentation of the in-syringe EkE of biological samples from the interfering proteins for the analysis of weakly acidic analytes using an acidic BGE, the metallic plunger of the EkE syringe served as a cathode and the metallic needle served as a ground. b, a single-frame time series for focusing of 200.0 µg/mL of Chromeo™ 488 labelled HSA to the plunger as a cathode using 50 mM formic acid in 30 % (v/v) acetonitrile and an applied voltage of - 2000 V. c, unfocused weak acidic dye (eosin B, 100.0 µg/mL) under the same conditions in Figure 3-2b.



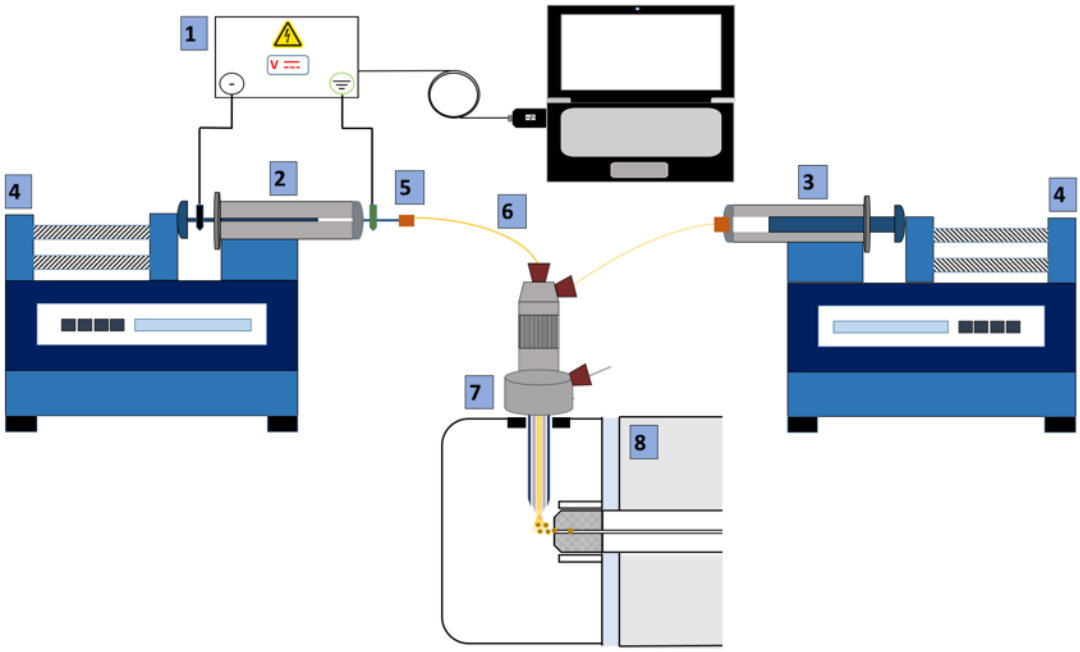
Naproxen
pKa: 4.15
Plasma protein binding: >99%

Paracetamol
pKa: 9.38
Plasma protein binding: 10-25%

Figure 3-3. Structure, pKa, and plasma protein binding of naproxen [31], and paracetamol [32].

3.4.1 System optimization and coupling with ESI-MS

The performance of the EkE syringe was investigated by monitoring of fluorescent labelled HSA (Chromeo^{TM} 488 labelled HSA) and ESI-MS experiments. The EkE syringe was interfaced to ESI-MS with a coaxial sheath sprayer as indicated in Figure 3-4.



1- USB power supply **2- EKE syringe** **3- Sheath liquid syringe**
4- Syringe pumps **5- MicroTight® fitting**
6- Capillary tube (ID 50 µm, length 250 mm)
7- Agilent triple tube ESI sprayer
8- Agilent 6330 Ion Trap mass spectrometer

Figure 3-4. Coupling of the EkE syringe with ESI-MS for the analysis of the weakly acidic compounds.

The best sensitivity was achieved by dispensing the syringe fluid at a flow rate of 4.0 µL/min. A sheath liquid of 80.0 % isopropyl alcohol was selected to attain the best sensitivity for a weak acidic analyte, naproxen, as illustrated in Figure 3-5a and Figure 3-5b. Moreover, Figure 3-5c shows that flow rate of 10.0 µL/min gives a good compromise between the sensitivity and the repeatability of the analysis expressed as the relative standard deviation of the average intensity (n=3).

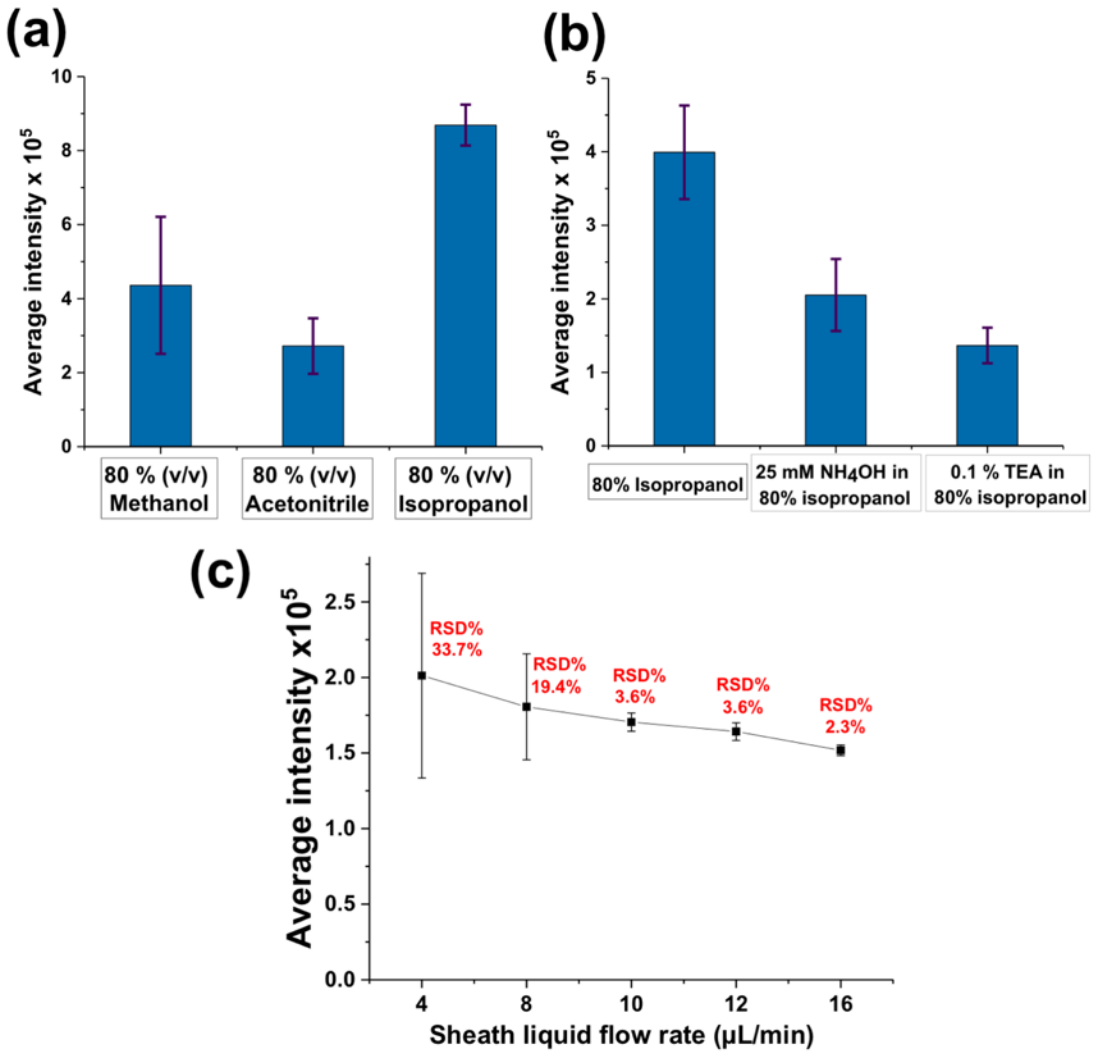


Figure 3-5. Influence of the sheath liquid composition and flow rate on the average signal intensity of naproxen infused to ESI-MS (4 µL/min) in a BGE composed of 50 mM formic acid in 30 % (v/v) acetonitrile. a, effect of adding different volatile solvents to the sheath liquid on the average signal intensity. b, effect of addition of different pH modifiers to the sheath liquid on the average signal intensity. c, effect of flow rate of the sheath liquid (80% isopropanol) on the sensitivity and the repeatability of the ESI-MS response.

The addition of an organic solvent proportion (30 % (v/v) acetonitrile) was essential to reduce electrolytic gases by decreasing the water content available for the electrolysis and to help in stabilizing the gas bubbles and decreasing their sizes [33], we couldn't increase the acetonitrile concentration more than 30 % (v/v) to avoid the precipitation of the serum proteins prior to the EkE.

The effect of different concentrations of formic acid (from 6.25 to 200.00 mM) covering the pH range of 2.3 to 3.0 on the position of the focused Chromeo™ 488 labelled HSA (200.0 µg/mL) was studied, determining the focusing time and the volume of the generated gas. Figure 3-6a shows that the generated gas volume increases with the molar strength of formic acid, also the higher gas generation is assumed to be the reason for expanding the focusing time due to the instability of the applied potential. On the other hand, concentrations below 50.0 mM formic acid didn't maintain the pH at the plunger side with the generated OH^- ions due to water electrolysis and the focused protein was shifted toward the middle of the barrel away from the plunger (cathode) end. A molar strength of formic acid up to 100 mM resulted in shifting of the aggregated proteins more toward the plunger as indicated in Figure 3-6a then there was no corresponding change by increasing the acid concentration further. Thus, 50.0 mM formic acid and 100.0 mM were selected for further study by ESI-MS to evaluate their effect on the signal intensity of naproxen in standard solution (4.00 µg/mL) with and without the EkE step application. The results presented in Figure 3-6b indicate that the best sensitivity was achieved by using 50.0 mM formic acid as a BGE under both conditions.

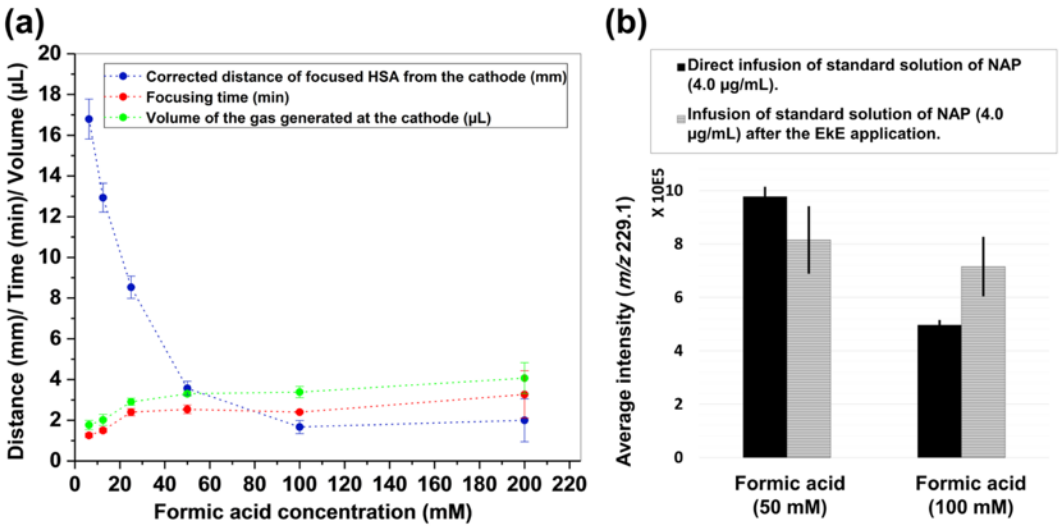


Figure 3-6. a, effect of the molar strength of formic acid on the position of the focused Chromeo^{TM} 488 labelled HSA (200.0 µg/mL) inside the syringe barrel, the focusing time, and the volume of hydrogen gas generated at the cathode due to water electrolysis, the distance measurement was done by ImageJ 1.52g software, the distance was corrected by subtracting the distance occupied by the generated gas, the error bars represent the SD of the mean values. b, analysis of naproxen (NAP) by ESI-MS using a BGE composed of 50 mM formic acid in 30 % (v/v) acetonitrile *versus* a BGE composed of 100 mM formic acid in 30 % (v/v) acetonitrile. The EkE step included -2000 V application for 320 s followed by infusion to ESI-MS (4.0 µL/min) with a coaxial flow of the sheath liquid while having -500 V applied. The error bars represent the SD of the mean value of the average intensities.

3.4.2 Ion suppression by serum proteins and the significance of the EkE

To experimentally verify the concept of serum protein elimination from a sample containing a weak acidic target compound using the EkE syringe, a sample containing naproxen and HSA was analysed by ESI-MS analysis with and without the EkE. Biochemical modification of HSA typically occurs beyond the first 30 residues from the albumin N terminus. To detect both modified and unmodified albumin [23], a method for the analysis of the first 30 residues was selected by raising the declustering potential to 350 V for source induced fragmentation of the first 30 residues [23]. The b_{24}^{4+} fragment ion (*m/z* 685.1) was the most abundant ion and selected to estimate the presence of HSA.

The combined EIEs of HSA (EIE: positive mode, *m/z* 685.1) and naproxen (EIE: negative mode, *m/z* 229.1) infused into the MS after electrokinetic extraction are shown in Figure 3-7a. The HSA in its cationic form concentrated

towards the plunger, facilitating a gain in signal intensity for naproxen early during the infusion period. Ion suppression of naproxen at the beginning of each run is due to the fact sample present inside the needle is not exposed to the clean-up field, and hence HSA has not been removed. The ion suppression at the last stages of the infusion are due to the infusion of the concentrated proteins into the MS.

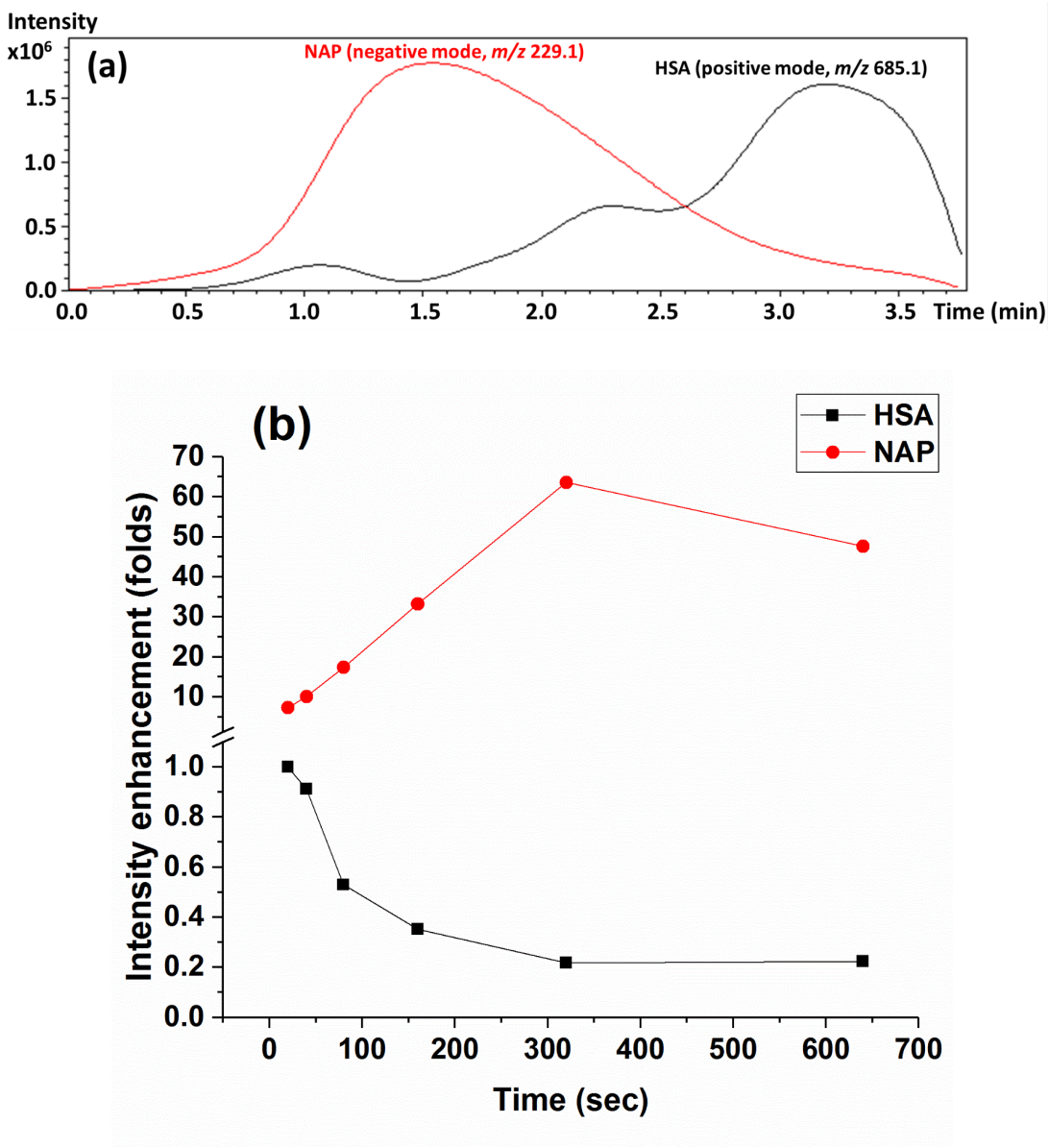


Figure 3-7. a, monitoring of naproxen (NAP) (8.0 µg/mL)/HSA (3.0 mg/mL) mixture using the developed EkE syringe-ESI/MS system with an applied voltage of -2000 V for 640 s. b, plot of intensity enhancement/reduction (ratio of the average intensity after each time interval of -2000 V application and the average intensity at time=0) for the molecular ion $[M+H]^{+1}$ of NAP and the fragment ion of HSA (*m/z* 685.1), average intensities of HSA and NAP were obtained from the mass spectra of the whole runs "0-3.75 min".

The MS data are summarised in Figure 3-7b as the intensity enhancement/reduction of NAP and of HSA *versus* the time. The naproxen signal increases with the time of the EkE step till 320 s indicating the gradual removal of the proteins, a maximum enhancement factor of 63.6 fold was realized at 320 s so it was selected as the optimum time for the EkE step. Also, Figure 3-7b clarifies the reverse proportional between the average intensities of naproxen and HSA signals due to the ionization suppression caused by HSA.

3.4.3 Analytical performance of the simultaneous determination of naproxen and paracetamol in serum samples by EkE syringe-ESI-MS

The developed EkE syringe-ESI-MS approach offered a very simplified workflow for the simultaneous analysis of naproxen and paracetamol in spiked serum samples using valproic acid as IS. The EkE syringe was used to dilute the serum sample by 15-fold with the BGE, to eliminate the serum proteins by EkE, and then to infuse the proteins-depleted sample into ESI-MS. By the virtue of the EkE syringe-ESI-MS system, the determination of naproxen and paracetamol in 1.0 µL spiked serum required only 9.08 minutes comprising 5.33 minutes (320 s) for the EkE and 3.75 minutes for the infusion to ESI-MS. Additionally, less than 5 µL of acetonitrile was consumed by the EkE syringe every run. This represents a significant enhancement compared to the current protein precipitation protocol prior to the LC-MS analysis of naproxen and paracetamol which requires 400 µL of serum sample, 800 µL of acetonitrile for proteins precipitation, and 10 minutes for only the centrifugation step to remove the precipitated proteins [34].

The EIEs representing the four sets including neat standard solutions and pre-spiked serum samples with and without the EkE step are indicated in

Figure 3-8. The mass spectra in Figure 3-9a and Figure 3-9b represent a comparison between spiked serum samples without and with the EkE to show the influence of the EkE on the signal intensities of the 2 drugs and the IS.

