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In-syringe electrokinetic protein removal from biological samples prior to electrospray ionization mass spectrometry

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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. Automation of protein removal protocols is challenging. Here, an electrokinetic extraction (EkE) syringe is presented allowing for on-line electrokinetic removal of serum proteins before ESI-MS. The method relies on the electrophoretic migration of charged serum proteins away from neutral target analytes in an electric field, applied across the syringe barrel utilizing the metallic syringe needle and plunger as electrodes. Under acidic conditions, most proteins are cationic facilitating their accumulation at an cathodic plunger. Similarly, under basic conditions, the anionic proteins aggregate near the anodic plunger. Actuation of the plunger enables infusion of the deproteinated sample remaining in the barrel into the ESI-MS for detection of acidic or basic molecules. The proposed concept is demonstrated by the determination of pharmaceuticals from human serum within minutes, with sample preparation limited to a 5x dilution of the sample in the background electrolyte (BGE) and application of voltage, both of which can be performed in-syringe. Signal enhancements of 3.6-32 fold relative to direct infusion of diluted serum and up to 10.8 fold enhancement, were obtained for basic and acidic pharmaceuticals, respectively. Linear correlations for the basic drugs by EkE-ESI-MS/MS were achieved, covering the necessary clinical range with LOQs of 5.3, 7.8, 6.1, and 17.8 ng/mL for clomipramine, chlorphenamine, pindolol, and atenolol, respectively. For the acidic drugs analysis, EkE-ESI-MS, the LOQs were 3.1 µg/mL and 2.9 µg/mL for naproxen and paracetamol, respectively. The EkE-ESI-MS and EkE-ESI-MS/MS methods showed good accuracy (%found of 81% to 120%), precision ($\leq$ 20%), and linearity (r $\geq$ 0.997) for all the studied drugs in spiked serum samples.

Introduction

Sample preparation is an indispensable part of the analytical procedure to prevent matrix effects that impact on the reproducibility and efficiency of analysis, and/or, to pre-concentrate the target analytes to enhance the sensitivity[1]. However, sample preparation is typically the most labor - intensive, expensive, and time-consuming part of the analytical workflow[2-3]. The use of off-line processes and manual handling also can result in loss of analyte which makes the sample preparation prone to errors and a source of variability[2]. Miniaturised, online, and automated sample preparation systems offer many merits to routine bioanalytical protocols such as low consumption of samples and solvents, high extraction efficiency, enhanced robustness, and high analysis speed[1], fueling intense efforts to automate and integrate sample preparation with the final detection step[4].

For the analysis of plasma and serum samples by ESI-MS, the serum albumins along with other abundant serum proteins can cause significant ion suppression and consequently compromise the detection of small-molecule analytes by competing for the charge available on the ESI droplets[5]. Protein precipitation, liquid-liquid extraction (LLE), solid phase extraction (SPE), and ultrafiltration are the most common methods used for the clean-up of these types of samples[6]. Protein precipitation is the simplest approach for protein removal where proteins are denatured using heat, organic solvents, or acids[6] but it suffers from variation in the recovery based on the precipitating agent[1]. Protein removal via LLE is hindered by having inferior selectivity, low recovery, emulsion formation, and low efficiency for extracting highly hydrophilic compounds, in addition to the difficulty in process automation[7-8]. Although SPE offers advantages over the other methods including higher selectivity and relatively easier automation[9], it is affected by limitations such as elaborate method development, high cost, poor inter - batch reproducibility, and the co-elution of the endogenous contaminants from the SPE cartridges such as polyethylene glycols and phthalates[2, 7] which can interfere with the target analytes and cause ion suppression[10]. Ultrafiltration methods require a relatively large volume of sample (>25 µL)[11] and in many instances, the removal of interfering compounds is incomplete, especially for high-molecular weight targets[12]. Importantly, automaton of all of these approaches is difficult and adds a considerable cost.

Recently, innovative strategies for analytes extraction from biological fluids have been developed and coupled with ESI-MS such as slug flow microextraction (SFME)[13-14], liquid extraction surface sampling (LESA)[15], and digital microfluidics[16]. They showed an ability to deal with micro-volume samples, 0.2 µL in LESA[15] and a high speed of extraction can be offered by these approaches ranging from dozens of seconds in SFME[14] to 5 minutes in DMF[16]. Moreover, SFME, and DMF are applied successfully for quantitative analysis[13-14, 16]. However, the drawbacks of SFME are represented in the manual transition from the preparation event to the MS event (non-automated electrospray) and the electrical hazard where the open ion-source has an exposed high voltage[17]. Also, DMF and LESA are unable to deal with wet samples to measure some biological and chemical properties like the enzymatic functions, used only in dried biofluid spots analysis[15-16].

Electrokinetic techniques present an alternative approach to treat biological samples based on the movement of charged species within an electric field, making it fundamentally different to all of the current widely used sample treatment approaches[18-19]. The power of electrokinetic processing has been demonstrated, for example through the electroextraction of acylcarnitines from plasma in 3-9 min using a three-phase system, yielding a 10-fold enhancement in detection and a protein-free ESI signal[20]. Also, a microfluidic electro membrane extraction (EME) platform was developed for the extraction of basic drugs in complex matrices like urine prior to the online ESI-MS detection[21]. Furthermore, an electrocapture cell was applied for the online enrichment of peptides at femtomole levels prior to MS analysis[22]. Despite the potential of these approaches, automation to increase the throughput is still difficult, nor are these approaches directly compatible with current high throughput laboratory instrumentation.

A syringe has been the most commonly used device for sample introduction to different analytical instruments for decades. Sample preparation within a syringe simplifies the workflow and enhances the analytical performance by integrating sample preparation into the existing liquid handling systems. Over the last few years, in-syringe systems were coupled with various analytical techniques such as electrothermal atomic absorption spectrometry[23], gas chromatography-mass spectrometry[24], and inductively coupled plasma spectrometry[25]. Recently, we electrokinetically created a pH boundary inside a syringe for the concentration and purification of amphoteric analytes, and demonstrated the detection of histidine in urine for the diagnosis of histidinemia[26]. The instrumental simplicity of this approach combined a DC power supply with a commercial glass syringe, applying the potential difference over the liquid in the syringe barrel using the syringe needle and metallic plunger which functioned as electrodes. Given the widespread use of syringes, the instrumental simplicity of this approach, and its potential for incorporation in an automated workflow, this novel approach is attractive for sample treatment in bioanalysis.