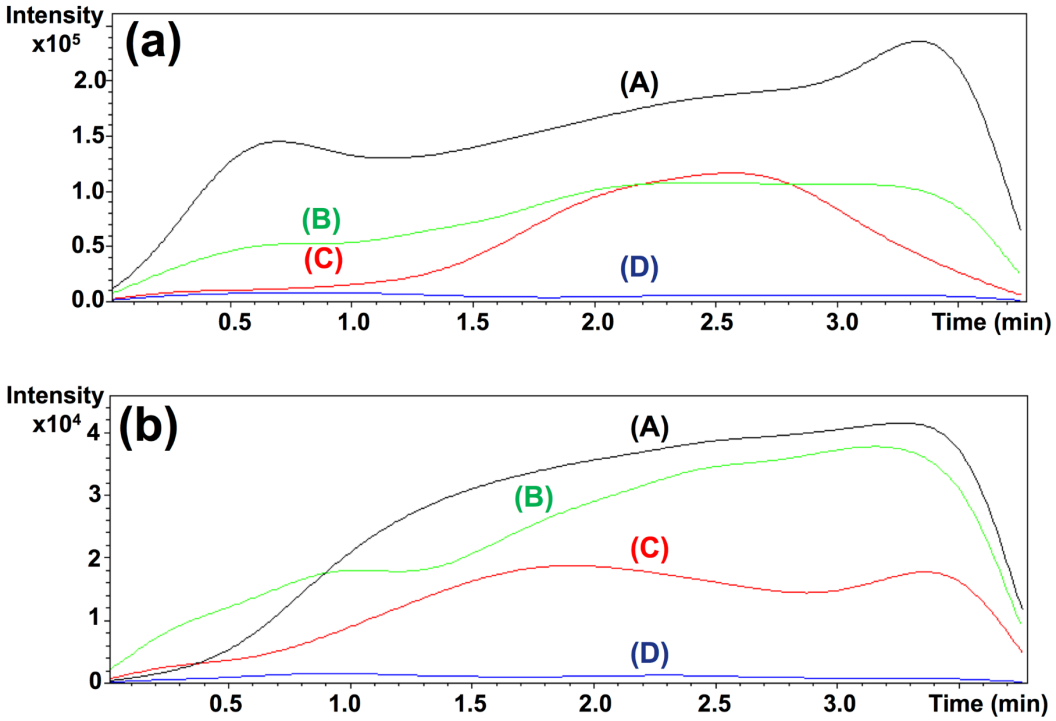


Figure 3-8. Comparison between neat standard solutions and pre-spiked serum samples with and without the EkE step. a, EIEs (*m/z* 229, $[M-1]^-$) of 16.0 µg/mL naproxen in neat standard solutions (A), neat standard solutions with the application of the EkE step before the infusion to ESI-MS (B), serum samples with the EkE step (C), and serum samples without the EkE step (D). b, EIEs (*m/z* 150, $[M-1]^-$) of 12.0 µg/mL paracetamol in the four sets. Each sample was 15-fold diluted by the BGE using the EkE syringe. The final composition of the BGE was 50 mM formic acid in 30 % (v/v) acetonitrile and the EkE step was done by -2000 V application for 320 s followed by infusion to ESI-MS (4.0 µL/min) with a coaxial flow of the sheath liquid while keeping -500 V applied.

The constructed calibration curves consisted of 0.00. 4.00, 8.00, 16.0, 32.0, and 64.0 µg/mL of naproxen and 0.00, 3.00, 6.00,12.0, 24.0, and 48.0 µg/mL of paracetamol in spiked serum samples. The LOD was found to be 1.0 µg/mL for both naproxen and paracetamol in spiked serum samples and the LOQs of naproxen and paracetamol were 3.1 µg/mL and 2.9 µg/mL, respectively, which were less than the peak plasma concentrations (C_{max}) of naproxen and paracetamol (76.6 [35] and 9.9 [36] µg/mL, respectively). A linear correlation was achieved by plotting the average intensity ratio (each drug/IS) *versus* the

added drug concentration. The corresponding regression equations were y=0.0279+0.0328*x and y=-0.0075+0.0099*x for naproxen and paracetamol, respectively, with a regression correlation coefficient (r) = 0.9994 for naproxen and with r = 0.9982 for paracetamol.

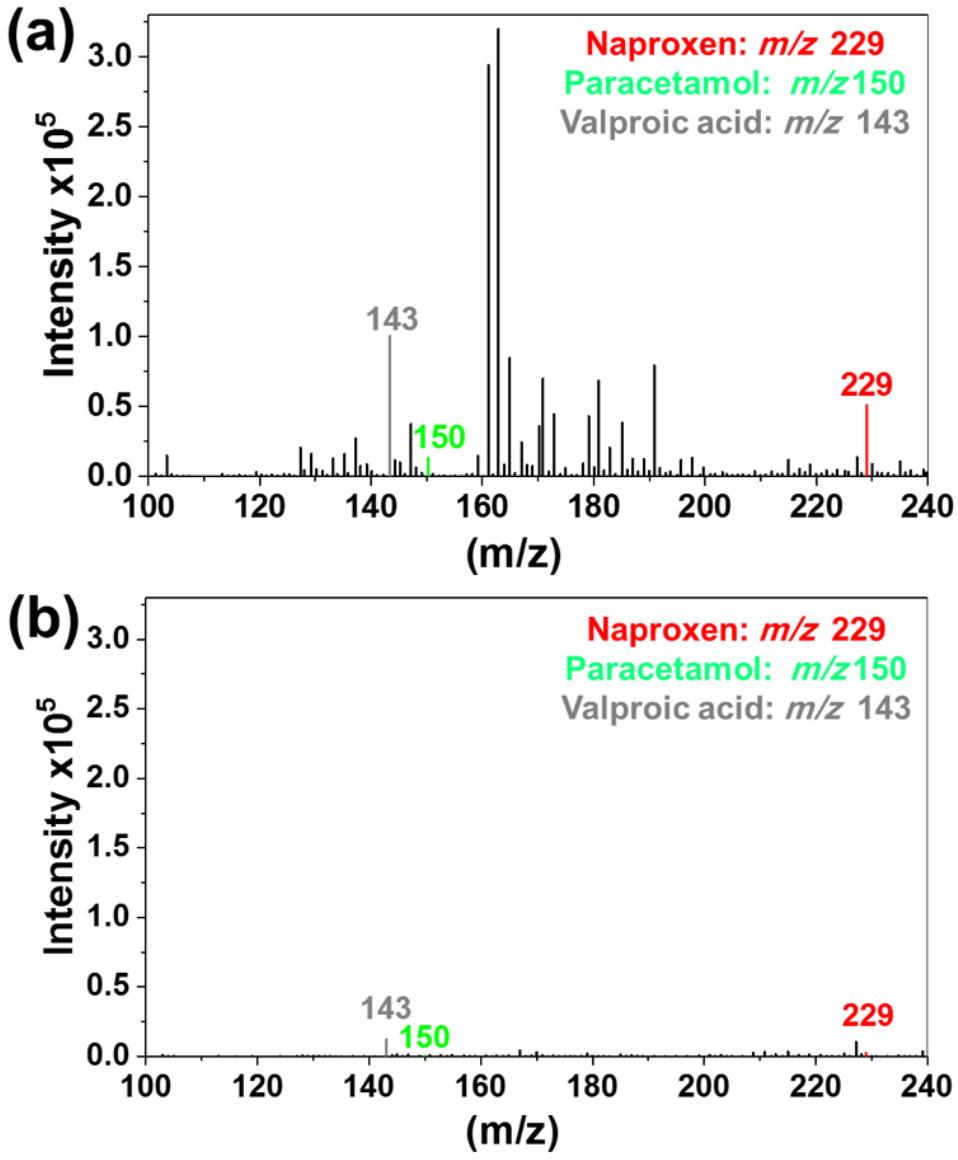


Figure 3-9. Comparison of mass spectra of the electrokinetic clean-up approach and the direct infusion approach for the analysis of weakly acidic compounds. a, mass spectrum (whole run, 0-3.75 minutes) of a spiked serum sample with added 16.0 µg/mL of naproxen (NAP), 12.0 µg/mL of paracetamol (PCM), and 160.0 µg/mL of valproic acid (IS) after 15-fold dilution and electrokinetic clean-up by the EkE syringe (-2000 V application for 320 s followed by infusion to ESI-MS (4.0 µL/min) while keeping -500 V applied). b, mass spectrum (whole run, 0-3.75 minutes) of a spiked serum sample with added 16.0 µg/mL of naproxen, 12.0 µg/mL of paracetamol, and 160.0 µg/mL of valproic acid (IS), after 15-fold dilution by the EkE syringe but without the EkE step. The final composition of the BGE was 50 mM formic acid in 30 % (v/v) acetonitrile.

The method accuracy and precision were determined using three replicates of each studied concentration with % found values from 81.2 % to 105 % and from 91.0 % to 116 % for naproxen and paracetamol, respectively. The method precision shows RSD less than 19 % for naproxen and less than 18 % for paracetamol as shown in Table 3-1.

Table 3-1. Accuracy and precision of the weakly acidic drugs determination in spiked serum by EkE syringe-ESI-MS

Drug	Concentration (µg/mL)	% Found	Repeatability *RSD (%) (n=3)	Intermediate precision *RSD (%) (n=3)
Naproxen	4.00	81.2	6.10	18.9
	16.0	105	10.5	14.3
	64.0	98.9	7.63	10.5
Paracetamol	3.00	116	11.2	17.3
	12.0	91.0	10.1	14.9
	48.0	103	9.15	10.6

*Relative standard deviation, RSD = 100 S/ $\overline{x}$

3.4.4 Process efficiency (PE)

The PE of the EkE and the ion suppression due to the serum matrix were evaluated at three concentration levels of each drug as summarized in Table 3-2. The average ion suppression for naproxen in serum without the EkE step was 94.1 % while the average PE of the EkE was found to be 39.9 % giving a signal 7.7-fold higher than the direct infusion of spiked serum at 16.0 µg/mL. For paracetamol, the average ion suppression was 93.9 % and the average PE was 36.1 % where the signal was enhanced by 10.8 fold after the EkE step compared to direct infusion at spiked concentration of 12.0 µg/mL.

Comparison between set A (neat standard solutions) and set B (neat standard solutions after the EkE) indicates that the average signal intensity was decreased due to the EkE step, the possible reason for this signal reduction is the gas generated due to the water electrolysis.

Table 3-2. Process efficiency (PE) data for the EkE of the weakly acidic drugs in spiked serum samples

Drug	Conc. (µg/mL)	Average signal intensity				**PE (%)	***Ion suppression without the EkE step (%)
		Set A	*Set B	Set C	Set D		
Naproxen	4.00	11233	8581	4094	905	36.4	91.9
	16.0	127272	94863	50786	6562	39.9	94.8
	64.0	354204	242285	153615	15992	43.4	95.5
						Mean ± SD	Mean ± SD
						39.9±3.5	94.1±1.9
Paracetamol	3.00	3353	2116	1229	240	36.7	92.8
	12.0	33004	26000	13168	1220	39.9	96.3
	48.0	55828	35541	17711	4140	31.7	92.6
						Mean ± SD	Mean ± SD
						36.1±4.1	93.9±2.1

* Set B includes the analyte standards with the EkE step.
** PE= C/A x 100
where C is the average signal intensity of the analyte in pre-spiked serum samples after the EkE (set C) and A is the average signal intensity of the neat standard (set A) [26].
*** Ion suppression = 100 – (D/A × 100)
where D is the average signal intensity of the analyte in pre-spiked serum samples without the EkE (set D) and A is the average signal intensity of the same neat standard (set A) [27].

3.5 Conclusions

The EkE syringe was used as a novel, simple, and time-saving approach for the clean-up of the biological samples from the interfering proteins without excessive use of solvents. This approach demonstrated the clean-up of human serum prior to the detection of weak acidic analytes by ESI-MS avoiding the ion suppression caused by the serum proteins. The process efficiency was evaluated and the signal intensity was 7.7 fold higher for naproxen molecular ion and 10.8 fold higher for paracetamol molecular ion after the clean-up step. The sample preparation with the EkE syringe rendered an accurate sample dilution, elimination of serum proteins, and sample infusion to ESI-MS with only one device. Furthermore, the sample clean-up was accomplished within ten minutes. Thus, this approach offers a very simplified platform for the automated analytical procedures.

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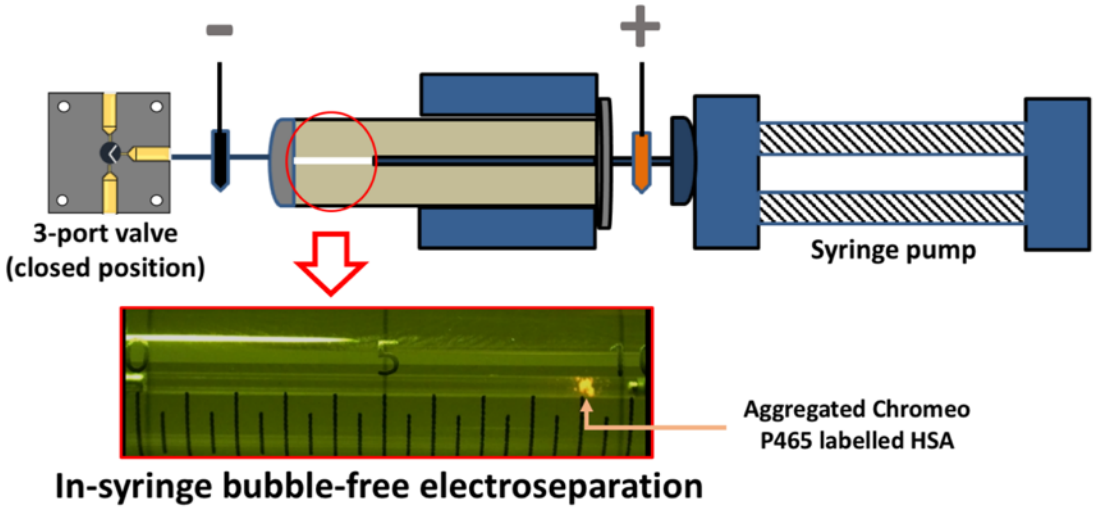
4. Bubble-free Electrokinetic Separation in One-Channel Chips, EkE Syringes

4.1 Abstract



Formation of uncontrolled bubbles due to the electrolysis gases can result in distortion of separation in the electrokinetic microfluidic systems. Different approaches for bubbles suppression in the electrokinetic extraction (EkE) syringes as an example of microfluidic devices were studied. These approaches included addition of quinhydrone to the background electrolyte to minimize the bubbles formation electrochemically, incorboration of acetonitrile for bubbles stabilization, and designing of a sealed system to pressurize the gases in the solution. A bubble-free pressurized in-syringe EkE was successfully achieved using 25-µL syringes and 100-µL syringes by connecting the needle of the syringe to a push-pull valve or a 3-port selector valve in a closed position and having a gas-tight plunger. The developed design was found effective using different electrodes polarities and electrolyte solutions.

4.2 Introduction

Gas bubble occurrence is one of the most challenging issues in the microfluidics field [1]. Unlike the macroscale systems, surface tension is dominant at the microscale which makes the air bubbles stick easier to the inner surfaces and very difficult to be removed [2]. Bubbles within the micro-dimensions separation channels can distort the electric field and analytes' separation [1], moreover, obstruction of the microfluidic paths and damage of the viable cells at the liquid–gas interface are frequently reported [3]. Generally, the translation of many microfluidic concepts to commercial products are hurdled by the spontaneous nature of uncontrolled gas bubbles [4].

Air bubbles have different origins and identifying the reason for their presence in the microfluidic devices is usually the first step to eliminate them. Gas bubbles may be introduced into the microfluidic systems from dead volumes of the fluidic lines, the interfaces, and interconnections, and/or improper sealing, also, they can be generated in the microfluidic device itself [5]. In miniaturized electrophoresis devices, the electric field can generate gas bubbles through two mechanisms [4]: 1) the electric currents due to the voltage difference in an ionic liquid drive non-spontaneous chemical reactions which, in many cases, have gas molecules as products, e.g. the electrolysis of water into oxygen at the anode and hydrogen at cathode; 2) the joule heating accompanying the high voltage application lowers the gas capacity of the solution and results in a bubble-prone supersaturation level according to Henry's law:

$c_s = H(T)\, p_g$

where c_s is the equilibrium concentration of the gas particles in the liquid

solution, p_g is the pressure of the gas in the vapor phase, and H(T) is Henry's law solubility constant which differs for every gas species and decreases with increasing the temperature [4, 6].

There are different approaches to prevent the introduction and/or to remove the gas bubbles from the microfluidic devices. One method is to install bubble trap(s) either as an external module or as an integrated component of the microfluidic system to ensure that all the introduced liquids are bubble-free. Typically, the traps utilize the air bubble buoyancy, and provide a section where the bubbles can float and be eliminated [2]. Also, hydrophobic surfaces can be used as bubble sinks for the floating bubbles [7]. However, the trapping approach has some drawbacks such as the need to stop the fluid flow and the need to external vacuum or pressure pumps [8].

To mediate the adverse effects of the electrolysis bubbles, many researchers prefer to isolate the electrode reservoirs from the separation chamber by controlling the channel depth [9] or by using ion permeable membranes [10, 11] but the main disadvantages of using the physical barrier, i.e. the membranes, are the voltage drop across the barrier due to the high electrical resistance [12] and the difficulty of the incorporation of the membranes into the microfluidics [13].

Other devices with electrodes not isolated from the separation channel like the system developed by Lu *et al.* [14] required low voltage (< 2V) to prevent bubble formation which made the separation times much longer. Macounova *et al.* [15] used a similar approach in their micro-free flow isoelectric focusing device, they found that the palladium electrodes are able to recombine O_2 and H_2 to H_2O avoiding the formation of gas bubble in the solution, however, the

capacity of palladium for gas adsorption is limited so the maximum applicable voltage can be used is 2.2 V [15].

Further, suppression of bubble generation *via* an electrochemical mean was presented by Kohlheyer *et al.* [16] utilizing the redox reaction of quinhydrone (complex of hydroquinone and *p*-benzoquinone) to replace the water electrolysis at the electrodes, this reaction prevented the gas generation and consequently improved the resolution of the three fluorescent compounds separation by 2.5 fold.

Electrostatic induction was also utilized for bubble-free electrophoresis where insulating walls were used to separate the electrodes from the separation channel when the potential is applied to the electrodes, the adjacent insulator will form a dipole with opposite charge toward the electrode, while the insulator–liquid interface would have the same polarity of the metal electrode, this approach is characterized by no current flow and consequently no electrolysis but it requires a continuous buffer flow to prevent the charged ions in the buffer from neutralizing the surface charge of the insulator [13].

In this Chapter, electrokinetic extraction (EkE) syringes are used to prepare the biological samples by removing the abundant proteins. This is achieved by charging the interfering proteins and aggregating them toward the opposite-charge electrode (the plunger) where the separation channel (syringe barrel) is in direct contact with the electrodes (metallic plunger and needle). This makes the elimination of the gas bubbles more challenging. Different approaches have been investigated to eliminate or to minimize the electrolysis bubbles during the EkE such as the incorporation of quinhydrone to react instead of water at the electrodes [16] and the addition of a percentage of organic solvent (acetonitrile)

to the background electrolyte (BGE) to decrease the water content available for the electrolysis and to help in stabilizing the gas bubbles and decreasing their sizes [17] but both approaches didn't eradicate the electrolysis induced bubbles. For a bubble-free EkE, a sealed system was designed by connecting the syringe needle to a push-pull valve or a 3-port selector valve in a closed position along with using a modified gas-tight plunger to dissolute the gas bubbles under elevated pressure conditions. The developed design was studied and found effective using different syringe volumes (25 µL and 100 µL syringes), different BGEs, and polarities of the electrodes.

4.3 Experimental Section

4.3.1 Chemicals and reagents

Albumin from human serum (HSA) (≥99 %, fatty acid free, globulin free), Chromeo™ P465, formic acid (≥98 %), and triethylamine (≥99.5%) were purchased from Sigma Aldrich (St. Louis, USA). Acetonitrile (LC/MS grade) was bought from Honeywell-Burdick & Jackson (Muskegon, USA). Chromeo™ 488 NHS-ester was purchased from Santa Cruz Biotechnology (Texas, USA). The fluorescent labelling of HSA by Chromeo™ 488 NHS-ester [18] and Chromeo™ P465 [19] was accomplished according to the vendor protocols [18, 19]. Deionized water was produced by a Milli-Q reagent water system (MA, USA).

4.3.2 Instruments

The EkE experiments were implemented by 100 µL syringe and 25 µL SGE analytical syringes, part number 005250 and 003250, respectively (Trajan Scientific and Medical, Australia). Push-pull valve, part no 031905, was given by Trajan Scientific and Medical, Australia. A 3-port valve (MV201-T132) was purchased from LabSmith, Inc. (Livermore, CA). A KDS 100 syringe pump

(Holliston, USA) was used to hold the syringe and the syringe plunger at a fixed position during the EkE and to inject the syringe contents with a constant flow rate. For the coupling of the syringe with the LabSmith 3-port valve, one-piece fitting has been used to connect the special syringe's stainless steel needle (OD 0.8 mm, ID 0.2 mm, length 35 mm, volume 1 µL). Voltage was applied using an in-house built USB DC voltage power supply (high voltage components: UltraVolt® 3V5-N0.5-EI-W and 3V5-P0.5-EI-W), unless otherwise mentioned, connected to the stainless-steel needle and plunger through alligator clips. PEEKsil® tubing (ID 25 µm, L 50 mm) was provided by Trajan Scientific and Medical and connected to one of the valves ports and served as a flow restrictor during the infusion step. Focusing of the fluorescent labelled HSA was visualized with a Dino-Lite Edge fluorescence microscope (AM4115T-GFBW; Taiwan).

4.3.3 Electrochemical method

0.4 % (w/v) of quinhydrone was added to a BGE composed of 50 mM formic acid and 10 µL of the solution were aspirated by a 25 µL syringe where a potential of -2000 V was applied using the plunger as a cathode and the needle as a ground for 5 min. The volume of gas bubbles generated at the cathode after adding quinhydrone was measured using ImageJ 1.52g software and compared with the volume of bubbles generated under the same conditions but without adding quinhydrone. The % reduction in bubbles was calculated according to the equation:

% reduction in bubbles = $(V_1-V_2)/ V_1 \times 100$

Where V_1 is the volume of bubbles at the cathode after the EkE without adding

quinhydrone and V_2 is the volume of bubbles at the cathode after the EkE with quinhydrone addition.