The focus of this work is to apply the EkE syringe for one of the most common and frequently used sample treatment processes, protein removal, and couple this on-line with ESI-MS(/MS) for the analysis of small molecules in serum and plasma. Two systems were investigated targeting weakly basic and weakly acidic compounds. For basic compounds, the sample was diluted in a basic electrolyte (300 mM NH_4OH, pH 11.4) such that the pH is higher than the isoelectric points *(pIs)* of the major serum proteins. This causes electromigration and concentration of the anionic proteins close to the anodic plunger leaving a protein-depleted region in the syringe and the targeted neutral weak bases distributed along the length of the syringe. Similarly, for acidic compounds, the sample is diluted in an acidic electrolyte (50 mM formic acid, pH 2.5) with pH below the *pIs* of the serum proteins such that they become cationic and focus near the cathodic plunger, this time leaving the neutralized weak acids distributed along the syringe. Subsequent actuation of the plunger allows for direct infusion of the protein-depleted syringe fluid into the ESI interface for MS detection of the bases (acids) with minimal ion suppression. An EkE syringe-ESI-MS/MS method was developed 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), spiked in serum using their corresponding deuterated isotopes as internal standards (ISs). A complementary EkE-ESI-MS method was developed using an acidic electrolyte for the analysis of naproxen and paracetamol from serum as examples for weakly acidic drugs, using valproic acid as an internal standard.

Results and Discussion

When a voltage is applied over the fluid inside the syringe, charged molecules move according to their electrophoretic mobility. Here, deproteination is achieved by selecting separation conditions under which the serum proteins are charged and the target analytes are neutral, allowing for protein removal through electromigration. Serum proteins include predominantly albumins (58%), globulins (38%), and fibrinogen (4%)[27], proteins with *pI*s of 4.7-4.9[28], 5.3-7.3[28], and 5.5-6.3[29], respectively. A 5x dilution of the sample with a basic electrolyte – 20% serum in 300 mM NH_4OH (pH 11.4) – means that nearly all of the serum proteins are negatively charged while weak bases including the target analytes with a pKa < 10.4 will be predominantly deprotonated and neutral. Application of a potential difference across the plunger and the needle using the plunger as anode result in the anionic serum proteins migrating towards and aggregating close to the plunger (the anode) while the weakly basic analytes remain homogenously distributed across the syringe volume, as shown in the schematic in Figure 1a. This concept was experimentally verified as presented in Figure 1b and Supplementary Video 1, showing the focusing of Chromeo™ P465 labelled human serum albumin (HSA) (λ_{ex} 486 nm; λ_{em} 650 nm) toward the plunger (anode). When rhodamine 6G (basic dye, pKa 7.5[30], λ_{ex} and λ_{em} of 526 nm and 555 nm, respectively[31]) is added to visualize the behavior of a weak bases, it remains unfocused while the HSA migrates towards and aggregates close to the plunger, as illustrated in Fig 1c and Supplementary Video 2. For weakly acidic compounds, an acidic BGE of 50 mM formic acid (pH 2.5) can be used to render the serum proteins positively charged, and the plunger is used as the cathode (Supplementary Figure 1 and Supplementary Video 3 utilizing Chromeo™ 488 HSA and eosin B as models for the serum proteins and acidic target analytes, respectively). The movement of the proteins towards the plunger creates a zone in the barrel that contains the analyte but is depleted in protein, making it suitable for analysis by ESI-MS. Not visualized is the migration of inorganic anions and cations towards the anode and cathode, respectively, providing a potential added advantage of desalting during deproteination.

The EkE syringe needle (OD 0.8 mm) was connected directly to a 3-port valve which was closed during the EkE of the weakly basic drugs. This provided a sealed system, allowing for the application of an increasing pressure through actuation of the gas-tight plunger, solubilising the electrolysis gases in solution and minimising the formation of gas bubbles in the syringe barrel during the electrokinetic deproteination step. The components of the bubble-free system are shown in Supplementary Figure 2a. A 25 µL SGE syringe - rated to withstand a maximum pressure of 1000 psi – could be used for up to 20 runs with a good efficiency in prevention of bubbles formation during the EkE before the PTFE seal of the syringe should be replaced. Supplementary Figure 2b exhibits different runs of bubble free

electrokinetic aggregation of Chromeo™ P465 labelled HSA using the developed platform. After the EkE process, the 3-port valve was rotated to connect the syringe with the ESI-MS outlet and the sample was infused slowly (5.0 µL/minute) through a triple tube ESI-MS sprayer *via* capillary tubing. 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/minute) to enhance the ionization of the weakly basic drugs in the positive mode. The EkE syringe-ESI-MS interface is indicated in Supplementary Figure 3.

A mixture of four weak bases and HSA was placed inside the syringe and EkE was performed with the application of a voltage of 800 V using the needle as a cathode before infusion of the mixture into the MS. The MS trace is shown in Figure 2a which shows the extracted ion electropherograms (EIEs) for each of the target analytes and their concentration profile during infusion of the syringe barrel content into the ESI-MS after the EkE process. HSA, as expected, is depleted from most of the infused volume, peaking at the end of the infusion owing to this fraction's origin being near the plunger. The majority of the syringe volume did not contain HSA and hence provides a good MS response for each of the drugs. The drug response at the start of the infusion is low, which we attribute to the elevated concentration of inorganic ions including sodium which will have migrated towards the cathodic needle, a phenomena contributing to the clean-up of the sample present in the syringe. Figure 2b indicates the gradual decrease of HSA average intensity by increasing the duration of voltage application (up to 90 seconds) and clarifies the proportional increase in signal intensity for the weakly basic drugs by this decrease in protein content.

Experiments were then undertaken with serum spiked with clomipramine, chlorphenamine, pindolol and atenolol, and diluted 5-times with NH_4OH (final concentration 300 mM). Application of a voltage of 800 V for 90 s purified the sample which was then infused into the MS (5 µL/minute, 200 V) over 96 s with a coaxial flow of the sheath liquid. The extracted ion electropherograms (EIEs) of the four drugs are shown in Figure 3 for spiked serum and pure standards with and without EkE, and MS/MS spectra are indicated in Figure 4a and Figure 4b. Figure 3 shows a slightly lower response of the 4 drugs in standards with the EkE step when compared to direct infusion of the standards without EkE. For the serum matrix, however, there is a considerable improvement for all of the 4 drugs when compared to direct infusion. Additionally, the EkE response for serum samples reached similar levels to that obtained in the standards.

A more quantitative evaluation of the EkE process is shown in Figure 5, Supplementary Table 1, and Supplementary Table 2 using four sets of solutions (neat standard solutions (A), neat standard solutions after the EkE (B), spiked serum samples after the EkE (C), and spiked serum samples without the EkE(D)), with data obtained from triplicate experiments at 3 different concentrations (10.0, 40.0, and 160.0 ng/mL for clomipramine, chlorphenamine, and pindolol and 75.0, 300.0, and 1200.0 ng/mL for atenolol). Figure 5a shows the reduction in MS response of standards 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[32-33]. Ion suppression due to the serum and inorganic ion matrix was found to be between 79-95% and the process efficiency– expressed as a percentage of the response for a spiked serum after EkE relative to the response for a standard[34]– ranged from 71-89% (Figure 5b and Supplementary Table 1). We believe variation in efficiency may be related to plasma protein binding as chlorphenamine which had the highest process efficiency (89.3%) has a protein binding of 72% while clomipramine had a lower process efficiency (74.5 %) and higher protein binding of 98 %. Pindolol and atenolol had process efficiencies of 71.3% and 78.6%, respectively, which is lower due to the proposed oxidation. The overall effect of the EkE process is a signal enhancement of 3.6-32 fold in comparison to the direct infusion of spiked serum (Figure 5c and Supplementary Table 2) which allowed the detection and determination of the studied drugs in serum samples at their therapeutic levels. For instance, the direct infusion approach did not provide the sensitivity to detect the C_{max} of chlorphenamine (12.6 ng/mL)[35] while owing to the 32-fold enrichment achieved by the in-syringe EkE approach the LOD was lowered to 2.6 ng/mL allowing for the detection of clinically relevant levels of this drug. The high enrichment factor of chlorphenamine may be related to the high process efficiency of its EkE compared to the other drugs as discussed above.