4.3.4 Addition of acetonitrile

Focusing of Chromeo™ 488 labelled HSA (200 µg/mL) toward the plunger of a 25 µL syringe using an applied voltage of -2000 was monitored to study the effect of adding different concentration of acetonitrile ranging from 0 % to 70 % (v/v) to the BGE (50 mM formic acid) on the volume of the gas generated at the cathode side, the focusing time, and the distance of the aggregated protein band from the cathode.

4.3.5 High pressure approach

A sealed system was designed for the EkE chamber (syringe barrel) by using a needle connected to the push-pull valve or connecting the needle to a LabSmith 3-port valve along with having a polytetrafluoroethylene (PTFE) coated gas tight plunger with an exposed metallic tip as indicated in Figure 4-1.

Two systems for the removal of proteins were developed using the push-pull valve; the first system was suitable for the analysis of the weakly acidic compounds employing a 50 mM formic acid as a BGE, the plunger as a cathode, and the needle as a ground, an external pressure was required by actuating the syringe pump with a flow rate 0.2 µL/min. Reversely, 1 % trimethylamine served as a BGE, plunger as a ground and the needle as a cathode for the system suitable for the weakly basic compounds, no external pressure was required however the syringe was placed on the syringe pump to keep the plunger at a fixed position. Both systems were applied using syringes with different volumes where 10 µL aliquots of the Chromeo™ P465 labelled

albumin (1 mg/mL) in the BGE were aspirated by the 25 µL EkE syringe and 40 µL aliquots of the protein solution were aspirated by the 100 µL syringe.

Another high voltage component, EMCO-E40 proportional DC to high voltage DC converter (XP Power, Singapore), was used for the high voltage application during the comparison between the design included the push-pull valve and the LabSmith 3-port valve system using the conditions of the weakly basic compounds analysis. By adjusting the input voltage to 15 V, the measured voltage across the syringe was found to be 600 V.

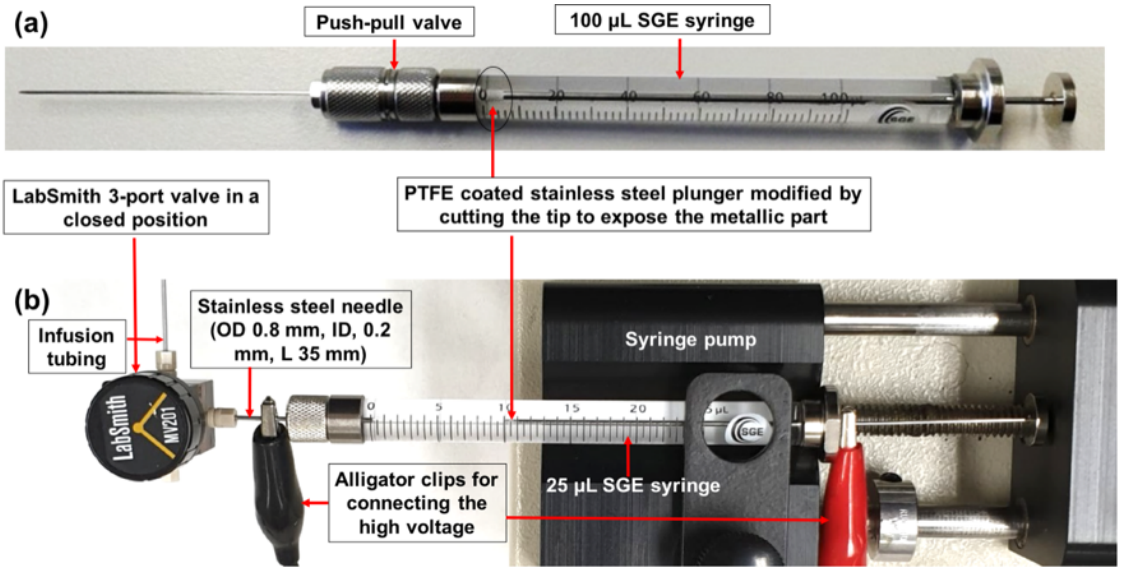


Figure 4-1. a, a 100 µL SGE syringe with a push-pull valve and a stainless steel PTFE coated plunger. b, a 25 µL EkE syringe connected to a LabSmith 3-port valve in a closed position.

4.3.6 Controlling of the bubbles during the infusion

A PEEKsil® tubing with an ID of 25 µm and a length of 50 mm was connected to the infusion port of the LabSmith 3-port valve to serve as a flow restrictor during the infusion. The syringe content was infused immediately with a flow rate of 5 µL/min through the flow restrictor after the EkE process (removal of the labelled albumin) using 1 % triethylamine as a BGE by applying the voltage (measured voltage of 600 V) for 1.5 min using the EMCO-E40 proportional DC converter. The solution was infused after switching the valve from the closed

position to the infusion position. The infusion process *via* the flow restrictor was compared to the infusion through an open port valve.

4.4 Results and Discussion

4.4.1 Electrochemical method

In an attempt to suppress the water electrolysis and consequently the electrolysis gases during the in-syringe EkE, a 0.4 % w/v of quinhydrone (a complex of hydroquinone and *p*-benzoquinone with water solubility= 0.4 g/100 g water) was added to the BGE to replace the water redox reactions at the electrodes [16]:

Anodic reaction $C_6H_4O_2H_2 \rightarrow C_6H_4O_2+2H^++2e^-$

Cathodic reaction $C_6H_4O_2+2H_2O+2e^- \rightarrow C_6H_4O_2H_2+2OH^-$

Using the BGE and the applied voltage of the system for weakly acidic compound's analysis "Chapter 3", the bubble generation reduced by 18.1% after the addition of quinhydrone and there were noticeable bubbles in the solution as indicated in Figure 4-2.

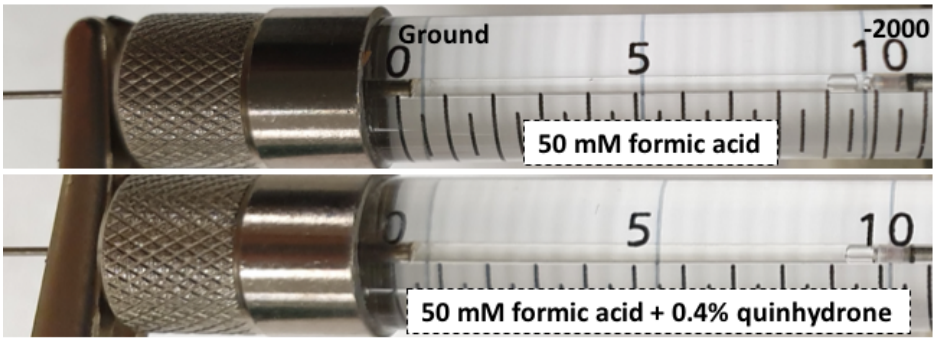


Figure 4-2. Comparison of the volume of the generated bubbles using a BGE of 50 mM formic acid and an applied voltage of -2000 V for 5 min without the addition of quinhydrone and after adding a 0.4 % (w/v) quinhydrone. The volume measurements were done by ImageJ 1.52g software.

4.4.2 Addition of acetonitrile

An organic solvent such as acetonitrile and methanol can stabilize the gas bubbles and decrease their sizes [17]. Figure 4-3 indicates the decrease of the

volume of the generated bubbles by increasing the concentration of acetonitrile up to 50 % without significantly affecting the focusing time or the distance of the focused albumin from the plunger (cathode), there was no further significant reduction in bubble volume by increasing the acetonitrile concentration to 70 %. Complete elimination of the bubbles generation during EkE was not achieved, and the addition of a high percentage of acetonitrile is not favorable to avoid the precipitation of the biological samples with high protein contents prior to the EkE.

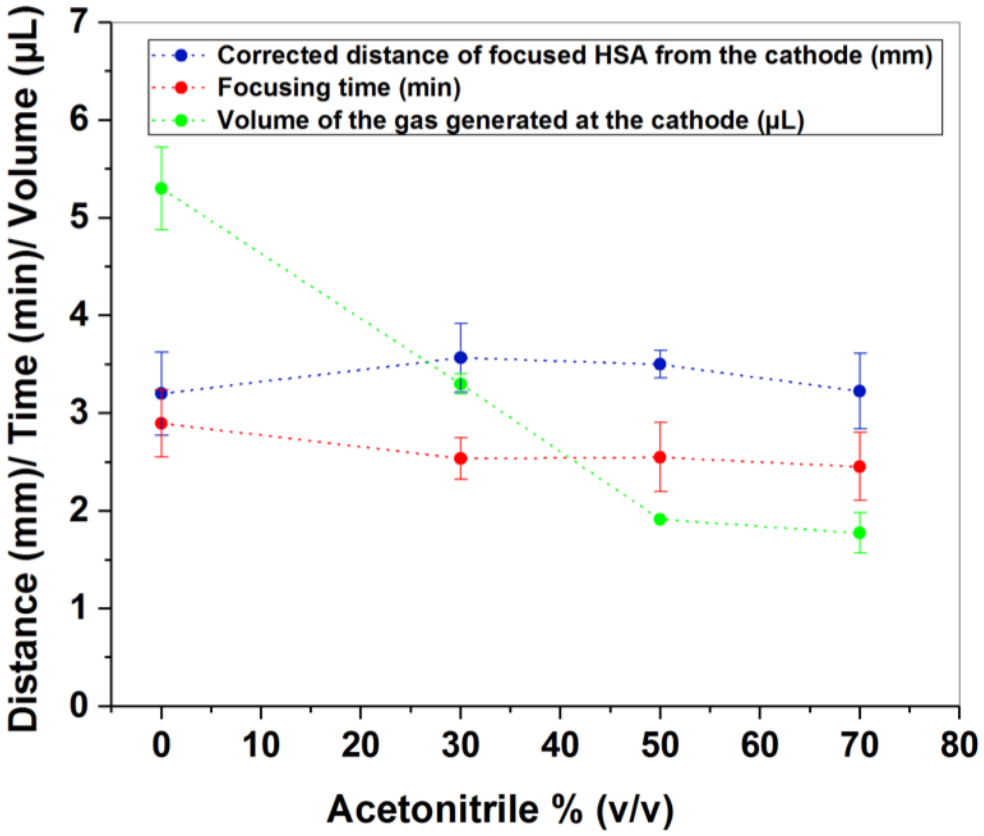


Figure 4-3: Effect of acetonitrile concentration on the distance of the aggregated protein band from the cathode, the focusing time, and the volume of the gas generated at the cathode side. The distance and volume measurements were done by ImageJ 1.52g software, the distance of the focused protein from the cathode was corrected by subtracting the distance occupied by the generated gas.

4.4.3 High pressure approach

A sealed system was designed to create a bubble-free in-syringe EkE system using a push-pull valve or a 3-port selector valve at the needle side and

a gas-tight plunger. For a liquid inside a closed system such as the sealed EkE syringe, any pressure applied is distributed homogeneously in the entire system so that any pressure gradient and gas supersaturation are avoided [4]. Zero flow owing to the sealed system will result in no pressure loss because of the hydraulic resistance according to Hagen–Poiseuille equation [20]:

$\Delta P=-8\mu LQ/\pi R^4$

Where ΔP is the pressure difference, μ is the viscosity, Q is the flow rate, L is the tube length, and R is the tube radius [20].

The generated high pressure inside the syringe can increase the solubility of gases and reduce their saturation levels in the liquid (see Henry's law [4, 6] in the Introduction section "Section 4.2" which results in the degassing of the bubbles.

Sealing the syringe with a push-pull valve and a gas tight plunger was examined first for the removal and aggregation of Chromeo™ P465 labelled HSA (isoelectric point 4.7-4.9 [21]). HSA was selected as a model abundant protein in blood. Frist, an acidic BGE (50 mM formic acid) was utilized to positively charge the albumin prior to its aggregation toward the cathodic plunger as indicated in Figure 4-4. The second system relied on using a basic BGE (1 % triethylamine) and the plunger as an anode for the focusing of the negatively charged albumin (Figure 4-5).

Figure 4-4a shows that simply sealing the syringe with the valve (i.e. no applied pressure by the syringe pump) was not sufficient to have a bubble free system for the acidic BGE system. In contrast, only sealing the system was enough for a bubble free EkE when the needle was used as a cathode in the system with a basic BGE as indicated in Figure 4-5a.

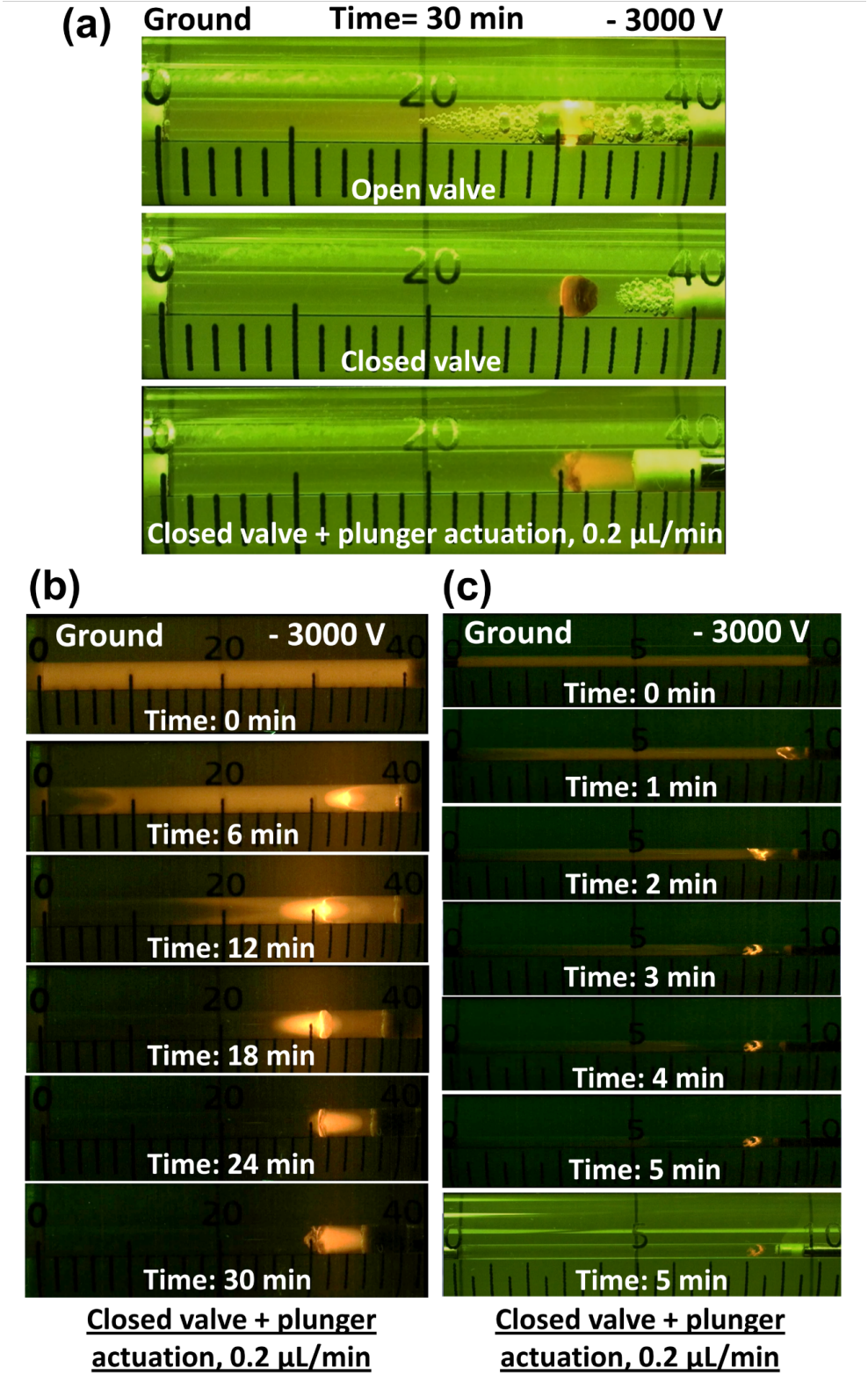


Figure 4-4. a, applying of a -3000 V on a solution of 1 mg/mL Chromeo^{TM} P465 labelled albumin in 50 mM formic acid using a 100 µL EkE syringe with the plunger as a cathode and the needle as a ground under different pressurizing conditions. b, a single-frame time series of the elimination of the Chromeo^{TM} P465 labelled albumin from the solution of 50 mM formic acid using a 100 µl EkE syringe connected to a closed push-pull valve and applying pressure by actuating the syringe pump with a flow rate 0.2 µL/min. c, a single-frame time series of the elimination of the labelled HSA in the acidic BGE using a 25 µl EkE syringe.

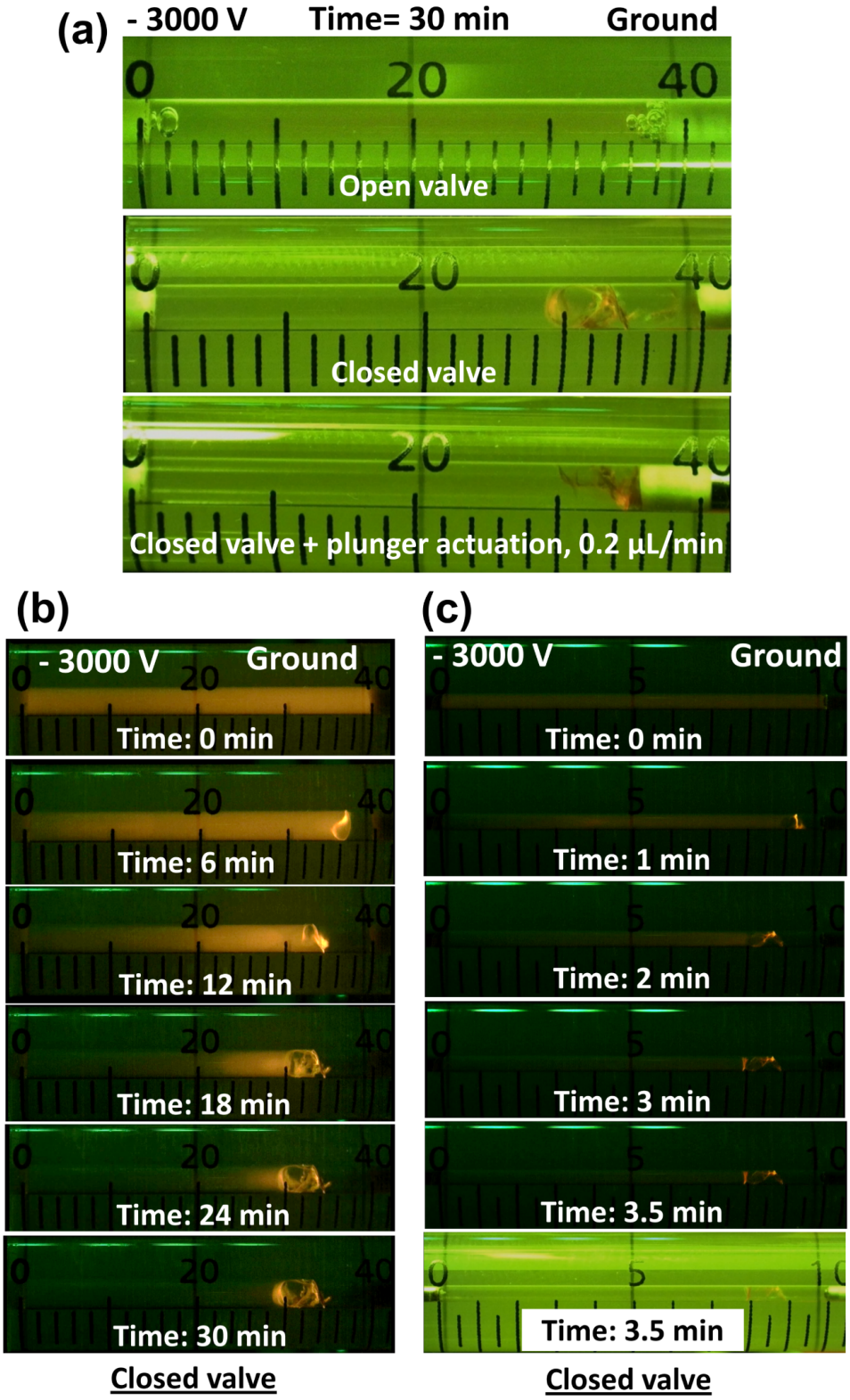


Figure 4-5. a, applying of a -3000 V on a solution of Chromeo™ P465 labelled albumin (1 mg/mL) in 1 % triethylamine using a 100 µL EkE syringe with the plunger as a ground and the needle as a cathode under different pressurizing conditions. b, a single-frame time series of the elimination of the Chromeo™ P465 labelled albumin from the solution of 1 % triethylamine using a 100 µl EkE syringe connected to a closed push-pull valve without any external pressure. c, a single-frame time series of the elimination of the Chromeo™ P465 labelled HSA in the basic BGE using a 25 µl EkE syringe.

In other words, the solution was free from any bubbles in the sealed basic BGE system whether the external pressure was applied by actuating the syringe pump during the EkE or not. The possible reason is the ease of H_2 gas leakage from the push-pull valve when the needle was utilized as a cathode in the basic BGE system. The addition of an external pressure by actuating the syringe pump with a very slow flow rate 0.2 µL/min, however, was able to eliminate the generation of the bubbles in the acidic BGE system (Figure 4-4a).

A bubble free EkE was achieved using 100 µL and 25 µL syringes as illustrated in Figure 4-4b and Figure 4-4c, respectively, for the acidic system. A much longer time was required to eliminate the albumin using the 100 µL syringe (6 times higher than the 25 µL syringe) due to the lower field strength and the higher amount of proteins to be eliminated accompanying the processing of a higher volume. In the same manner of the acidic system, the EkE using the basic system was accomplished in 100 µL and 25 µL syringes (Figure 4-5b and Figure 4-5c, respectively).

Given the limitations of the push-pull system such as not allowing different infusion ports, an alternative approach of using a 3-port valve was investigated using an EMCO E40 DC converter as a high voltage component. This converter offered higher power than the UltraVolt® 3V5-N0.5-EI-W power supply, 3 Watts for the EMCO E40 compared to 0.5 Watt for the Ultravolt® power supply. The EMCO E40 DC converter system should be less affected by the applied voltage auto-regulation so it can consequently accelerate the protein aggregation.

The bubble free EkE was accomplished within 90 s as shown in Figure 4-6 in both push-pull valve system and 3-port valve system using the EMCO E40 DC converter with a voltage of 600 V measured across the syringe. Although

the focusing times were very close in the push-pull valve system and the 3-port valve systems (78 s and 90 s, respectively), the 3-port valve can offer many advantages over the push-pull valve such as being convenient for the coupling with the mass spectrometers through the infusion port and also this coupling represents the first step toward the full-automation where more ports can be added with a suitable multi-port valve.

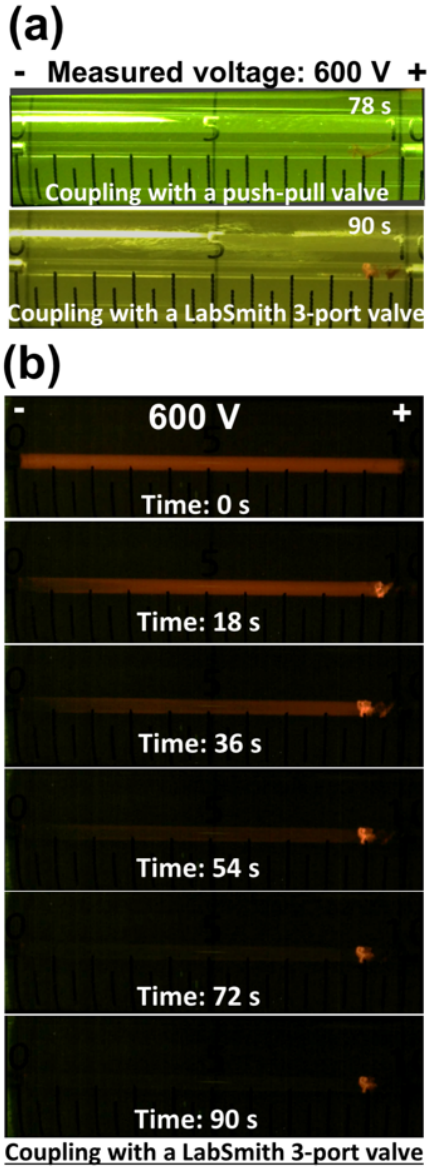


Figure 4-6. a, elimination of Chromeo™ P465 labelled albumin (1 mg/mL) from a solution of 1 % triethylamine using a 25 µL EkE syringe after coupling with the push-pull valve and the 3-port LabSmith valve in closed position. b, a single-frame time series of the elimination of the Chromeo™ P465 labelled albumin in 1 % triethylamine using a 25 µL EkE syringe coupled with a 3-port LabSmith valve in a closed position utilizing the plunger as an anode and the needle as a cathode.

4.4.4 Controlling of the bubbles during the infusion

To keep a high pressure applied after the valve is switched to the injection port for infusion, a PEEKsil tubing (L 50 mm and ID 25 µm) was connected as a flow restrictor to the injection port and was found to be efficient in minimizing bubble generation during infusion (5 µL/min) as shown in Figure 4-7. After infusing 3 µL of the syringe content, less than a 0.25 µL of bubbles was noticed in the system connected to the flow restrictor while 1.7 µL of gas bubbles were generated if the infusion step was accomplished without it.

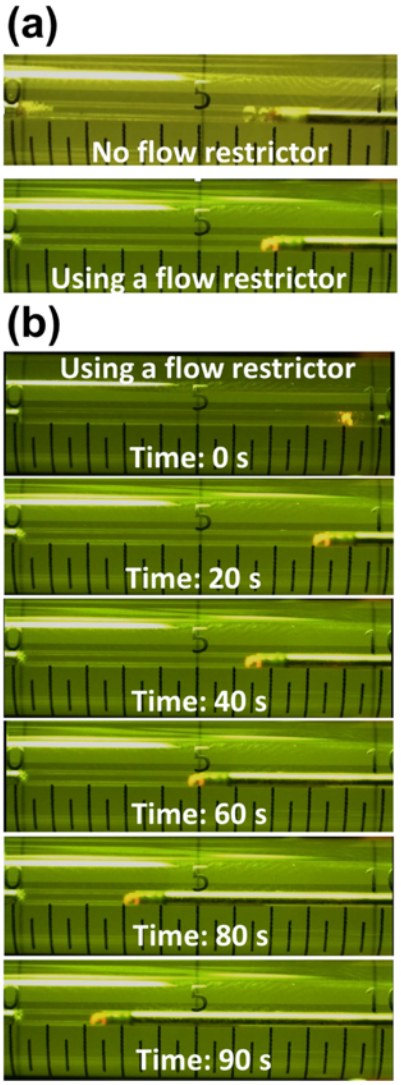


Figure 4-7. a, the bubbles generated after the EkE of the Chromeo™ P465 labelled albumin (1 mg/mL) and the infusion of 3 µL of the syringe contents with a flow rate 5 µL/min with and without a flow restrictor. b, a single-frame time series of the infusion of the syringe content for 90 s with a flow rate of 5 µL/min *via* a PEEKsil tubing (L 50 mm and ID 25 µm) connected to the infusion port of the 3-port LabSmith valve.

4.5 Conclusions

Different approaches for designing a bubble free in-syringe EkE have been investigated including the electrochemical methods, adding a % of organic solvents, and applying pressure. The high pressure approach was found to be the most effective for bubble elimination during the EkE (100% elimination of the bubbles) followed by adding 50% acetonitrile to the BGE (~60 % reduction in the generated bubbles volume) and the least effective was the electrochemical approach (< 20 % reduction in the generated bubbles volume).

An efficient bubble free system was developed using the high pressure approach, the simple design is based on sealing the syringe during the EkE by connecting a push/pull valve or a 3-port valve in the closed position at the needle side along with using a gas-tight plunger. The developed design was successfully applied for the electrokinetic elimination of albumin from the solution using two different BGE systems to offer suitable options for the clean-up of either the acidic or the basic target analytes. The first system was based on positively charging the HSA using 50 mM formic acid and elimination of the protein toward the plunger (cathode). The other system is based on the same concept but 1 % triethylamine was used as a BGE and the plunger served as an anode. The coupling of the bubble free system with electrospray ionization mass spectrometry for the basic compounds' analysis is explained in details in the next Chapter "Chapter 5".

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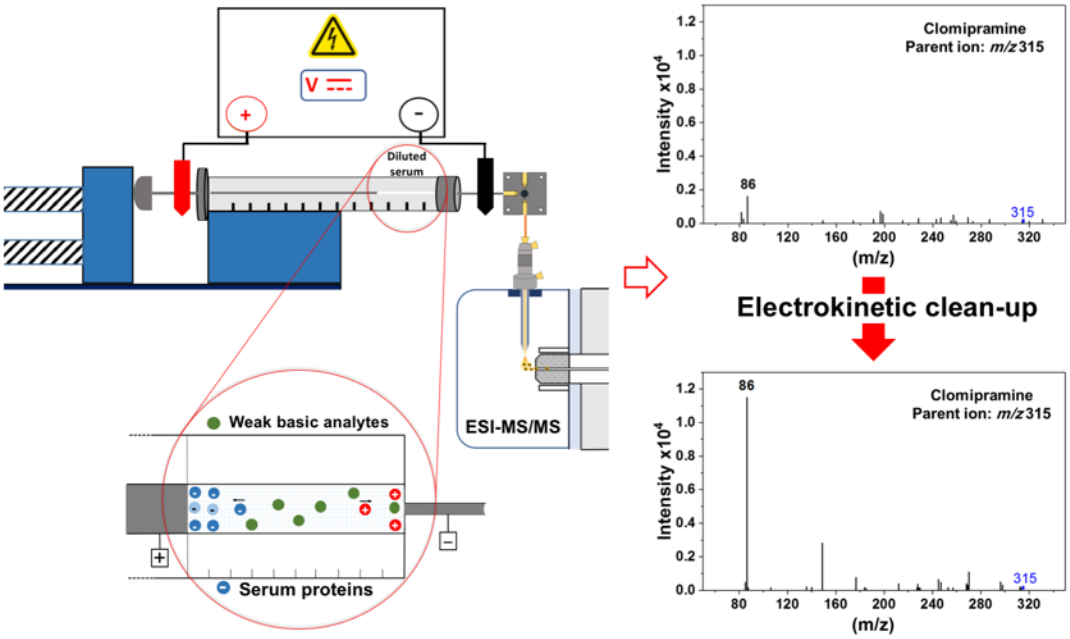
5. In-Syringe Electrokinetic Preparation of Serum Samples for Analysis of Weak Basic Compounds *via* ESI-MS

This chapter has been included in an accepted research article in Angewandte Chemie Int. Ed. [https://doi.org/10.1002/anie.202006481].

5.1 Abstract



The approach of the in-syringe electrokinetic extraction (EkE) was applied for the analysis of weakly basic compounds. Under basic conditions, the anionic proteins aggregate near the anodic plunger allowing for the infusion of the deproteinated sample into ESI-MS for basic molecules detection. Signal enhancements of 3.6-32 fold relative to direct infusion of diluted serum were obtained for basic pharmaceuticals, namely, clomipramine, chlorphenamine, pindolol, and atenolol. The sample preparation for the EkE-ESI-MS/MS method for the basic pharmaceutical determination in spiked serum was limited to a 5x

dilution of the sample in the background electrolyte (BGE) followed by vortex-mixing for 30 s. The BGE after the serum dilution was composed of 300 mM NH_4OH in 30 % (v/v) acetonitrile and the in-syringe EkE was accomplished by applying 800 V for 90 s on a 10 µL of the diluted serum using the needle and the plunger as a cathode and an anode, respectively. A bubble-free system was maintained during the EkE by connecting the needle to a 3-port valve in a closed position and using a gas tight plunger. After the EkE, the valve was switched and 8.0 µL of the syringe content were Infused into the ESI sprayer (flow rate: 5.0 µL/min) along with a coaxial infusion of methanol/water/formic acid, 75:24.5:0.5 (v/v) as a sheath liquid at a flow rate of 5.0 µL/min. The applied voltage was reduced to 200 V during the infusion step. The calibration graphs of the EkE-ESI-MS/MS method were constructed for clomipramine, chlorphenamine, pindolol, and atenolol using the corresponding deuterated isotopes as internal standards. Linear correlations for the basic drugs were achieved with a correlation coefficient (r) > 0.997, covering the necessary clinical range. LOQs of 5.3, 7.8, 6.1, and 17.8 ng/mL were attained for clomipramine, chlorphenamine, pindolol, and atenolol, respectively. Moreover, acceptable accuracy (% found of 82% to 120%) and precision ($\leq$ 20 %) were attained for all the spiked basic drugs.

5.2 Introduction

The same concept of the in-syringe electrokinetic extraction (EkE) for the analysis of weakly acidic drugs in serum samples (Chapter 3) was adapted in this chapter for the analysis of weakly basic compounds. A basic background electrolyte (BGE) composed of 300 mM NH_4OH with a pH 11.4, a pH higher than the serum proteins' *pIs,* directs protein electromigration towards the

plunger (anode) leaving a protein-depleted region in the syringe while the neutral weakly basic compounds will mainly remain dispersed across the syringe at this high pH. Subsequent actuation of the plunger infuses neutral molecules into the ESI interface, allowing for their MS detection with no influence from the proteins.

A bubble free EkE syringe was coupled with ESI-MS/MS for the analysis of four weakly basic drugs with different levels of protein binding, namely, clomipramine, chlorphenamine, pindolol, and atenolol (plasma protein binding: 98 %, 72 %, 40 %, and 6-16 %, respectively) in spiked serum using their corresponding deuterated isotopes as internal standards (ISs).

Accomplishing the sample preparation in a liquid handling device - syringe - simplified the workflow and can create new opportunities for the automated analysis.

Time of only 3.1 min (1.5 min for EkE, 1.6 min for infusion) is required for the analysis of a sample of 2 µL serum with a consumption of 3.5 µL of acetonitrile in the BGE used to dilute the sample in the syringe. This is not only a time-efficient analysis but also cost-effective and environmentally friendly.

5.3 Experimental Section

5.3.1 Chemicals and reagents

Albumin from human serum (HSA) (≥99 %, fatty acid free, globulin free), sterile-filtered human serum, Chromeo™ P465, ammonium hydroxide (NH_4OH) solution (28-30 %), clomipramine hydrochloride (≥98 %), chlorpheniramine maleate salt (≥99 %), pindolol (≥98 %), atenolol (≥98 %), rhodamine 6G (dye content = 99 %), formic acid (≥98 %), and methanol (HPLC grade, ≥99.9 %), were purchased from Sigma Aldrich (St. Louis, USA). Clomipramine-d6

hydrochloride, chlorpheniramine-d6 maleate salt, pindolol-d7, and atenolol-d7 were obtained from Toronto Research Chemicals (Ontario, Canada). Acetonitrile (LC/MS grade) was bought from Honeywell-Burdick & Jackson (Muskegon, USA). Deionized water was produced by a Milli-Q reagent water system (MA, USA). Labelling of HSA by Chromeo™ P465 was accomplished according to the vendor protocol [1].

5.3.2 Instruments

The same instruments mentioned in Chapter 3 “Section 3.3.2”.

5.3.3 System optimization and coupling of the EkE syringe with ESI-MS

A 25 µL SGE analytical syringe (P/N 003250) was modified to develop a bubble-free EkE system. The syringe had a gas-tight plunger with polytetrafluoroethylene (PTFE) plunger tip. The upper 1.2 mm of the PTFE tip was cut to have an electric connection between the stainless steel plunger and the solution in the syringe barrel. A special stainless steel needle (OD 0.8 mm, ID 0.2 mm, length 35 mm, volume 1 µL) was used and coupled with a 3-port valve (LabSmith, Inc., Livermore, CA) with a tubing interface of 0.8 mm directly by a one-piece fitting. The EkE syringe was placed on a syringe pump and connected to the 3-port valve adjusted to the closed position during the EkE.

An EMCO-E10CT proportional DC to high voltage DC converter (XP Power, Singapore) was used for the high voltage application where the plunger served as an anode and the needle served as a cathode. After accomplishing the EkE, the 3-port valve was actuated to direct the flow of the clean sample to the triple tube sprayer through the capillary tubing (250 mm length, 50 µm ID, 360 µm OD, and 0.5 µL volume). ESI-MS analysis was performed in the positive mode of ionization with the following source settings: nebulizer gas pressure, 12.5

psi; dry gas temperature, 250°C; dry gas flow rate, 6.0 L/min; electrospray voltage of the ion source, -4500 V, with the use of ion charge control; smart target, 100000; maximum accumulation time, 200 milliseconds. DataAnalysis software was utilized to integrate the mass spectra and to produce the corresponding extracted ion electropherograms (EIEs). For the ESI-MS/MS analysis, fragmentation was carried out by the collision induced dissociation (CID) for 50 milliseconds in multiple reaction monitoring (MRM) mode. Fragmentation amplitude was set to 0.5 V for all the drugs and the deuterated isotopes except pindolol and pindolol-d7 where the fragmentation amplitude was adjusted to 0.4 V and the mass cut-off selection was set to 24 % of the precursor mass.

The EkE syringe was washed after every sample, the precipitated proteins were removed from the EkE syringe by N_2 gas after removing the needle and plunger, then, the EkE syringe was assembled and flushed with Milli-Q water, acetonitrile 50 % (v/v), and the BGE, respectively. Also, the valve and the capillary to the ESI-MS were purged with Milli-Q water, acetonitrile 50 % (v/v) and the BGE, 25 µL each, after every run.

A co-axial flow of the sheath liquid was enabled *via* the Agilent triple tube ESI sprayer and both the sample and the sheath liquid were infused accurately by two separate syringe pumps to the ESI sprayer. The sheath liquid was composed of 0.5 % formic acid in 75 % (v/v) methanol (5 µL/min).

Optimized experimental conditions such as the composition of the BGE, the applied voltage, the EkE time, and the serum dilution were selected by ESI-MS experiments and monitoring of the Chromeo™ P465 labeled HSA focusing *via* the fluorescence microscope.

5.3.4 Investigation of the ion suppression by serum proteins and significance of the EkE

A mixture of clomipramine, chlorphenamine, pindolol, atenolol (50.0 ng/mL of each drug), and HSA (8.0 mg/mL) as the most abundant protein in serum was analysed by ESI-MS without the EkE step (t=0) and after application of 800 V for different time intervals (t=10, 30, 60, 90, and 120 s) using 300 mM NH_4OH in 30 % (v/v) acetonitrile as a BGE. Every EkE syringe was filled with 10 µL of the drug/proteins mixture in the BGE and infused to ESI-MS with a flow rate of 5.0 µL/min after each time interval. The sheath liquid was composed of 0.5 % formic acid in 75 % (v/v) methanol and infused at a flow rate of 5.0 µL/min. For the weakly basic drugs, EIEs were obtained for the molecular ion $[M+1]^{+1}$ using the source conditions mentioned in section ”5.3.3 System optimization and coupling of the EkE syringe with ESI-MS”. For HSA analysis, the method of Babic *et al*. [2] (source-induced fragmentation method) was used by adjusting the voltage difference between the capillary exit and the skimmer to 335 V. The resulting b_{24}^{4+} fragment ion (most abundant fragment, *m/z* 685.1) was detected utilizing the positive mode of ionization and selected for the HSA monitoring. Finally, the average signal intensities of the weakly basic drugs and HSA at every time interval were divided by the corresponding intensities at t=0 and plotted *versus* time.

5.3.5 Determination of weakly basic drugs in spiked serum samples

Research using biological fluids was performed under the ethics approval by the Tasmanian Health and Medical Human Research Ethics Committee (approval number, H0016575). 1.00 mg/mL standard solutions of clomipramine, chlorphenamine, pindolol, atenolol, and the internal standards (clomipramine-

d6, chlorpheniramine-d6, pindolol-d7, and atenolol-d7) were prepared separately in methanol. The working solutions including all the drugs and the internal standards were prepared by appropriate dilution of the standard solutions with methanol. Sterile-filtered human serum was obtained from Sigma Aldrich (St. Louis, USA) and divided into 1.5 mL Eppendorf™ tubes, serum tubes were kept at -20 °C until the analysis. Aliquots of 20.0 µL serum samples were spiked by adding specific volumes of the working solutions to obtain spiked concentrations of 10.0, 20.0, 40.0, 80.0, and 160.0 ng/mL of clomipramine, chlorphenamine, and pindolol, 75.0, 150.0, 300.0, 600.0, and 1200.0 ng/mL of atenolol, 80.0 ng/mL of clomipramine-d6, chlorpheniramine-d6, pindolol-d7, and 600.0 ng/mL of atenolol-d7. Each serum sample was diluted 5 times with the BGE where the final composition of the BGE was 300 mM NH_4OH in 30 % (v/v) acetonitrile, then the diluted spiked serum was vortex-mixed for 30 s.

The EkE step was achieved by aspirating 10 µL of the diluted spiked serum into the EkE syringe followed by application of 800 V using the plunger as an anode and the needle as a cathode for 90 s while the syringe was placed on the syringe pump and connected to the 3-port valve in the closed position. After EkE, the valve was opened to the ESI sprayer and 8.0 µL of the EkE syringe content were Infused at a flow rate of 5.0 µL/min while keeping 200 V applied along with a coaxial flow of a sheath liquid (5.0 µL/min) composed of methanol:water:formic acid 75:24.5:0.5.

Multiple reaction monitoring (MRM) scanning was done using the transitions (molecular ion to the most abundant daughter ion) in Table 5-1. The calibration graphs were constructed by plotting the average intensity ratio between each

drug and the deuterated internal standard (n=3) from the integrated MS/MS spectra for the time interval of 1.4-1.6 minutes *versus* the corresponding drug concentration in ng/mL and the corresponding regression equations were derived.