Moving to acidic targets under acidic conditions, serum samples were diluted in-syringe to demonstrate the potential of this approach as part of an automated workflow. Following the EkE approach under acidic conditions and with reversed polarity compared to the method developed for the basic drugs, signal intensities of the acidic drugs in serum increased by 7.7 and 10.8 fold for naproxen (16.0 µg/mL) and paracetamol (12.0 µg/mL), respectively. Figure 6 illustrates a comparison between the mass spectra of the spiked serum samples after the EkE and without the EkE to show the effect of the electrokinetic clean-up on the MS signal of the studied acidic drugs.

The constructed calibration curves consisted of 5 concentration levels ranging between 10.0-160.0 ng/mL for clomipramine, chlorphenamine, and pindolol and 75.0-1200.0 ng/mL for atenolol. A linear correlation was achieved by plotting the average intensity ratio of each drug to a deuterated standard for the interval 1.4-1.6 minutes *versus* the added drug concentration, the linear regression equations and the correlation coefficients (r) have been indicated in Figure 7 and Supplementary Table 3. The limit of detection (LOD) and the limit of quantitation (LOQ) of the four drugs in serum samples were below their peak plasma concentrations (C_{max}) (Figure 7 and Supplementary Table 3).

The LOQs of clomipramine, chlorphenamine, and atenolol were found to be 5.3, 7.8, and 17.8 ng/mL, respectively, using the developed in-syringe EkE-ESI-MS/MS. These are higher than the quantitation limits of the corresponding published HPLC-ESI-MS/MS methods (0.5 ng/mL for clomipramine[36], 0.1 ng/mL for chlorphenamine[37], and 5.1 ng/mL for atenolol[38]) however these methods used QQQ MS instruments, which offer better quantitative performance and higher sensitivity in the multiple reaction mode (MRM) than the quadrupole Ion Trap mass analyzer used here[39-40], and chlorphenamine method utilized a LLE step in which the extract was evaporated to dryness and reconstituted in a smaller volume[37]. A better sensitivity was enabled for pindolol by the in-syringe EkE approach (LOQ of 6.1 ng/mL in human serum) compared to the HPLC-ESI-MS/MS method (LOQ of 10 ng/mL in human serum[41]) where the latter used protein precipitation. Overall, the developed in-syringe EkE-ESI-MS/MS method provided the sensitivity required for the

determination of the four basic drugs at their therapeutic levels in plasma.

The method accuracy and precision were evaluated for the EkE syringe-ESI-MS/MS method and the results are summarized in Supplementary Table 4; acceptable accuracy (% found of 82% to 120%) and precision (≤ 20 %) were attained for all the spiked basic drugs. For the acidic drugs determination by EkE-ESI-MS method, LOQs of 3.1 µg/mL and 2.9 µg/mL were achieved for naproxen and paracetamol, respectively. Moreover, the % found of the studied drugs using EkE-ESI-MS method ranged from 81 % to 116 % with a precision ≤ 19. The calibration data of the acidic drugs determination in spiked serum is discussed in more detail in the Supporting Information.

The developed EkE syringe offers a very simple protocol for protein removal and desalting prior to direct infusion ESI-MS/MS. With the method requiring 1.5 min for EkE and 1.6 min for infusion, only 3.1 min is demanded for processing a 2 µL serum sample. Realising this using 3.5 µL of acetonitrile makes the approach also cost-effective and environmentally friendly, while the use of the same syringe for dilution, EkE, and infusion into the MS showcases its potential for incorporation in an automated workflow.

In conclusion, the online preparation of samples using a syringe, an established liquid handling device, allows for combining of different steps of the analytical method in a single platform. With liquid handling driven by a syringe pump, this approach opens up new opportunities for integrated and automated analytical workflows. The potential of the EkE syringe to process complex samples is demonstrated with the clean-up of spiked serum samples prior to direct quantitative analysis by ESI-MS. Different applications and implementations can be investigated in the future by modifying the chemistry of the system, integration of other components such as membranes, and alteration of the syringe dimensions and design. Not only biofluids but also proteomics samples and environmental samples could be enriched, processed, and desalted in the future applications. Furthermore, coupling of the EkE syringes with advanced instruments such as high-resolution accurate-mass mass spectrometry (HRAM-MS) and ion mobility mass spectrometry (IM-MS) instruments can boost the applications of EkE syringes in bioanalysis and omics studies and lessen the need of a hyphenated chromatographic separation.

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References

[1] Z. Niu, W. Zhang, C. Yu, J. Zhang, Y. Wen, *Trends Anal. Chem.* **2018**, *102*, 123-146.

[2] N. Weng, W. Jian, *Wiley Series on Pharmaceutical Science and Biotechnology: Practices, Applications, and Methods; Targeted biomarker quantitation by LC-MS*, John Wiley & Sons, Inc., New Jersey, **2017**.

[3] D. M. Pavlović, S. Babić, A. J. M. Horvat, M. Kaštelan-Macan, *Trends Anal. Chem.* **2007**, *26*, 1062-1075.

[4] M. Abdel-Rehim, M. L. Lee, B. Bojko, *J. Chromatogr. B* **2017**, *1043*, 1-2.

[5] G. H. Terence, S. Inese, R. B. Karen, W. Yunxia, M. David, E. S. Ronald, in *Tandem Mass Spectrometry - Applications and Principles*, InTech, **2012**, pp. 389-420.

[6] C. Bylda, R. Thiele, U. Kobold, D. A. Volmer, *Analyst* **2014**, *139*, 2265-2276.

[7] L. Novakova, H. Vlckova, *Anal. Chim. Acta* **2009**, *656*, 8-35.

[8] J. Pawliszyn, H. L. Lord, in *Handbook of Sample Preparation*, John Wiley & Sons, Inc, New Jersey, **2010**, pp. 285-311.

[9] V. P. Miller, *Bioanalysis* **2012**, *4*, 1111-1121.

[10] M. J. Rardin, *J. Am. Soc. Mass Spectrom.* **2018**, *29*, 1327-1330.

[11] Y. Zhao, Y. Hou, J. Ji, F. Khan, T. Thundat, D. J. Harrison, *Anal. Chem.* **2019**, *91*, 7570-7577.