Table 5-1. Analytical performance for the determination of the weakly basic drugs in spiked serum by EkE syringe-ESI-MS

Drug	Transition (*m/z*)	C_{max} (ng/mL)	Regression equation y=a+bx	Correlation coefficient (r)	LOD (ng/mL)	LOQ (ng/mL)
Clomipramine	315 > 86	41.0 to 81.0 [3]	y=0.0178+0.0091x	0.9975	1.8	5.3
Clomipramine-d6 (IS)	321 > 92					
Chlorphenamine	275 > 230	12.6 [4]	y=0.0282+0.0091x	0.9993	2.6	7.8
Chlorphenamine -d6 (IS)	281 > 230					
Pindolol	249 > 116	79.0 [5]	y=-0.0013+0.0052x	0.9998	2.0	6.1
Pindolol-d7 (IS)	256 > 123					
Atenolol	267 > 190	1070.0 [6]	y=-0.0391+0.0017x	0.9975	5.9	17.8
Atenolol-d7 (IS)	274 > 190					

5.3.6 Method validation

The developed method was validated according to the ICH Q2 (R1) guidelines [7]. The accuracy of the developed method for the determination of the weakly basic drugs in the spiked serum samples was evaluated at three concentrations levels; 10.0, 40.0, and 160.0 ng/mL for clomipramine, chlorphenamine, and pindolol and 75.0, 300.0, 1200.0 ng/mL for atenolol by calculating the ratio of the found and the added concentrations. Repeatability and intermediate precision were expressed by the RSD of the assayed triplicates of each concentration level [8].

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated according to the equations [7]:

LOD=3.3 σ/b [7]

LOQ=10 σ/b [7]

where σ is the standard deviation of the blank samples response and b is the slope of the calibration curve.

5.3.7 Process efficiency

Four samples sets (neat standards, neat standards after the EkE, spiked serum samples, and spiked serum samples after the EkE) were prepared as explained in details before in Chapter 3 “3.3.7” to evaluate the significance of the developed EkE which was applied according to the experimental conditions mentioned in section “5.3.5 Determination of weakly basic drugs in spiked serum samples”.

5.4 Results and Discussion

Using a basic BGE at pH 11.4 (300 mM NH_4OH), almost all serum proteins were negatively charged: albumins (58 % of total protein amount), globulins (38 %), and fibrinogen (4 %) [9] which have isoelectric points (*pI*s) of 4.7-4.9 [10], 5.3-7.3 [10], and 5.5-6.3 [11], respectively. Upon application of the voltage, the anionic serum proteins migrate, focus, and aggregate close to the plunger (the anode) while weakly basic analytes are neutral and don’t migrate electrokinetically at this high pH as indicated in Figure 5-1a. This was experimentally verified as shown in Figure 5-1b which shows the focusing of Chromeo™ P465 labelled human serum albumin (HSA) toward the plunger (anode) while Figure 5-1c clarifies that rhodamine 6G (basic dye, pKa 7.5) remains unfocused using an electrolyte composed of 300 mM NH_4OH in 30 % (v/v) acetonitrile and an applied voltage of 200 V. The structures, pKa values, and plasma proteins binding levels of the studied weakly basic analytes are summarized in Figure 5-2.

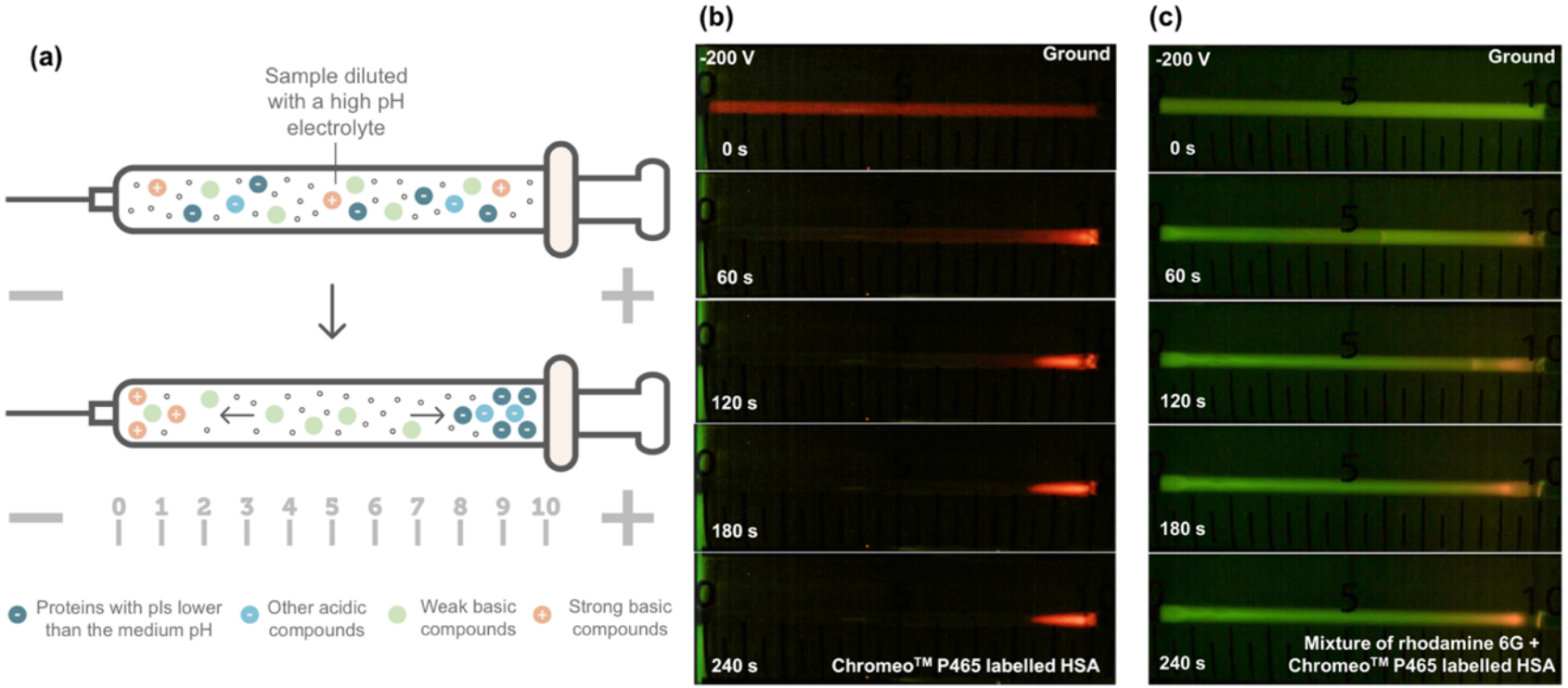


Figure 5-1. Electrokinetic clean-up mechanism. a, a schematic presentation of the EkE of biological samples from the interfering proteins by the EkE syringe for the analysis of weakly basic analytes using a basic BGE, the metallic plunger of the EkE syringe served as an anode and the metallic needle served as a cathode. b, a single-frame time series for focusing of 1.0 mg/mL of Chromeo™ 488 labelled human serum albumin (HSA) to the plunger as an anode using 300 mM NH_4OH in 30 % (v/v) acetonitrile and an applied voltage of 200 V. c, a single-frame time series for the separation of 1.0 mg/mL of Chromeo™ P465 labelled human serum albumin toward the plunger from its mixture with the weakly basic dye rhodamine 6G (2.0 µg/mL) using 300 mM NH_4OH in 30 % (v/v) acetonitrile and an applied voltage of 200 V.

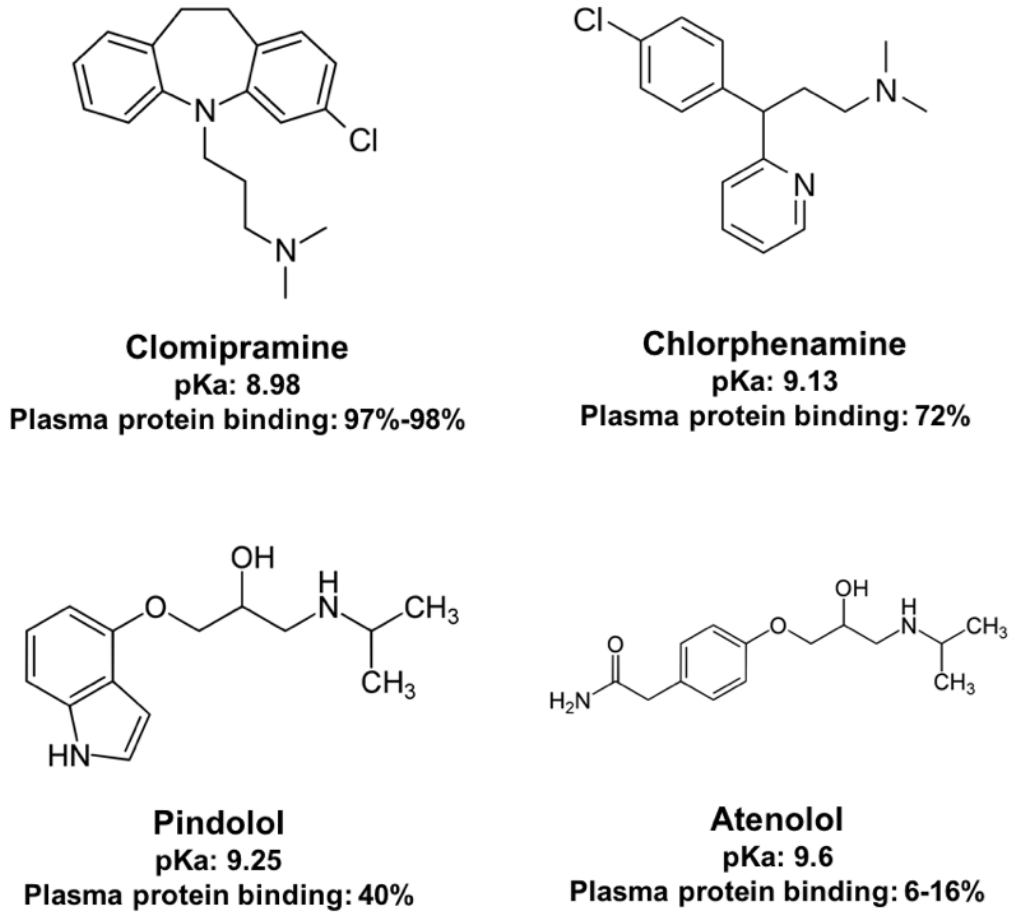
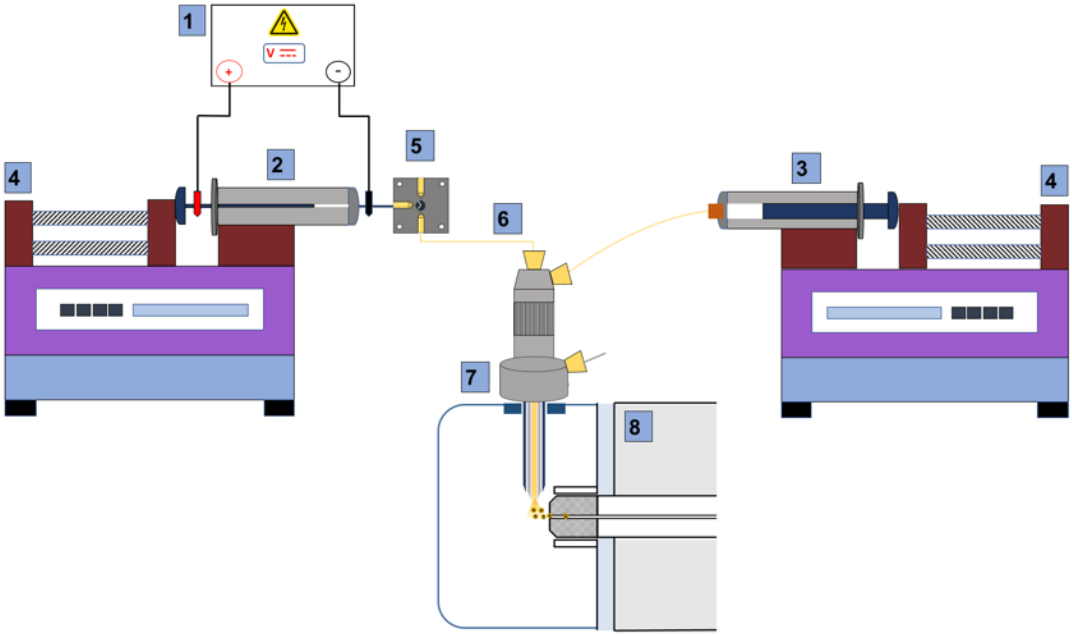


Figure 5-2. Structure, pKa, and plasma protein binding of clomipramine[12], chlorphenamine [13], pindolol [14], and atenolol [15].

5.4.1 Coupling of the EkE syringe with ESI-MS for the analysis of weakly basic analytes

The customized EkE syringe's needle (OD 0.8 mm) was connected directly to the 3-port valve which was adjusted to the closed position during the EkE step to provide a sealed system along with the gas-tight plunger, the generated high pressure in the syringe barrel due to the sealed system is necessary to solubilize the electrolysis gases into the solution and prevents the formation of gas bubbles in the separation channel (syringe barrel) during the EkE. Once the EkE was accomplished, the 3-port valve was positioned to the ESI-MS outlet and the clean-sample was infused to the triple tube ESI-MS sprayer *via* the capillary tubing, using the syringe pump with a flow rate (5.0 µL/min). The

triple tube sprayer allows for the coaxial infusion of the sheath liquid (0.5 % formic acid in 75 % (v/v) methanol, flow rate 5.0 µL/min) to enhance the ionization of the weakly basic drugs in the positive mode. The EkE syringe-ESI-MS Interface is indicated in Figure 5-3.



1- High voltage power supply **2- EkE syringe** **3- Sheath liquid syringe**
4- Syringe pump **5- 3-port valve**
6- Capillary tube (ID 50 µm, length 250 mm)
7- Agilent triple tube ESI sprayer
8- Agilent 6330 Ion Trap mass spectrometer

Figure 5-3. Coupling of the EkE syringe with ESI-MS (EkE syringe-ESI-MS interface).

5.4.2 Ion suppression by serum proteins and the significance of the EkE

To experimentally investigate the effect of the presence of serum proteins on the ionization of the weakly basic compounds, a mixture containing 8.0 mg/mL HSA (the most abundant serum protein at a concentration resembles the normal concentration of albumin in human serum after the 5-fold dilution with the BGE) and 50.0 ng/mL of clomipramine, chlorphenamine,

pindolol, and atenolol was infused to ESI-MS without the EkE and after different time intervals of 800 V application.

To detect HSA by ESI-MS, a source induced fragmentation method [2] was adapted by adjusting the voltage difference between the capillary exit and the skimmer to 335 V. The b_{24}^{4+} fragment ion (*m/z* 685.1) was the most abundant ion and selected to estimate the presence of HSA where the biochemical modification of HSA typically occurs beyond the first 30 residues from the albumin N terminus [2].

The average intensity of the molecular ion $[M+H]^{+1}$ of each drug and the fragment ion of HSA (*m/z* 685.1) were measured after each time interval of the EkE step and the ratio of the average intensity to the average intensity at time=0 (no EkE) was plotted *versus* time (Figure 5-4).

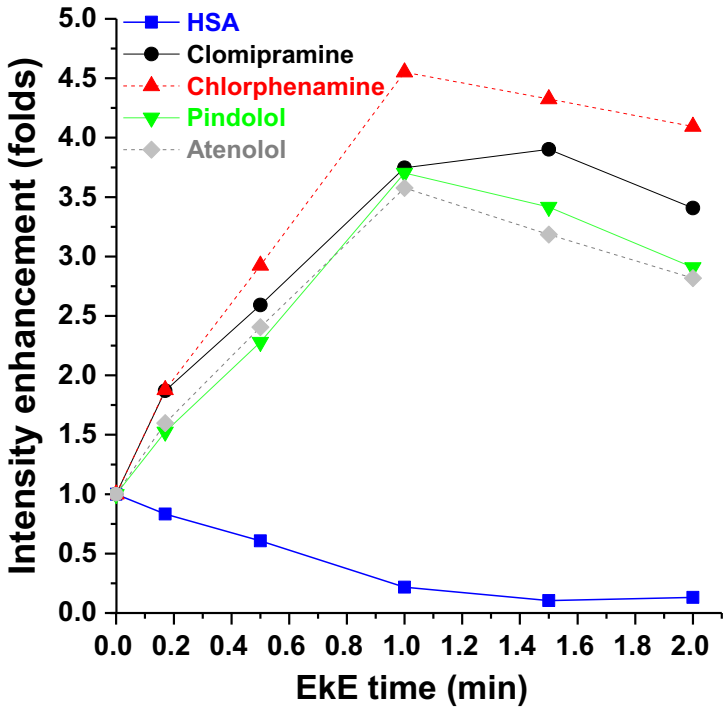


Figure 5-4. The ion suppression by serum proteins and the significance of the in-syringe EkE. Plot of the intensity enhancement/reduction (ratio of the average intensity after each time interval of 800 V application and the average intensity at time=0) for the molecular ion $[M+H]^{+1}$ of each drug and the fragment ion of HSA (*m/z* 685.1) *versus* EKE time, the studied mixture composed of 50.0 ng/mL of each drug and 8.0 mg/mL HSA in a BGE composed of 300 mM NH_4OH in 30 % (v/v) acetonitrile. The contents of the EkE syringe were infused to ESI-MS with a flow rate of 5.0 µL/min after each time interval and the sheath liquid was composed of 0.5 % formic acid in 75 % (v/v) methanol (5.0 µL/min).

Figure 5-4 indicates the gradual decrease of HSA intensity by time (up to 90 s) due to the aggregation and the removal of HSA by the EkE step and clarifies the reverse proportional between the signal intensities of weakly basic drugs and the HSA due to the elimination of the proteins' ion suppression. The Intensities of the four weak bases were enriched by increasing the time of the EkE up to 60 s after that there was no significant enhancement in intensity.

5.4.3 System optimization

NH_4OH was preferred over other electrolytes such as triethylamine which can contaminate the mass spectrometer and cause ion suppression in the positive mode of ESI-MS [16]. Solutions with different concentrations of NH_4OH (from 10.0 to 600.0 mM) were studied to serve as a BGE. Figure 5-5 shows that increasing the concentration of NH_4OH from 10 to 75 mM resulted in shifting of the focused Chromeo^{TM} P465 labeled HSA toward the plunger and decreasing the focusing time. The concentration of NH_4OH and the buffer capacity should be high enough to prevent the pH change and to avoid the electrical conductivity gradients due to the H^+ and OH^- fluxes from water electrolysis. The conductivity gradient can lead to flow instabilities [17]. There was no further enhancement by increasing the concentration from 75 up to 600 mM NH_4OH. However, increasing of NH_4OH concentration to 300 mM was necessary to keep the aggregated proteins as close as possible to the plunger (5.2 mm away from the plunger) after addition of 20 % (v/v) human serum to the BGE solution, as shown in Figure 5-5 by providing a buffer capacity of 11.1 mM.

Increasing the applied voltage from 100 V to 600 V decreased the focusing time of Chromeo^{TM} P465 labeled HSA from 4.0 minutes to 1.5 minutes after which the voltage *versus* focusing time graph reached a plateau as indicated in

Figure 5-6. 800 V was selected as the optimum applied voltage for the EkE step as the higher voltage can increase the electrophoretic velocities of the analytes, but at the same time results in higher current and Joule heating which is able to disperse the electrokinetic-based separation processes due to the temperature gradients in the separation channel [18].

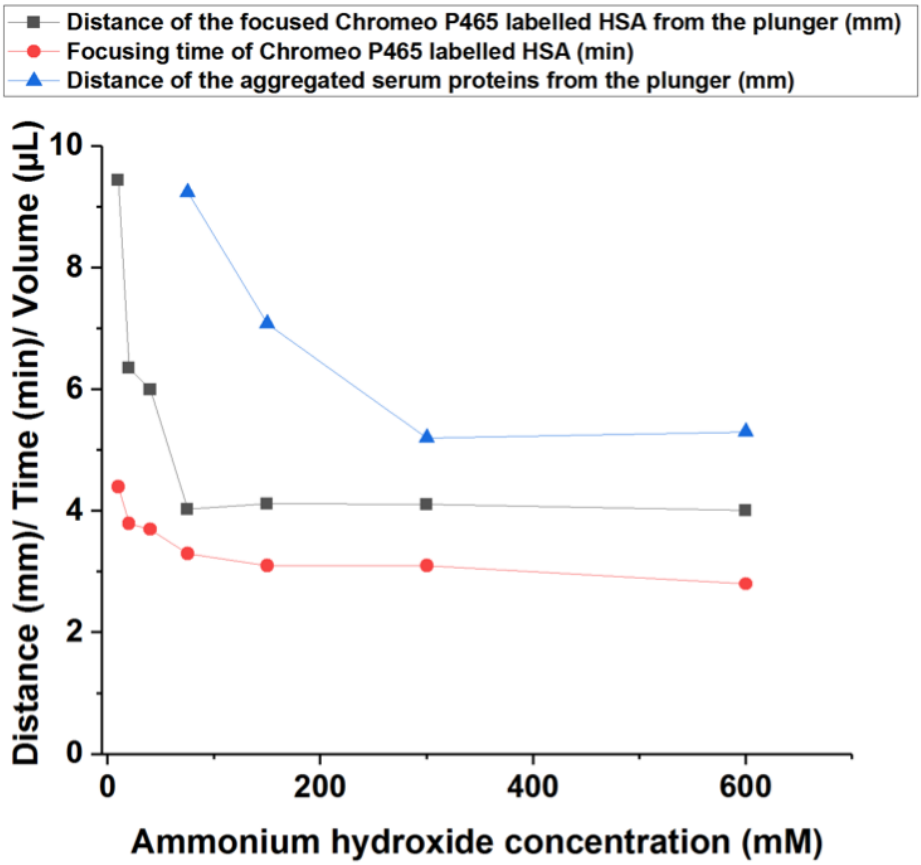


Figure 5-5. Effect of the molar strength of NH_4OH on the position of the focused Chromeo™ P465 labelled HSA (1.0 mg/mL) inside the syringe barrel, its focusing time, and the position of the aggregated serum protein after addition of 20 % (v/v) serum to the BGE using an applied voltage of 200 V, the distance measurement was done by ImageJ 1.52g software.