[12] W. Frenzel, I. Markeviciute, *J. Chromatogr. A* **2017**, *1479*, 1-19.

[13] Y. Ren, M. N. McLuckey, J. Liu, Z. Ouyang, *Angew. Chem. Int. Ed.* **2014**, *53*, 14124-14127.

[14] J. Deng, W. Wang, Y. Yang, X. Wang, B. Chen, Z.-P. Yao, T. Luan, *Anal. Chim. Acta* **2016**, *940*, 143-149.

[15] M. Bailey, E. C. Randall, C. Costa, T. L. Salter, A. M. Race, M. de Puit, M. Koeberg, M. Baumert, J. Bunch, *Anal. methods* **2016**, *2016*, 3373-3382.

[16] A. E. Kirby, N. M. Lafrenière, B. Seale, P. I. Hendricks, R. G. Cooks, A. R. Wheeler, *Anal. Chem.* **2014**, *86*, 6121-6129.

[17] C. W. Klampfl, M. Himmelsbach, *Anal. Chim. Acta* **2015**, *890*, 44-59.

[18] A. Wuethrich, P. R. Haddad, J. P. Quirino, *Trends Anal. Chem.* **2016**, *80*, 604-611.

[19] A. I. Shallan, R. M. Guijt, M. C. Breadmore, *Bioanalysis* **2014**, *6*, 1961-1974.

[20] R.-J. Raterink, P. W. Lindenburg, R. J. Vreeken, T. Hankemeier, *Anal. Chem.* **2013**, *85*, 7762-7768.

[21] N. J. Petersen, S. T. Foss, H. Jensen, S. H. Hansen, C. Skonberg, D. Snakenborg, J. P. Kutter, S. Pedersen-Bjergaard, *Anal. Chem.* **2011**, *83*, 44-51.

[22] S. Vollmer, J. Astorga-Wells, G. Alvelius, T. Bergman, H. Jörnvall, *Anal. Biochem.* **2008**, *374*, 154-162.

[23] G. Giakisikli, A. N. Anthemidis, *Talanta* **2017**, *166*, 364-368.

[24] L. Guo, S. Tan, X. Li, H. K. Lee, *J. Chromatogr. A* **2016**, *1438*, 1-9.

[25] R. Sánchez, B. Horstkotte, K. Fikarová, H. Sklenářová, S. Maestre, M. Miró, J.-L. Todolí, *Anal. Chem.* **2017**, *89*, 3787-3794.

[26] I. E. Mikhail, M. Tehranirokh, A. A. Gooley, R. M. Guijt, M. C. Breadmore, *Anal. Chem.* **2019**, *91*, 8259-8266.

[27] K. M. Waterstram-Rich, P. E. Christian, *Nuclear Medicine and PET/CT - E-Book: Technology and Techniques*, Elsevier Health Sciences, **2013**.

[28] E. Poehler, C. Herzog, C. Lotter, S. A. Pfeiffer, D. Aigner, T. Mayr, S. Nagl, *Analyst* **2015**, *140*, 7496-7502.

[29] H.-O. Ho, C.-C. Hsiao, T. D. Sokoloski, C.-Y. Chen, M.-T. Sheu, *J. Control. Release* **1995**, *34*, 65-70.

[30] M. Li, R. L. Harbron, J. V. Weaver, B. P. Binks, S. Mann, *Nat. Chem.* **2013**, *5*, 529-536.

[31] K. Hardeep, N. Verma, *J Biosens. Bioelectron.* **2015**, *6*, 166. doi:10.4172/2155-6210.1000166.

[32] R. H. Patil, R. N. Hegde, S. T. Nandibewoor, *Ind. Eng. Chem. Res.* **2009**, *48*, 10206-10210.

[33] S. Smarzewska, W. Ciesielski, *Anal. Methods* **2014**, *6*, 5038-5046.

[34] B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng, *Anal. Chem.* **2003**, *75*, 3019-3030.

[35] S. U. Yasuda, P. Zannikos, A. E. Young, K. M. Fried, I. W. Wainer, R. L. Woosley, *Br. J. Clin. Pharmacol.* **2002**, *53*, 519-525.

[36] M. Vandenbosch, T. Somers, E. Cuypers, *Drug Test. Anal.* **2019**, *11*, 1083-1093.

[37] Q. Liao, Z. Xie, B. Pan, C. Zhu, M. Yao, X. Xu, J. Wan, *Chromatographia* **2008**, *67*, 687-694.

[38] R. R. Kallem, J. K. Inamadugu, M. Ramesh, J. V. L. N. Seshagirirao, *Biomed. Chromatogr.* **2013**, *27*, 349-355.
[39] G. Hart-Smith, S. J. Blanksby, *Mass Analysis. In Mass Spectrometry in Polymer Chemistry (eds C. Barner-Kowollik, T. Gruendling, J. Falkenhagen, S. Weidner)*, Wiley-VCH, Verlag GmbH & Co., Germany, **2012**, pp. 5-32.
[40] C. Soler, B. Hamilton, A. Furey, K. J. James, J. Mañes, Y. Picó, *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2151-2164.
[41] M. E. Abdel- Hamid, O. A. Phillips, *J. Liq. Chromatogr. Relat. Technol.* **2003**, *26*, 1937-1957.
[42] D. L. Buhrman, P. I. Price, P. J. Rudewiczcor, *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 1099-1105.