To optimize the ionization of the studied basic drugs in the ESI-MS experiments, sheath liquids of different composition were infused at different flow rates and the average intensities of the analytes were monitored. Best sensitivity for all the drugs was attained by infusing a sheath liquid composed of 0.5 % formic acid in 75 % (v/v) methanol at a flow rate of 5.0 µL/min as illustrated in Figure 5-7.

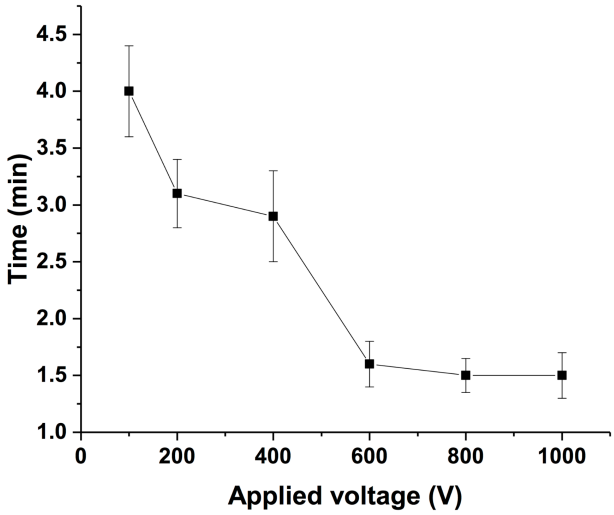


Figure 5-6. Effect of the applied voltage on the focusing time of Chromeo^{TM} P465 labelled HSA (1.0 mg/mL) by the EkE syringe using a BGE composed of 300 mM NH_4OH.

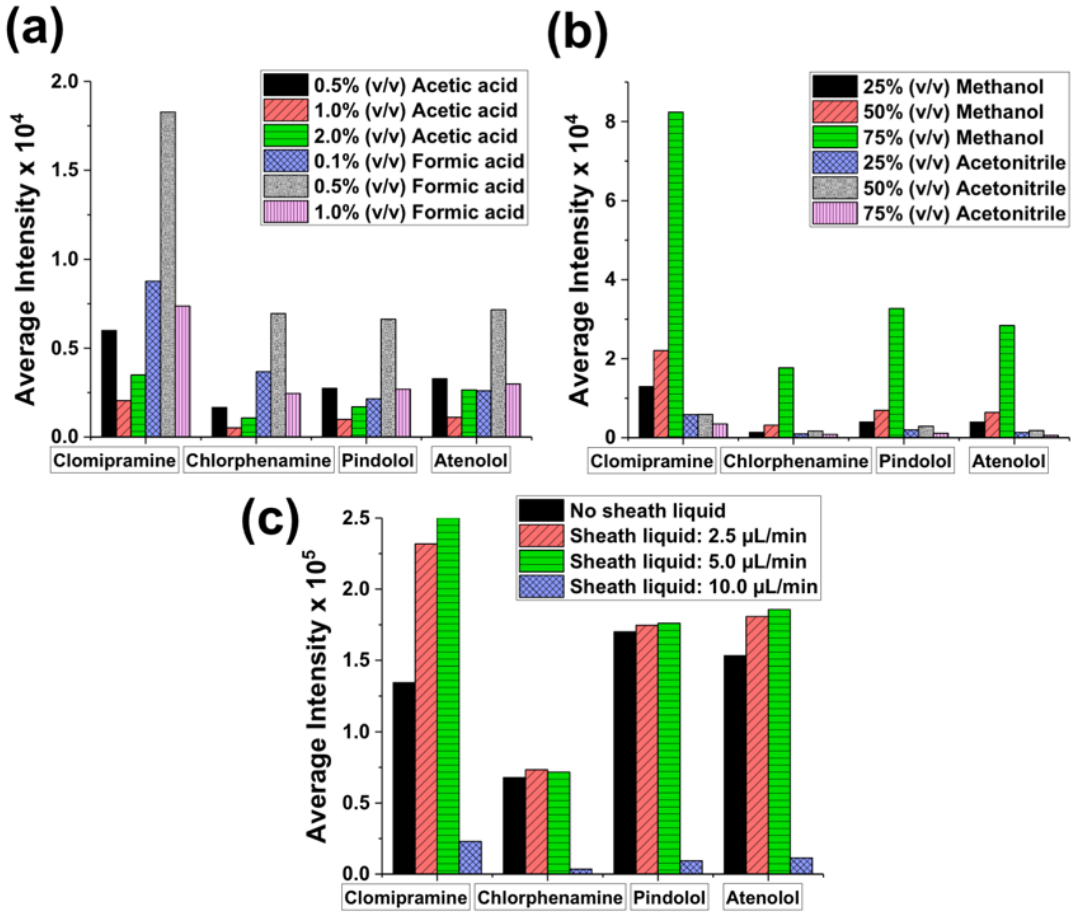


Figure 5-7. Influence of the sheath liquid composition and flow rate on the average signal intensity of clomipramine, chlorphenamine, pindolol, and atenolol (250.0 ng/mL of each drug) infused into ESI-MS (5 µL/min) in a BGE composed of 300 mM NH_4OH in 30 % (v/v) acetonitrile. a, effect of addition of different acids to the sheath liquid (50 % methanol) on the average signal intensities of the studied drugs. b, effect of adding different volatile solvents to the sheath liquid (0.5% formic acid) on the average signal intensities of the studied drugs. c, effect of the flow rate of the sheath liquid (0.5% formic acid in 75% methanol) on the sensitivity of the ESI-MS response.

Selection of a precise time window to integrate the average intensity for the experimental parameters optimization by ESI-MS and construction of the calibration graphs was accomplished by dividing each electropherogram into 0.2-minute segments and measuring the area percentage of each segment. The plot of the area percentage against time (Figure 5-8) indicates that the highest percentage of all drugs were infused to ESI-MS at the 1.4-1.6 minutes' segment. This shift toward the end of the infusion is related to the drug-protein binding where the drugs were partially focused toward the plunger due to the electrokinetic migration of serum proteins. Clomipramine (highest serum protein binding 98 %) showed the highest drug percentage at the 1.4-1.6 minutes' segment and the 1.6-1.8 minutes' segment and atenolol with lowest serum protein binding (6-16 %) exhibited the least drug percentage at the same segments.

Figure 5-9 illustrates the average intensities obtained from the integration of different segment widths around 1.5 minutes (midpoint of 1.4-1.6 segment) and compares between adding different percentages of the spiked serum in the BGE. For the four drugs, adding 20 % spiked serum to the BGE and using of 1.4-1.6 minutes window resulted in the best sensitivity without adversely affecting the repeatability (the SD indicated in Figure 5-9). The high average intensity obtained by adding 20 % serum is related to the compromise between the sample dilution and the ion suppression by serum matrix.

90 s was selected as the optimum time for the EkE step, the high signal intensity of all the drugs and the lowest SD indicate the complete removal of serum proteins (Figure 5-10).

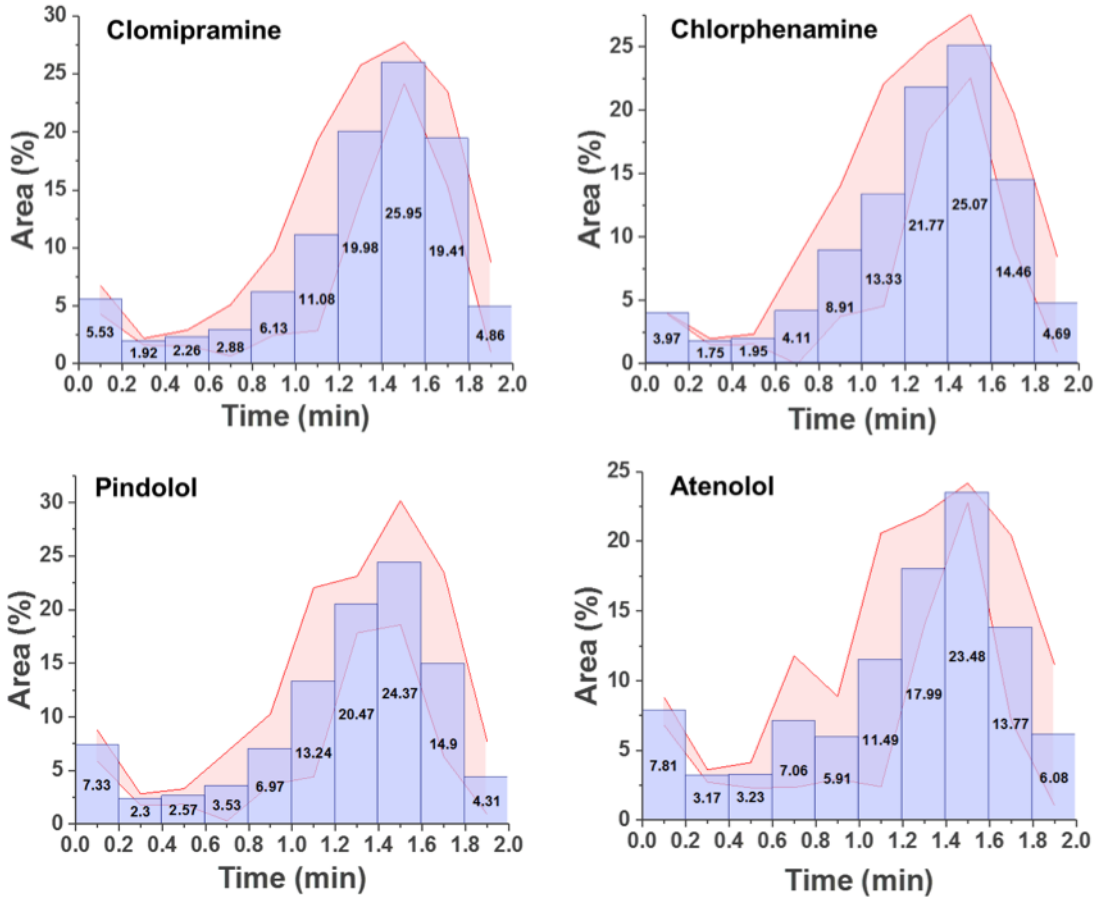


Figure 5-8. Distribution of the infused drugs by the EkE syringe. The area percentage of the 0.2-minute segments of the EIEs of clomipramine, chlorphenamine, pindolol, and atenolol (250.0 ng/mL of each drug) in spiked serum samples. 800 V was applied by the EkE syringe for 1.5 minutes on 10 µL of the x5 diluted spiked diluted serum in 300 mM NH_4OH and 30 % (v/v) acetonitrile followed by infusion of 8 µL to the ESI-MS (5.0 µL/minute) with a coaxial flow of the sheath liquid while keeping 200 V applied by the EkE syringe. The distance between the two red lines represents +/- SD of the mean area percentage.

Addition of acetonitrile was necessary to prevent the aggregation of the drugs along with the focused proteins where acetonitrile can assist in liberation of the bound drugs by breaking the hydrophobic interactions and ionic interactions with the serum proteins [19], this effect was very clear for clomipramine (protein binding 98 %) where the average signal intensity was more dependent on the acetonitrile percentage in the medium (Figure 5-11), however, we couldn't increase the concentration of acetonitrile more than 30 % (v/v) to avoid the serum proteins precipitation before the EkE. Although residual bound drugs might be aggregated with the serum protein during the

EkE, addition of acetonitrile can maximize the portion of the free drugs to be determined.

The potential was applied during infusion to keep the aggregated proteins close to the plunger, 200 V was selected as the optimum voltage for the infusion step to achieve the best sensitivity as indicated in Figure 5-12, the higher voltages resulted in more generated bubbles which can interrupt the infusion to the ion source, on the other hand, the signal related to the infusion without voltage application was suppressed due to the diffusion of the focused proteins which consequentially suppress the ionization.

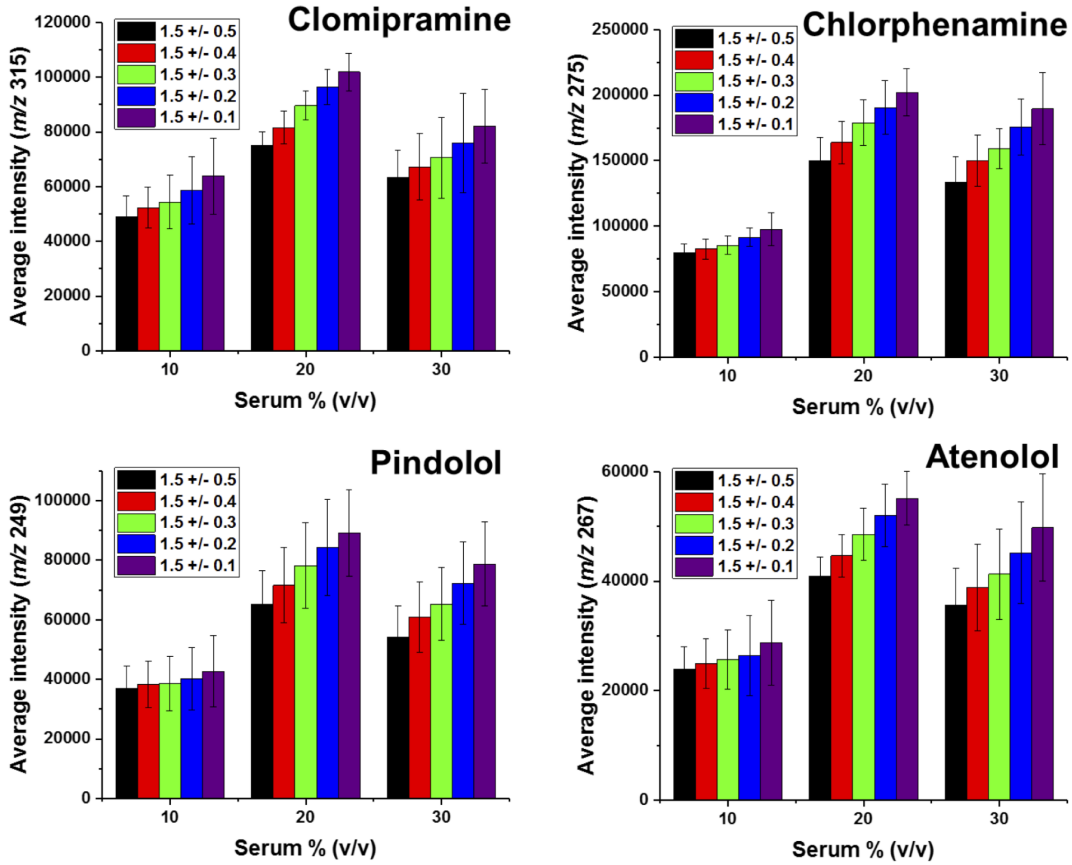


Figure 5-9. The average intensities of the weakly basic drugs (250.0 ng/mL of clomipramine, chlorphenamine, pindolol, and atenolol) in spiked serum samples obtained from the integration of different segment widths around the point of 1.5 minutes, the spiked serum was added to 300 mM NH_4OH in 30 % (v/v) acetonitrile at 3 different levels 10 %, 20 %, 30 % (v/v). The EkE was accomplished by applying 800 V for 1.5 minutes followed by infusion of 8 µL to the ESI-MS (5.0 µL/min) with a coaxial flow of the sheath liquid while keeping 200 V applied by the EkE syringe. The error bars represent the SD of the mean average intensities.

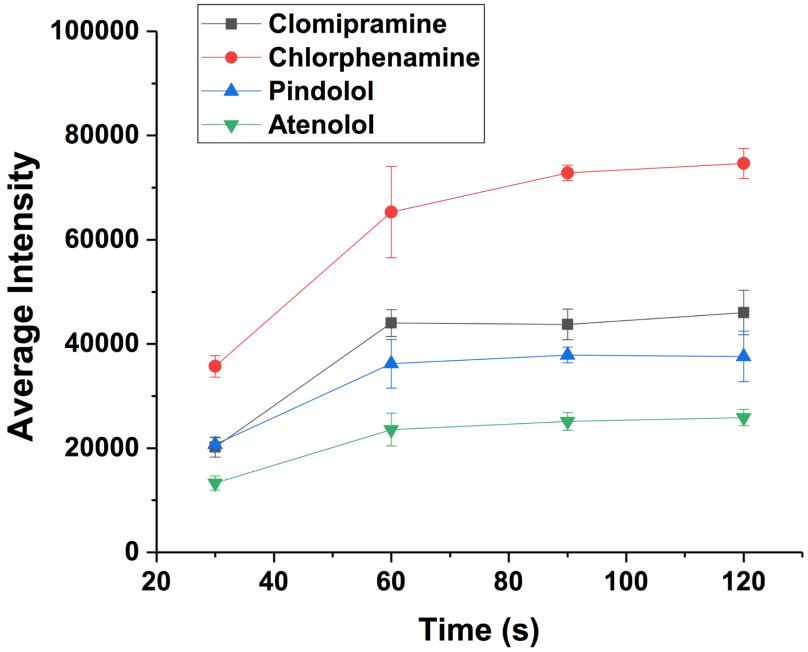


Figure 5-10. Effect of the time of the EkE step on the average intensity of 250.0 ng/mL of clomipramine, chlorphenamine, pindolol, and atenolol in spiked serum samples. Each serum sample was diluted five times by the BGE (final concentration 300 mM NH_4OH in 30 % (v/v) acetonitrile) and the EkE was done by application of 800 V. The error bars represent the SD of the mean average intensities.

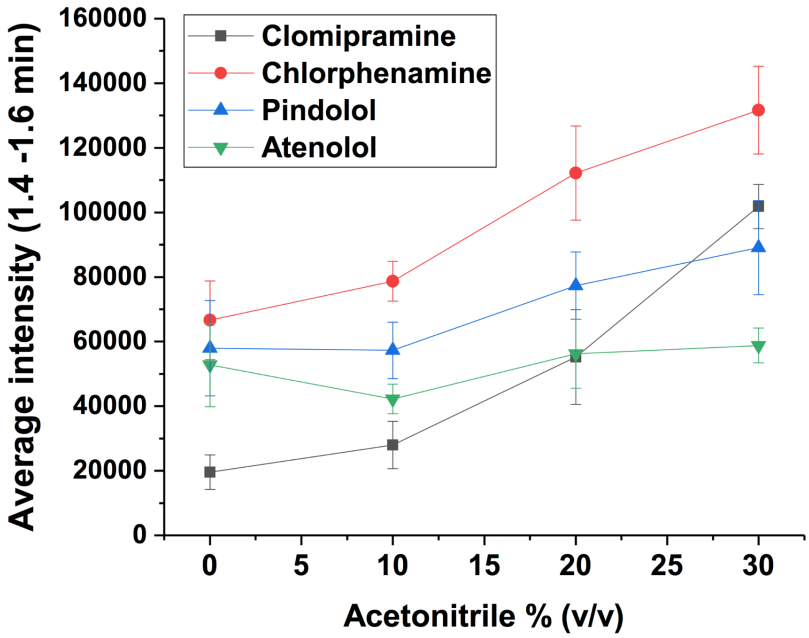


Figure 5-11. Effect of acetonitrile concentration on the average intensity (1.4-1.6 minutes) of 250.0 ng/mL of clomipramine, chlorphenamine, pindolol, and atenolol in spiked serum samples. Each serum sample was diluted five times by the BGE and the final concentration of the BGE was 300 mM NH_4OH. The EkE was accomplished by applying 800 V for 1.5 minutes followed by infusion of 8 µL to the ESI-MS (5.0 µL/min) with a coaxial flow of the sheath liquid while keeping 200 V applied by the EkE syringe. The error bars represent the SD of the mean average intensities (1.4-1.6 minutes).

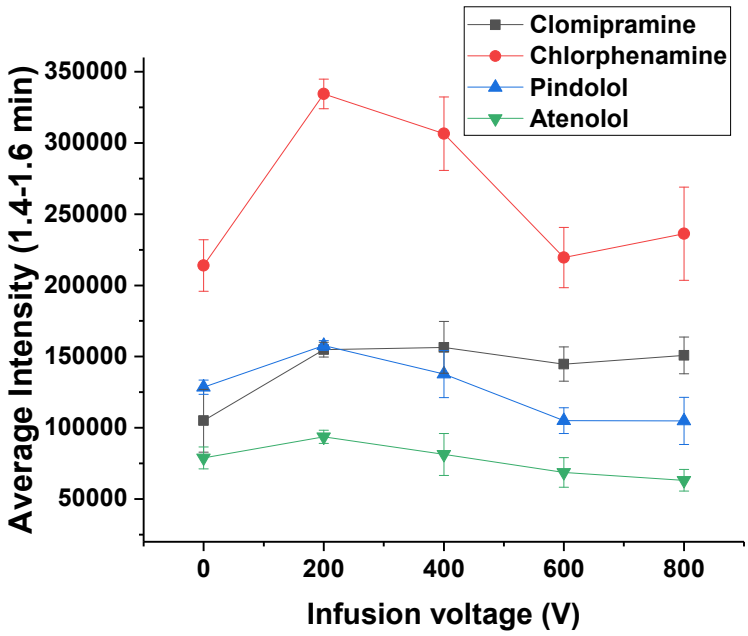


Figure 5-12. Effect of the voltage applied during the infusion step on the average intensity (1.4-1.6 minutes) of 250.0 ng/mL of clomipramine, chlorphenamine, pindolol, and atenolol in spiked serum samples. Each serum sample was diluted five times by the BGE and the final concentration of the BGE was 300 mM NH_4OH in 30 % (v/v) acetonitrile. The EkE was accomplished by application of 800 V for 90 s. The error bars represent the SD of the mean average intensities (1.4-1.6 minutes).