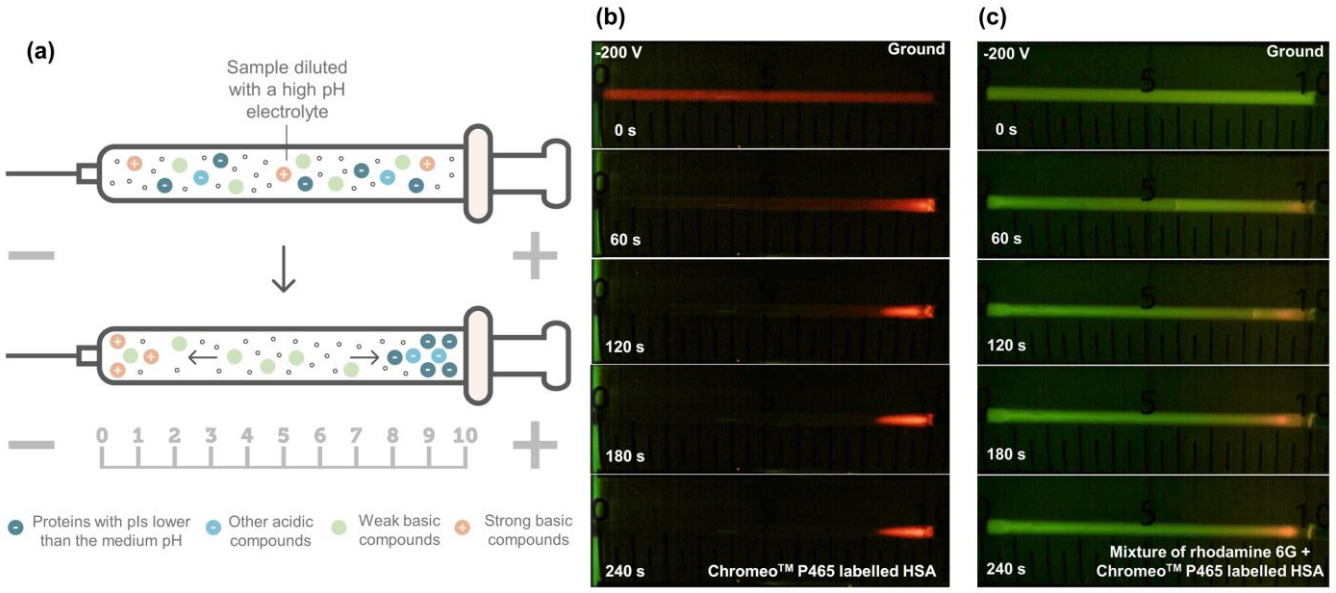

Figure 1. Mechanism of electrokinetic sample preparation in a syringe. (a) A schematic presentation of the in-syringe EkE of biological samples, removing interfering proteins for the analysis of weakly basic analytes using a basic BGE. The metallic plunger of the EkE syringe served as an anode, attracting the anionic proteins, and the metallic needle served as a cathode**. (b)** A single-frame time series showing the concentration of 1.0 mg/mL of Chromeo™ P465 labelled human serum albumin (HSA) close to the anodic plunger using 300 mM NH_4OH in 30% (v/v) acetonitrile as BGE. **(c)** A single-frame time series visualising the removal of 1.0 mg/mL of Chromeo™ P465 labelled HSA from the weakly basic dye rhodamine 6G (2.0 µg/mL) using 300 mM NH_4OH in 30% (v/v) acetonitrile as BGE. Experiments were conducted in a 10 µL of a glass syringe, with the 0-10 graduation visible in black in the images corresponding to the volume of the barrel ranging from 0-10 µL depending on the plunger position (every µL corresponds to approximately 2.4 mm).

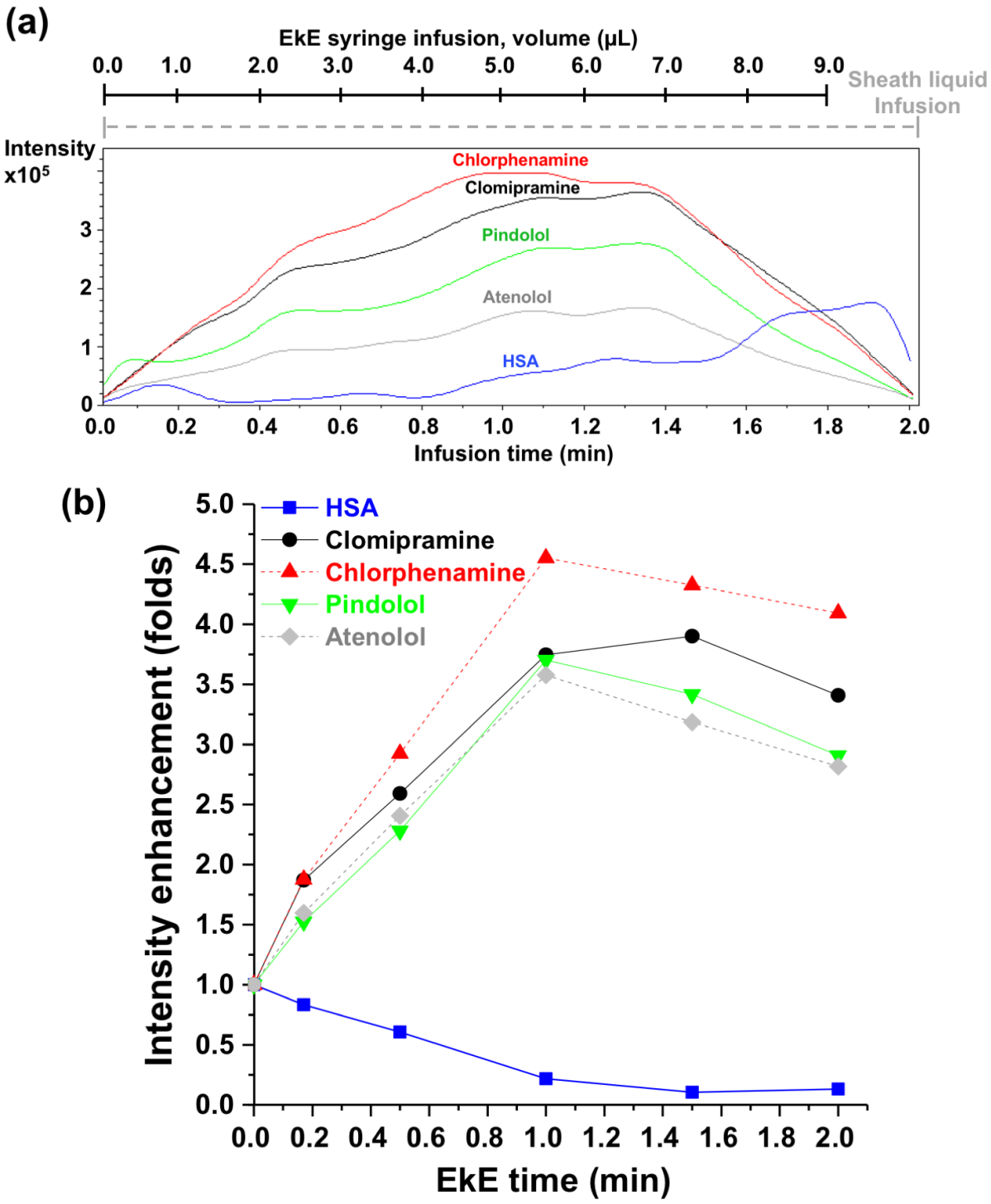

Figure 2. MS data of the in-syringe electrokinetic removal of HSA from weakly basic pharmaceuticals: clomipramine, chlorphenamine, pindolol, and atenolol. (a) EIEs of the molecular ion $[M+H]^{+1}$ of each weakly basic drug (50.0 ng/mL) and the fragment ion (*m/z* 685.1) of HSA (8.0 mg/mL) in 300 mM NH_4OH in 30% (v/v) acetonitrile after the in-syringe EkE for 90 s by applying 800 V. The contents of the EkE syringe were infused into ESI-MS at a flow rate of 5.0 µL/minute and a sheath liquid (5.0 µL/minute) composed of formic acid 0.5% in 75% (v/v) methanol**. (b)** 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) of each drug and HSA.

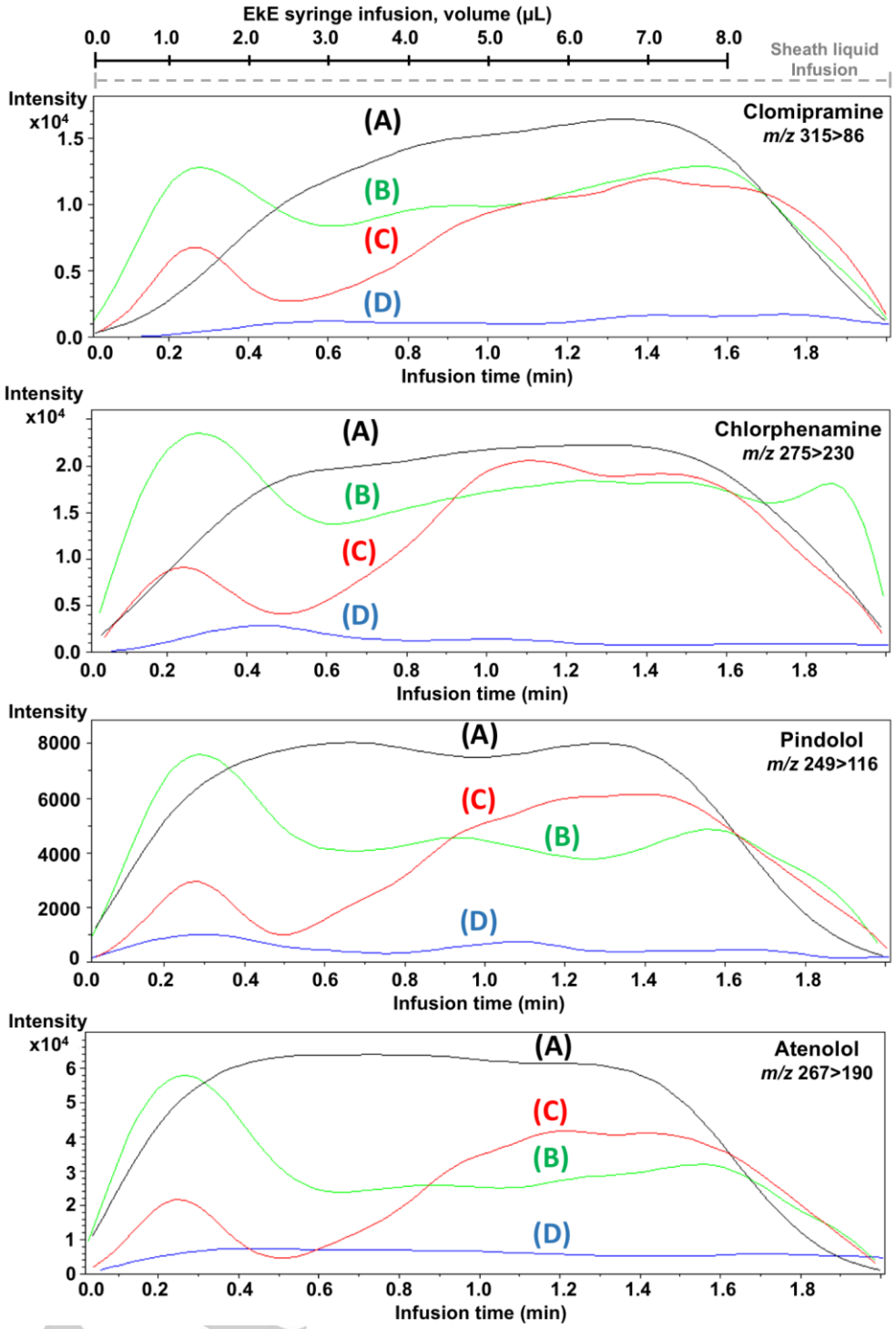

Figure 3. Effect of EkE processing on standard solutions and serum samples containing weakly basic pharmaceuticals. EIEs in MRM are presented for neat standard solutions without EkE **(A)**, neat standard solutions with EkE **(B)**, spiked serum samples with EkE **(C)**, and spiked serum samples without EkE **(D)**. Samples were diluted 5 times to yield a final BGE concentration of 300 mM NH_4OH in 30 % (v/v) acetonitrile. Samples contained 40.0 ng/mL of clomipramine, chlorphenamine, and pindolol and 300.0 ng/mL of atenolol, respectively.