5.4.4 Determination of weakly basic drugs in spiked serum samples

The developed EkE syringe offered a very simplified protocol for the determination of clomipramine, chlorphenamine, pindolol, and atenolol in spiked serum samples by direct infusion ESI-MS/MS using the corresponding deuterated isotopes as internal standards. The EkE syringe was used to aspirate the spiked serum 5-times diluted with the BGE, to eliminate the serum proteins by EkE, and then to infuse the clean sample into the ESI-MS/MS. By the virtue of the EkE syringe-ESI-MS system, a time of only 3.1 minutes is required for every 2 µL serum sample analysis (1.5 minutes for the EkE and 1.6 minutes for the sample infusion) and less than 3.5 µL of acetonitrile was consumed using the EkE syringe for every EkE run.

The EIEs representing the four sets including neat standard solutions and pre-spiked serum samples with and without the EkE are indicated in Figure 5-

13. Additionally, the MS/MS spectra in Figure 5-14a and Figure 5-14b represent a comparison between spiked serum samples without the EkE and after the EkE to show the significance of the EkE on the signal intensities of the 4 drugs.

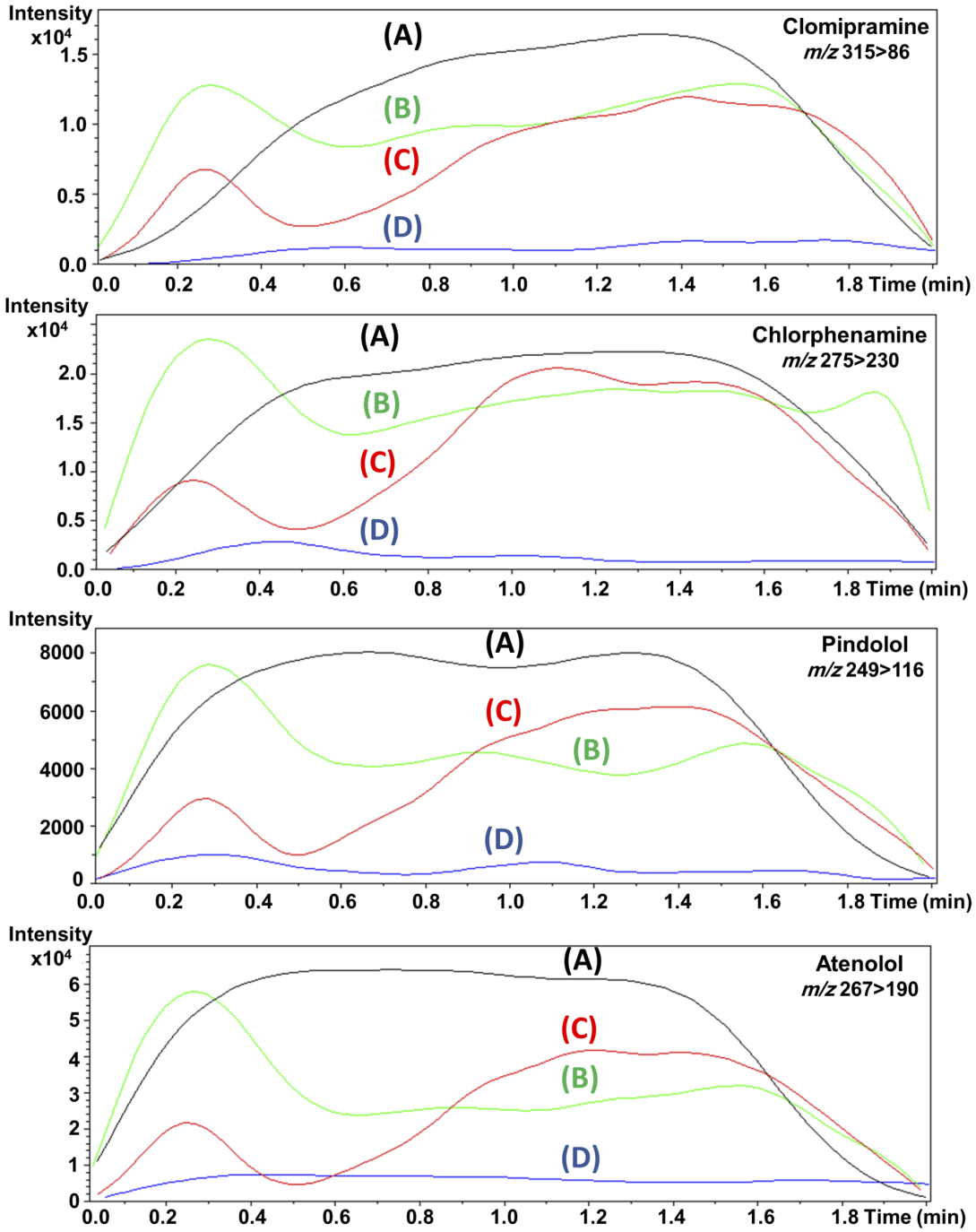


Figure 5-13. Comparison between neat standard solutions and pre-spiked serum samples with and without the EkE. The EIEs in MRM mode of the four weakly basic drugs (40.0 ng/mL of clomipramine, chlorphenamine, and pindolol and 300.0 ng/mL of atenolol) spiked in: neat standard solutions (A), neat standard solutions with the application of the EkE step before the infusion to ESI-MS (B), serum samples with the application of the EkE step (C), and serum samples without the EkE step (D). Every set was diluted 5 times with the BGE and the final composition of the BGE was 300 mM NH_4OH in 30 % (v/v) acetonitrile.

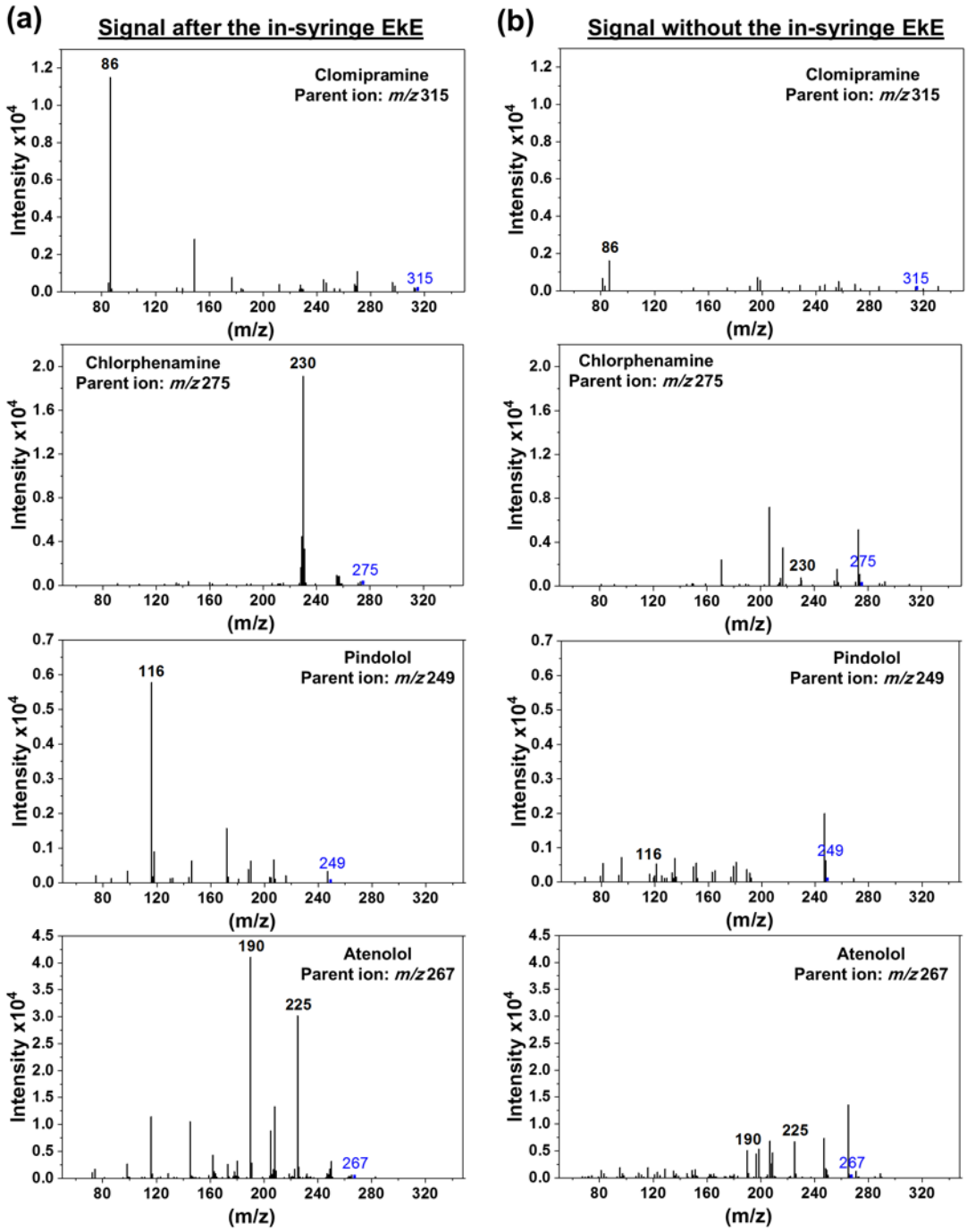


Figure 5-14. Comparison of MS/MS spectra of the electrokinetic clean-up approach and the direct infusion approach. a, MS/MS spectra (1.4 to 1.6 minutes) showing the molecular ions to the most abundant daughter ions' transitions of a spiked serum sample with added 40.0 ng/mL of clomipramine, chlorphenamine, and pindolol and 300.0 ng/mL of atenolol after the 5-times dilution with the BGE and the application of the EkE. b, MS/MS spectra (1.4 to 1.6 minutes) of the spiked serum sample without the EkE step.

The constructed calibration curves consisted of 5 concentration levels to give added concentration ranges of 10.0-160.0 ng/mL for clomipramine, chlorphenamine, and pindolol and 75.0-1200.0 ng/mL for atenolol. Linear

correlations were achieved by plotting the average intensity ratio (each drug/IS) for the interval 1.4-1.6 minutes *versus* the added drug concentration, the linear regression equations and the regression correlation coefficients (r) have been added to Table 5-1 and Figure 5-15. The limit of detection (LOD) and the limit of quantitation (LOQ) of the four drugs in spiked serum samples were below their peak plasma concentrations (C_{max}) (Table 5-1 and Figure 5-15), the LOQs of clomipramine, chlorphenamine, pindolol, and atenolol were 5.3, 7.8, 6.1, and 17.8 ng/mL, respectively.

The method accuracy and precision were evaluated for the EkE syringe-ESI-MS/MS method and the results are summarized in Table 5-2, method precision shows RSD $\leq$ 20.0 % for all the drugs.

Table 5-2. Accuracy and precision of the weakly basic drugs determination in spiked serum

Drug	Concentration (ng/mL)	% Found	Repeatability *RSD (%) (n=3)	Intermediate precision *RSD (%) (n=3)
Clomipramine	10.0	82.5	14.0	13.5
	40.0	89.8	9.9	5.3
	160.0	98.4	6.3	12.7
Chlorphenamine	10.0	86.0	19.6	17.7
	40.0	108	11.3	16.1
	160.0	99.2	15.1	18.2
Pindolol	10.0	93.6	18.6	10.4
	40.0	94.6	15.4	19.2
	160.0	99.4	6.3	8.2
Atenolol	75.0	120	15.2	18.5
	300.0	88.0	7.6	7.8
	1200.0	101	11.2	9.2

*Relative standard deviation, RSD = 100 S/ $\overline{x}$

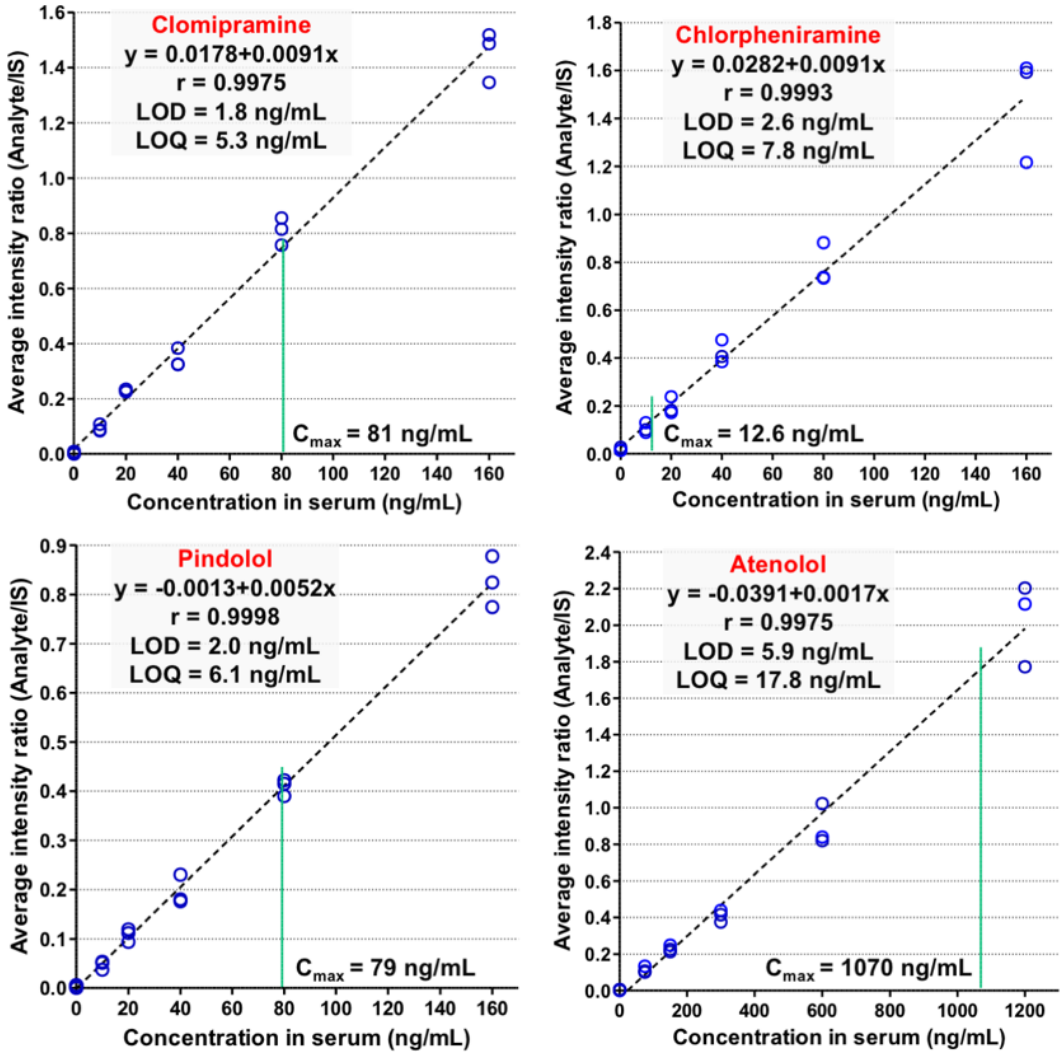


Figure 5-15. Determination of pharmaceuticals in spiked serum using the in-syringe EkE-ESI-MS/MS method. Quantitative analysis of serum spiked with clomipramine (10 to 160 ng/mL), chlorphenamine (10 to 160 ng/mL), pindolol (10 to 160 ng/mL), and atenolol (75-1200 ng/mL) using their corresponding isotopologues as internal standards (ISs), clomipramine-d6 (80 ng/mL), chlorphenamine-d6 (80 ng/mL), pindolol-d7 (80 ng/mL), and atenolol-d7 (600 ng/mL). The calibration graphs were constructed by plotting the average intensity ratio for the time interval 1.4-1.6 minutes *versus* the corresponding drug concentration. Three replicates were run for each concentration level.

5.4.5 Process efficiency

Quantitative evaluation of the EkE process is shown in Figure 5-16, from triplicate experiments at 3 different concentrations (10.0, 40.0, 160.0 ng/mL for clomipramine, chlorphenamine, and pindolol and 75.0, 300.0, 1200.0 ng/mL for atenolol). Figure 5-16a shows the reduction in MS response with voltage, with clomipramine and chlorphenamine being reduced by 10-20 % while pindolol and atenolol were reduced by nearly 50 % possibly due to an irreversible

electrochemical oxidation reaction [20, 21]. The ion suppression due to the serum and inorganic ion matrix was found to be between 79-95 % and the process efficiency– a percentage of the response in pre-spiked serum after the extraction to the response in neat standards [22]– ranged from 71-89 % (Figure 5-16b). This percentage may be related to plasma protein binding as chlorphenamine (protein binding of 72 %) had the highest process efficiency of 89.3 % while clomipramine which (the highest protein binding, 98 %) had a relatively lower process efficiency of 74.5 %. Pindolol and atenolol had process efficiencies of 71.3 % and 78.6 %, respectively, which are lower due to the proposed oxidation. The overall net outcome of the EkE process is a signal enhancement of 3.6-32 fold, compared to the direct infusion of spiked serum without the EkE (Figure 5-16c).

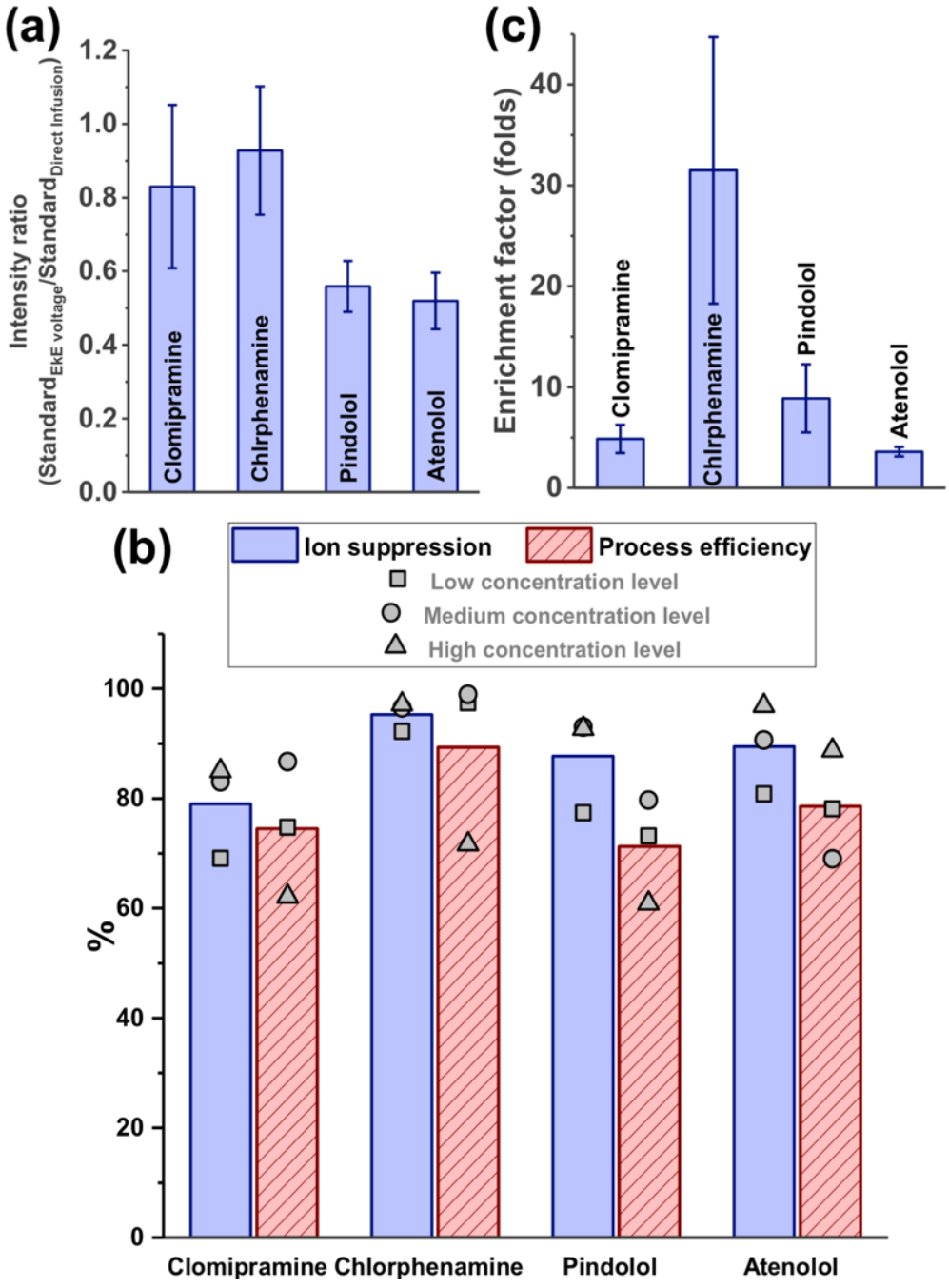


Figure 5-16. Quantitative assessment of EkE. a, Effect of EkE processing on the signal intensity of standards, 40.0 ng/mL of clomipramine, chlorphenamine, and pindolol and 300.0 ng/mL of atenolol, (B/A) with n=3 for each set, the error bars represent the SD of the average intensity ratio where $SD=[(SD_A/Mean_A)^2+(SD_B/Mean_B)^2]^{1/2}x|$average intensity ratio|. b, the ion suppression due to the serum matrix (Ion suppression = 100 – (D/A × 100) [23]) and the process efficiency of the EkE (PE (%) = C/A × 100 [22]), PE and Ion Suppression were evaluated at three concentration levels and each concentration level was run in triplicate. c, The enrichment factors (C/D) obtained by the EkE of serum samples (n=3 for each set) spiked with 40.0 ng/mL of clomipramine, chlorphenamine, and pindolol and 300.0 ng/mL of atenolol, the error bars represent the SD of the average intensity ratio where $SD=[(SD_C/Mean_C)^2+(SD_D/Mean_D)^2]^{1/2}x|$average intensity ratio|.
A is the average intensity of neat standard solutions, B is the average intensity of neat standard solutions after the in-syringe EkE, C is the average intensity of spiked serum samples after the in-syringe EkE, and D is the average intensity of spiked serum samples without the EkE. All the average intensities were obtained for the time interval 1.4-1.6 minutes.