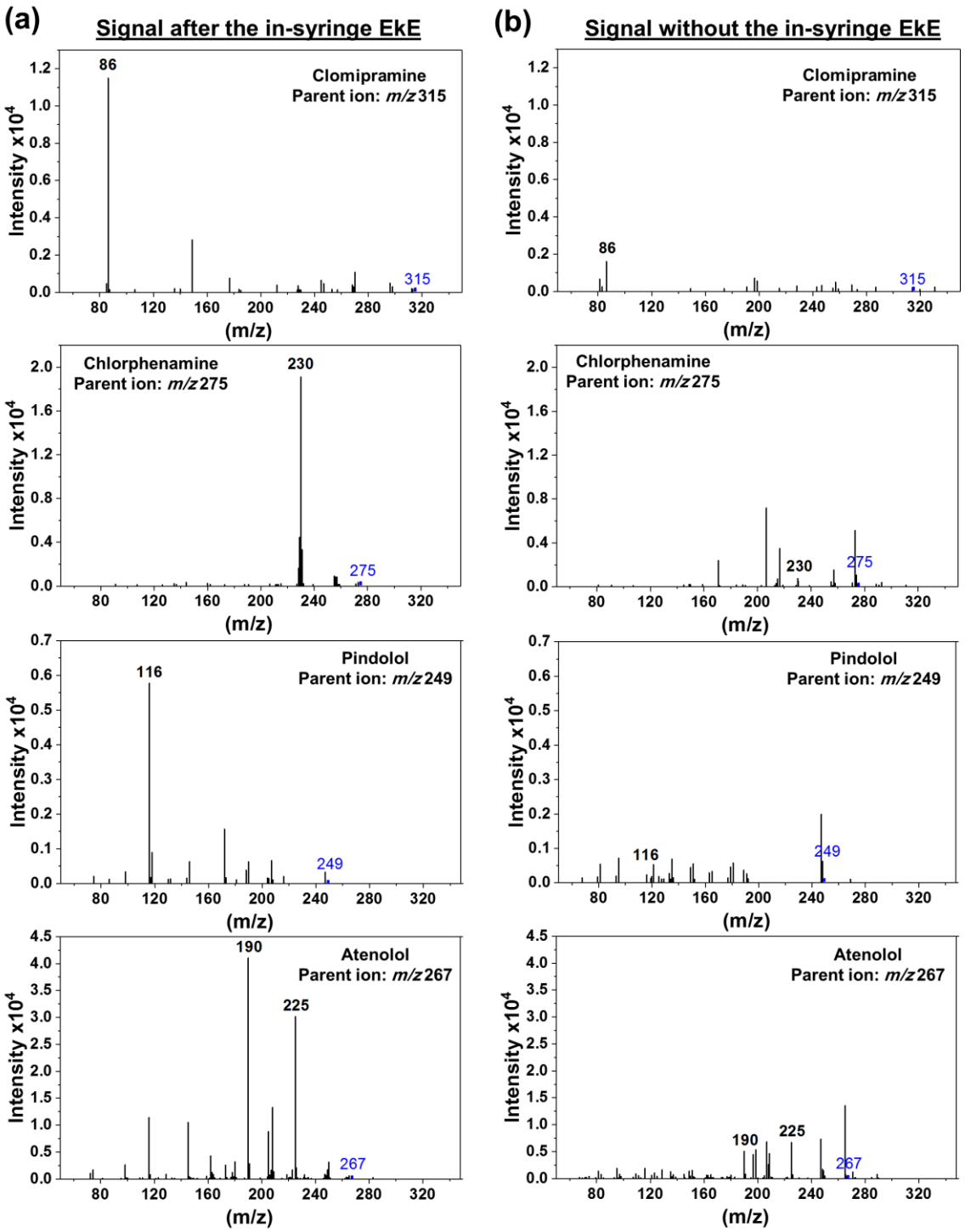

Figure 4. MS/MS spectra of the weakly basic pharmaceuticals in serum using in-syringe EkE approach and the direct infusion approach. (a) MS/MS spectra (1.4 to 1.6 minutes) showing the transitions of molecular ions to the most abundant daughter ions of 40.0 ng/mL of clomipramine, chlorphenamine, and pindolol and 300.0 ng/mL of atenolol in spiked serum after 5-time dilution with the BGE and application of the EkE**. (b)** MS/MS spectra (1.4 to 1.6 minutes) of the spiked serum sample without the EkE step.

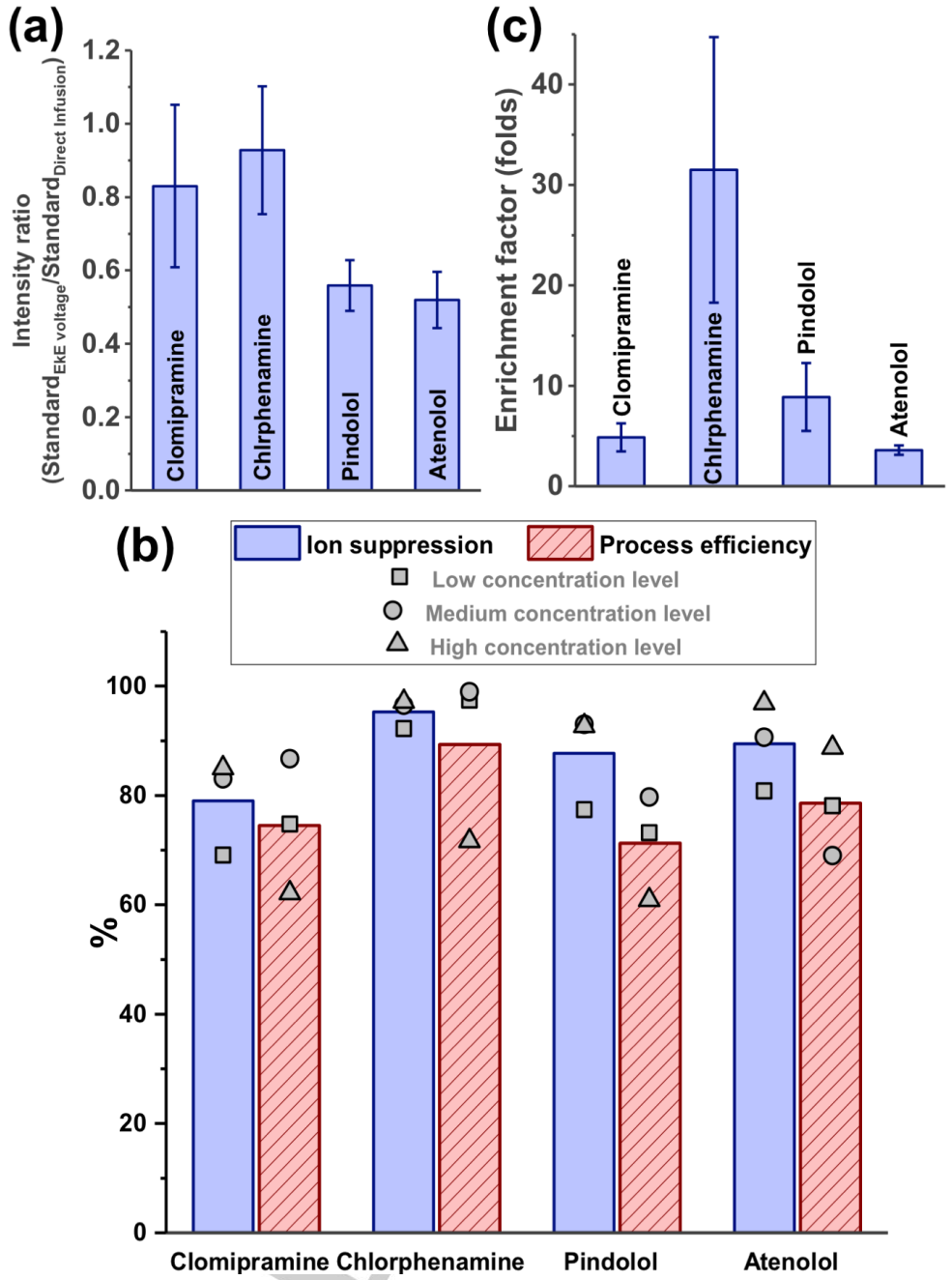

Figure 5. Quantitative assessment of the EkE process. (a) Effect of EkE processing on the signal intensity of standards, 40.0 ng/mL of clomipramine, chlorphenamine, and pindolol and 300.0 ng/mL of atenolol (B/A), n=3 for each set, the error bars represent the SD of the average intensity ratio where $SD=[(SD_A/Mean_A)^2+(SD_B/Mean_B)^2]^{1/2}x|$average intensity ratio$|$ **(b)** Realized reduction in ion suppression and gain in process efficiency of the EkE process in comparison with direct infusion of diluted (5x) serum samples. Ion suppression due to the serum matrix calculated as 100 – (D/A × 100)[42]; process efficiency calculated as PE (%) = C/A × 100[34]. 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.