5.5 Conclusions

The EkE syringe was used as a novel, simple, and time-saving approach for the clean-up of the biological samples from the interfering proteins without excessive use of solvents. This approach demonstrated the clean-up of human serum prior to the direct infusion ESI-MS avoiding the ion suppression caused by the serum proteins. A bubble-free EkE design was applied on the analysis of the weakly basic compounds in serum using ESI-MS/MS. The process efficiency was evaluated at different concentration levels of the weakly basic drugs. Moreover, the signal intensity was enriched by more than 30 fold in case of chlorphenamine analysis by the virtue of the in-syringe EkE process. The developed system has been proved to be suitable for the analysis of compounds with different degrees of plasma-protein binding and there is no need for tedious method development as in the traditional solid phase extraction protocols where we developed only one method/system suitable for a wide range of weakly basic analytes. The sample preparation by the EkE syringe rendered accurate sampling, elimination of serum proteins, and sample infusion to ESI-MS in an online system using only one device. Furthermore, the sample clean-up was accomplished within 1.5 minutes and requires less than 3.5 µL of acetonitrile per run. Thus, this approach offers a very simplified platform for automated analytical procedures.

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6. Future Direction: Design of Automatic EkE Syringes for ESI-MS

Despite the potential of performing the sample preparation within the electrokinetic extraction (EkE) syringe was demonstrated, automation is needed to increase the throughput and usability of the device. Full automation of the platform would let us evaluate/enhance the EkE syringe's robustness and lifetime and minimize the carryover.

The EkE-in-a-syringe system can be modified within the MAST (modular analytical separation technology developed in Trajan Scientific and medical, Ringwood, VIC, Australia) platform architecture. MAST is an open-source Arduino controlled system for the digital control and automation of a series of hardware components. The MAST platform would be able to control the analytical syringe drive (pump), the selector valve, and the high voltage (HV) component. It would be programmable from a graphical user interface (GUI), allowing a series of sequential steps to be automated. It would allow, for example, dilution of the sample with background electrolyte (BGE), application of the voltage, dispensing/collecting fractions of the syringe fluids, and automated washing of the syringe between samples. We are here indicating the requirements, preliminary design, and workflow of the proposed system.

6.1 System Requirements

6.1.1 Functional requirement

- The EkE-in-a-syringe unit would be used for the biological and pharmaceutical sample preparation where the target analytes can be amphoteric, neutral, or charged molecule.

6.1.2 Physical requirements

- The EkE would comprise a barrel having a discharge end and a receiving end; wherein the cathode and the anode can be configured to provide a voltage across a solution contained in the barrel.
- The EkE syringe would have metallic ends to serve as electrodes.
- The metallic ends of the EkE syringe would be resistant to corrosion.

6.1.3 Operational requirements

- The developed operation unit would provide applied voltages ranging from -5000 V to 5000 V at the EkE step and at the infusion step.
- The time of the EkE would be adjusted based on the nature of the sample and the target analytes.
- The syringe drive would be able to compress the solution in the syringe barrel with a flow rate ranging from 0 nL/min to 200 nL/min during the EkE.
- The syringe drive would infuse liquids with a flow rate ranging from 10 nL/min to 1000 µL/min.
- The interface between the EkE-in-a-syringe unit and the analyser would involve the least possible dead volume.

6.1.4 Safety features

- The EkE-in-a-syringe unit would comply with local electrical safety standards.
- The syringe drive body would be isolated from any electrical connection.
- The plunger and the needle would be protected against access during the voltage application.
- Glass components would remain contained if they are broken during the procedures.

6.1.5 Environmental considerations

- The EkE-in-a-syringe unit would be operable at ambient conditions.
- The EkE-in-a-syringe unit design would take into account its placement in a “wet” chemistry laboratory.

6.1.6 Maintenance and servicing

- Any wear factor parts would be easily accessible for replacement by laboratory staff.
- Any wear factor parts would withstand a specified number of operations between replacements.
- A service manual providing full details for maintenance and service would be supplied.

6.2 Design of the EkE-in-a-Syringe Unit

The mainboard of the EkE-in-a-syringe platform is proposed to be an Arduino board controlling and synchronizing the work of the main components of the unit, the HV power supply, the syringe pump, and the 8-port selector valve. A portable HV DC converter would be utilized to apply the HV to the metallic ends of the EkE syringe to accomplish the EkE and to avoid any distortion of the focused bands during the infusion to ESI-MS by applying optimized voltages, the polarity of the electrodes can be reversed through a manual button or an option in the GUI to make the EkE system suitable for the analysis of acidic, basic, or amphoteric analytes. E series (http://www.emcohighvoltage.com/proportional/eseries.php) or F series (https://www.xppower.com/Product/F-Series) of the XP EMCO proportional HV DC converters represent suitable options for the HV component of the system due to their small dimensions, versatility, and ability to provide up to 8 kV at

10 W continuous output power (F series) with an option for the polarity reversing.

The syringe drive and the 8-port selector valve would be programmed to allow the aspiration and dispensing of the different washing solutions, the BGE, and the raw sample in the washing and EkE cycles and to enable the infusion of the prepared sample plug to the ESI-MS and the contaminants to the waste. uProcess automated 8-position selector valve AV801 from LabSmith (https://products.labsmith.com/uprocess-automated-8-position-selector-valve-av801/#.XdMRCL9xXIE) can connect the EkE syringe to 8 different ports with an internal volume of only 370 nL. Furthermore, all PEEK, PEEKsil®, and fused silica tubings can be optimized for a bubble-free filling of the EkE syringe and infusion to ESI-MS with a minimum in-line dead volume. The proposed design of the EkE-in-a-syringe unit is indicated in Figure 6-1.

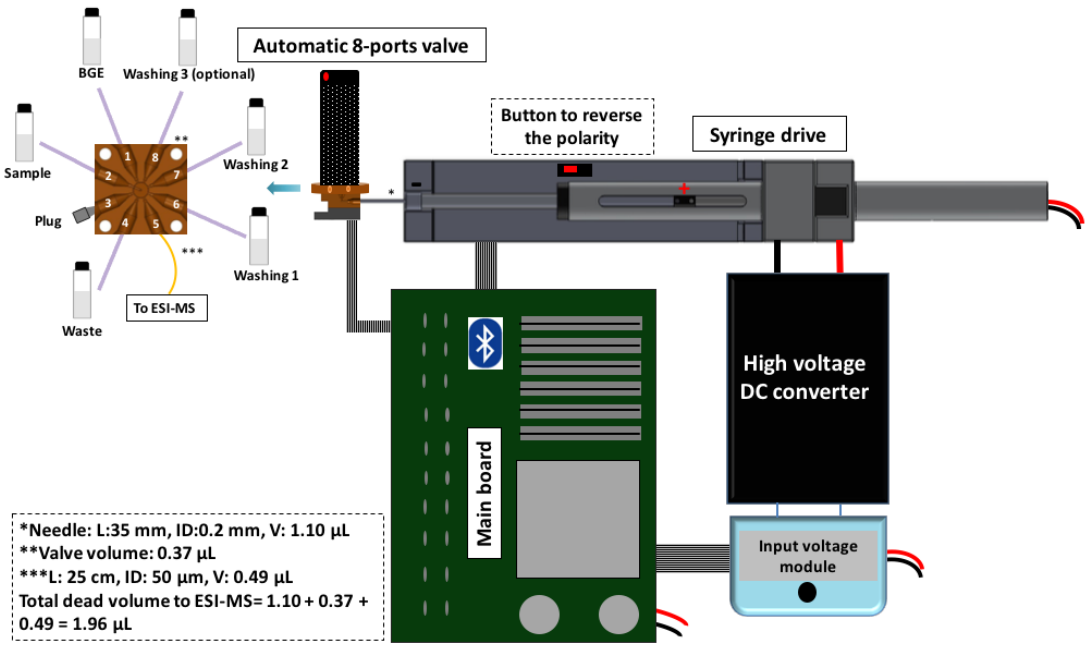

Figure 6-1. The proposed design of the EkE-in-a-syringe unit for the biological samples preparation and its coupling with ESI-MS.

6.3 Automated Sequence and Workflow Using the EkE-in-a-Syringe Unit

This automated workflow has been designed for the clean-up of biological samples using a 25-µL EkE syringe but the EkE syringe's volume should be selected *via* the GUI. The workflow includes two cycles: the washing cycle and the EkE cycle which can be initiated separately or as a one automated sequence. The primed syringe filled with the BGE or the washing liquid would be placed at the syringe pump at the home position (the plunger adjusted to the 25 µL mark) to have a constant starting point every cycle and to make sure that the syringe is free from air bubbles before the EkE, this step is also added at the end of each automated sequence to be ready for the new sequence. The adjustable parameters at the GUI would include: volume of the BGE to be added to the sample (for the sample dilution), EkE voltage, EkE time, syringe drive flow rate during the EkE, infusion voltage, flow rate of the final infusion step, volume to be infused to the waste, and volume to be infused to the mass spectrometer.

6.3.1 Washing cycle

1-Switch the valve to 4 (waste)

2-Dispense 25 µL, flow rate 1000 µL/min

3-Switch the valve to 6 (washing liquid 1)

4-Aspirate 25 µL, flow rate 1000 µL/min

5-Switch the valve to 4 (waste)

6-Dispense 25 µL, flow rate 1000 µL/min

7-Switch the valve to 6 (washing liquid 1)

8-Aspirate 25 µL, flow rate 1000 µL/min

9-Switch the valve to 5 (ESI-MS)

10-Dispense 25 µL, flow rate 25 µL/min

11-Switch the valve to 6 (washing liquid 1)

12-Aspirate 25 µL, flow rate 1000 µL/min

13-Switch the valve to 2 (empty sample vial)

14-Dispense 25 µL, flow rate 1000 µL/min

15-Switch the valve to 7 (washing liquid 2)

16-Aspirate 25 µL, flow rate 1000 µL/min

17-Switch the valve to 5 (ESI-MS)

18-Dispense 25 µL, flow rate 25 µL/min

19-Switch the valve to 7 (washing liquid 2)

20-Aspirate 25 µL, flow rate 1000 µL/min

21-Switch the valve to 2 (empty vial)

22-Dispense 25 µL, flow rate 1000 µL/min

23-Switch the valve to 1 (BGE)

24-Aspirate 25 µL, flow rate 1000 µL/min

25-Switch the valve to 5 (ESI-MS)

26-Dispense 25 µL, flow rate 25 µL/min

27-Switch the valve to 1 (BGE)

28-Aspirate 25 µL, flow rate 1000 µL/min

29-Switch the valve to 2 (empty vial)

30-Dispense 25 µL, flow rate 1000 µL/min

31-Switch the valve to 1 (BGE)

32-Set to home position

6.3.2 EkE cycle

1-Swith the valve to 1 (BGE)

2-Dispense 25 µL, flow rate 1000 µL/min

3-Aspirate 25 µL, flow rate 1000 µL/min

4-Switch the valve to 2 (sample)

5-Dispense 25 µL, flow rate 1000 µL/min

6-Switch the valve to 1 (BGE)

7-Repeat (steps 3 to 6) to add certain volume of the BGE to the sample (volume of the BGE to be added to the sample).

8-Switch valve to 2 (sample)

9-Aspirate 25 µL, flow rate 25 µL/min

10-Dispense 25 µL, flow rate 1000 µL/min

11-Repeat 9 and 10 one more time

12-Aspirate 10.1 µL, flow rate 25 µL/min

13-Switch the valve to 3 (plug)

14-Dispense 0.1 µL, flow rate of 1 µL/min

15-Apply a certain voltage (EkE voltage) for a certain time (EkE time) while keeping the pump dispensing with a certain flow fate (syringe drive flow rate during the EkE, 0.0 to 200 nL/min).

16-Apply voltage for the infusion step (infusion voltage)

17-Switch the valve to 4 (waste)

18-Dispense with a certain flow rate (flow rate of the final infusion step) a certain volume (volume to be infused to the waste)

19-Switch the valve to 5 (ESI-MS) Immediately after 19

20-Dispense with a certain flow rate (flow rate of the final infusion step) a certain volume (volume to be infused to the mass spectrometer)

21-Stop voltage

22-Switch the valve to 6 (washing liquid 1)

23-Adjust to home position

7. Concluding Remarks

This thesis presents the first implementation of an electrokinetic technique within a syringe exploiting the embedded stainless steel syringe needle and plunger as electrodes. The preparation of complex biological samples in a syringe, an established liquid handling device, allows for sample dilution, electrokinetic focusing of the targeted compounds and/or removing the interfering molecules, and finally infusion to the analyser using a single platform. With liquid handling driven by a syringe pump, this approach opens up new opportunities for integrated and automated workflows for complex samples analysis, for example, the online coupling with electrospray ionization-mass spectrometry (ESI-MS).

In the first example of the electroseparation-in-a-syringe systems presented in Chapter 2, the generation of hydronium at the plunger and hydroxyl ions at the needle of a 25 µL analytical glass syringe due to water electrolysis facilitated the formation of a neutralization reaction boundary (NRB) with a step gradient from pH 3 to pH 11 to focus the different amphoteric compounds such as bovine serum albumin, R-phycoerythrin and hemoglobin. No carrier ampholytes (CAs) were used which are expensive and not compatible with ESI-MS. Different experimental parameters affecting the in-syringe carrier ampholytes free-isoelectric focusing (CAF-IEF) system were optimized, and the IEF syringe was coupled with ESI-MS using an interface with a triple tube ESI sprayer to incorporate a co-axial flow of 0.2% (v/v) formic acid as a sheath liquid for enhancing the ionization efficiency. A background electrolyte (BGE) composed of 1.0 mM NH_4Ac (pH 4.0) in 70.0% (v/v) acetonitrile was used for the IEF

of histidine by applying −200 V for 5.0 min prior to the infusion to ESI-MS while having -400 V applied. A nonlinear quadratic fit calibration curve was constructed for histidine over the range of 4.0−64.0 μg/mL with a limit of detection (LOD) of 2.2 μg/mL. Histidine analysis in spiked urine samples was demonstrated by the IEF syringe-ESI-MS system with an accuracy ranged from 88.25% to 102.16%, a relative standard deviation (RSD) less than 11%, and an enhancement factor of 8.5 compared to the direct infusion approach providing a useful tool for the diagnosis of histidinemia.

Another implementations of the analytical syringe as an electrokinetic platform were the electrokinetic extraction (EkE) syringes which were developed for the on-line electrokinetic removal of amphoteric compounds (serum proteins) from serum samples prior to the ESI-MS to allow the analysis of the weakly acidic and basic analytes as indicated in Chapter 3 and Chapter 5, respectively. The clean-up mechanism relies on the electrophoretic migration of the serum proteins away from the target analytes when a potential difference is applied across the syringe, utilizing the syringe metallic needle and plunger as electrodes**.** This design permitted the aggregation of the serum proteins toward the plunger before the direct infusion of the deproteinized sample into the ESI-MS.

For the analysis of the weakly acidic compounds (Chapter 3), the serum is drawn into the EkE syringe together with an electrolyte comprises formic acid (pH 2.5) to positively charge the serum proteins while the weakly acidic compounds will remain neutral at this low pH. The electrokinetic purification process is initiated by applying -2000 V across the syringe, utilizing the syringe stainless steel plunger and needle as cathode and ground, respectively. The

EkE syringe-ESI-MS method was applied for the determination of naproxen and paracetamol in spiked serum using valproic acid as internal standard (IS). The LOQs of naproxen and paracetamol in spiked serum samples were 3.1 µg/mL and 2.9 µg/mL, respectively, which are less than the peak plasma concentrations of naproxen and paracetamol, 76.6 and 9.9 µg/mL, respectively. A linear correlation was achieved by plotting the average intensity ratio (each drug/IS) *versus* the added drug concentration with a regression correlation coefficient (r) = 0.9994 for naproxen and with r = 0.9982 for paracetamol. The evaluation of the method precision showed a RSD less than 19 % for naproxen and less than 18 % for paracetamol. By the virtue of the EkE syringe, the signal intensities of naproxen and paracetamol were enhanced by 7.7 fold and 10.8 fold, respectively, after the removal of the serum proteins.

Different approaches were explored to eliminate the electrolysis bubbles generated during the EkE in Chapter 4, the most efficient design exploited a sealed system to pressurize and dissolute the gas bubbles into the solution where the needle end was closed *via* a push-pull valve or a LabSmith 3-port valve in a closed position and a gas-tight plunger was used at the other side. The developed design was successfully applied using different syringe volumes (25 µL and 100 µL syringes), BGEs (acidic and basic), and polarities of the electrodes.

The analysis of the basic drugs in serum (Chapter 5) depended on using a basic BGE of 300 mM NH_4OH in 30% acetonitrile to negatively charge the serum proteins prior to their aggregation close to the plunger (the anode) by application of 800 V for 90 seconds. In this system, we achieved a bubble-free EkE to enhance the system performance. The applicability of the proposed

method was demonstrated by the determination of different pharmaceuticals in 10 µL of diluted human serum within a few minutes, with sample preparation limited to conducting a 5x dilution. Four weakly basic drugs with different levels of protein binding, namely, clomipramine, chlorphenamine, pindolol, and atenolol were determined in spiked serum by the EkE-ESI-MS/MS system using their corresponding deuterated isotopes as ISs. The ion suppression due to the serum proteins and the process efficiency (PE) of the EkE were evaluated at 3 different concentration levels of each drug, the average ion suppression without the clean-up step was found to be 79.1 %, 95.3 %, 87.7 %, and 89.5 %, while after the EkE, the average PEs were 74.5 %, 89.3 %, 71.3 %, and 78.6 %, for clomipramine, chlorphenamine, pindolol, and atenolol, respectively, with an enrichment factor > 30 fold for chlorphenamine. LOQs of 5.3, 7.8, 6.1, and 17.8 ng/mL, for of clomipramine, chlorphenamine, pindolol, and atenolol, respectively, in spiked serum were achieved using the EkE syringe-ESI-MS system. Not only a simplified workflow is presented by the EkE syringe but a significant reduction in the required sample volume, the analysis time, and the organic solvent consumption, where only a sample volume of 2 µL, a total analysis time of 3.1 min, and a 3.5 µL of acetonitrile were required for every electrokinetic clean-up run.

Although the potential of the novel approach of performing the sample preparation within the EkE syringe was demonstrated, the current version of the device is totally operated in a manual manner. Given the fact that other advanced technologies with analysis speed of few seconds per sample are already available such as the robotic SPE, RapidFire system, it is essential to boost the throughput of the electrokinetic in-syringe systems to be considered

in the sample preparation market. The next stage of this project will be the full automation of the platform to achieve a high throughput stand-alone MS analysis of the complex biological samples. The proposed automated system requirements, preliminary design, and workflow were explained in detail in Chapter 6.

One of the limitations of the current EkE syringe is that it can only concentrate ampholytes around their isoelectric points, while matrix components were only removed without a significant pre-concentration of the target weakly acidic or weakly basic analytes. More focus should be given in the future on the ways by which weak acids and bases can be concentrated inside the syringe through the investigation of different EkE chemistries.

Furthermore, the relatively wide diameter (0.73 mm) of the current syringe barrel adversely affects the strength and consistency of the applied voltage, the resolution of the separation, and the throughput of the analysis due to the joule heating generation. In contrast, using syringes with a narrow ID (i.e. nano-volume syringes) for the EkE requires a specially designed interface with MS. In the future, a detailed study should be dedicated to the syringe design including the diameter of the syringe barrel to achieve a higher resolution separation and an enhanced throughput.

As a novel bioanalytical technique, the in-syringe EkE should be fully validated using large number of samples to be translated in the regulated laboratory environments. Detailed precision studies including within-run precision and between-run precision with different analysts, equipments, and laboratories should be accomplished. Additionally, the developed methods should be cross-validated with original validated methods as references.