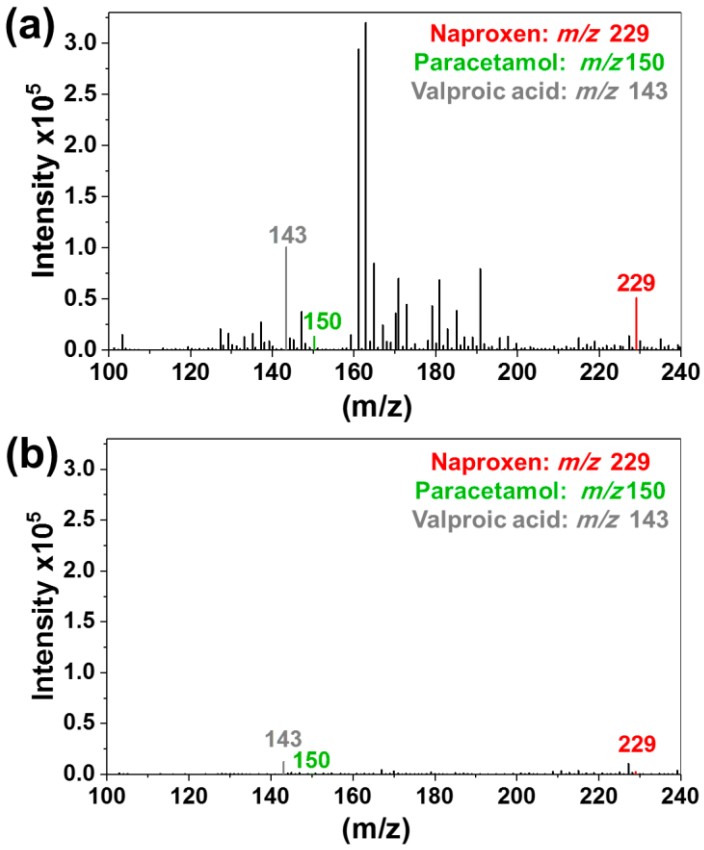

Figure 6. Mass spectra of the weakly acidic pharmaceuticals in serum using in-syringe EkE approach and the direct infusion approach. (a) Mass spectrum (whole run, 0-3.75 minutes) of a spiked serum sample with added 16.0 µg/mL of naproxen (NAP), 12.0 µg/mL of paracetamol (PCM), and 160.0 µg/mL of valproic acid (IS) after the in-syringe 15-fold dilution and EkE (-2000 V application for 320 seconds). **(b)** Mass spectrum of the spiked serum sample after 15-fold dilution but without the EkE step. The final composition of the BGE was 50 mM formic acid in 30% (v/v) acetonitrile.

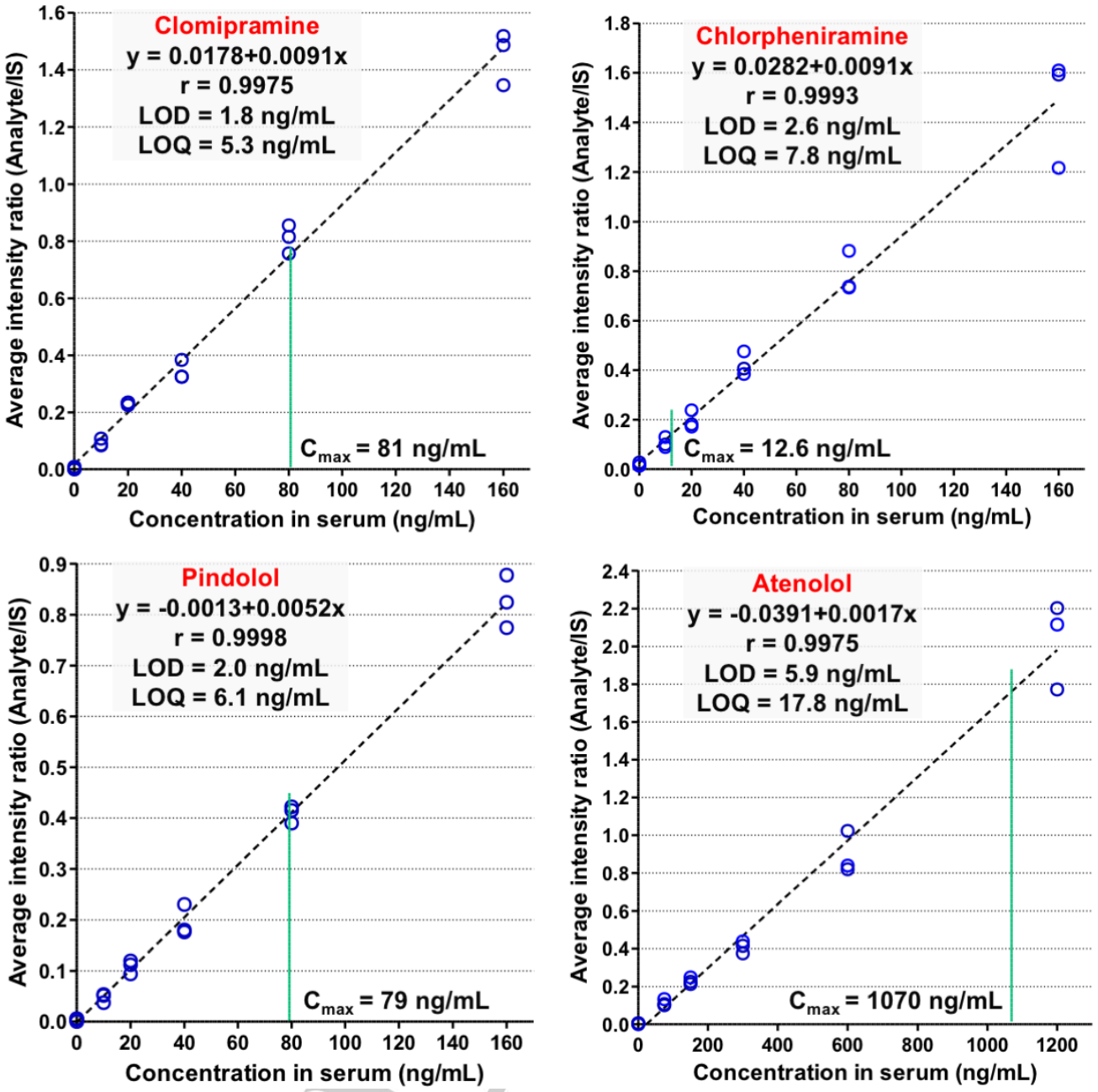

Figure 7. Determination of pharmaceuticals in spiked serum using the in-syringe EkE-ESI-MS/MS method. Quantitative analysis of serum spiked with clomipramine (10 to 160 ng/mL), chlorphenamine (10 to 160 ng/mL), pindolol (10 to 160 ng/mL), and atenolol (75-1200 ng/mL) using their corresponding isotopologues 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.

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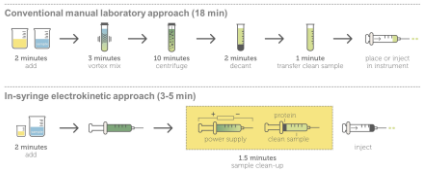

In-syringe electrokinetic protein removal is introduced and hyphenated with electrospay ionization mass spectrometry (ESI-MS) for the direct analysis of biological samples. The novel approach can simplify the bioanalytical workflow, minimize the use of organic solvent to few µLs, and allow the whole analysis - in-syringe sample preparation and online ESI-MS determination - to be accomplished in less than five minutes.

((The Table of Contents text should give readers a short preview of the main theme of the research and results included in the paper to attract their attention into reading the paper in full. The Table of Contents text **should be different from the abstract** and should be no more than 450 characters including spaces.))

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